Examiner 1

1a) A summary of the specific glucocorticoid effects mimicked by annexin I.

In Vitro Systems	Annexin	GC Effect mimicked by Annexin I	Reference
Isolated guinea pig lung	Recombinant human annexin I	Inhibited agonist-induced production of eicosanoids	Cirino et al., 1987.
Isolated macrophages	Purified annexins	Inhibited release of arachidonic acid	Errasfa et al., 1988.
Rat brain	1. Annexin I peptide 1-188 2. Annexin I antiserum	1. Appeared protective against neuronal death and oedema induced by focal cerebral ischaemia. 2. Significantly exacerbate damage	Relton et al., 1991.
Rat stratium	Annexin I peptide, 1-188	Inhibit neuronal damage	Black et al., 1992.
Alveolar macrophages	Annexin I	Inhibit superoxide generation by NADPH oxide	Maridonneau-Parini et al., 1989.
Human peripheral blood neutrophils	Annexin I peptide, 2-26	Suppress initial rate and maximal response of FcγR-mediated superoxide release and phagocytosis	Goulding et al., 1988.
Erythrocytes	Annexin I	Inhibits formation of aggregates between erythrocytes sensitised with anti-erythrocytes IgG and leukocyte expression Fc receptor for IgG.	Goulding and Guyre, 1993b.
In Vivo Systems			
Rat carrageenan-induced pleurisy	Partly purified annexins	Reduced exudate volume and leukocyte infiltration	Blackwell et al., 1982.
Rat carrageenan-induced paw oedema	Human recombinant annexin I	Inhibits paw oedema induced by carrageenan	Cirino et al., 1989.
Animal carrageenan-induced paw oedema	Annexin I peptide, ac2-26	Inhibits carrageenan paw oedema and air pouch neutrophil accumulation	Cirino et al., 1993.
Rat carrageenan oedema model	Antiserum to annexin I peptide, 1-188	Reverse inhibitory effects of dexamethasone	Duncan et al., 1993.
rats	Human recombinant annexin I	Inhibit pyrogenic actions of cytokines and other agents	Davidson et al., 1991.
In mice	Anti-serum to annexin I	Normalised impaired febrile responses to murine IL-1β	Strijbos et al., 1993.
Rats, mice	Annexin I peptide, 1-188	Inhibit pyrogenic effects of cytokines	Carey et al., 1990; Strijbos et al., 1992a.
Conscious rats	1. Annexin I peptide, 1-188 2. Annexin I peptide, 1-188 3. Anti-serum to annexin I	1. Reduced acute increases in colonic temperature and oxygen consumption, resulting from IL-1β and IFNγ injections 2. Did not affect response to PGE_2 and its activity was prevented by heat treatment or by pretreatment of animals with annexin I peptide antiserum 3. Enhanced thermogenic responses to IL-1β in dexamethasone-treated rats	Carey et al., 1990.
Conscious rats	Recombinant annexin 1-188 peptide	Produced small increases in the thermogenic effects	Strijbos et al., 1992b.
Murine pouch model	Annexin I	Inhibited migration of IL-1-induced	Perretti et al.,

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		migration of neutrophils	1993b.
Murine pouch model	Annexin I monoclonal antibody	Inhibit glucocorticoid action in IL-1-induced neutrophil migration	Perretti & Flower, 1993b; Getting et al., 1997.
Hamster cheek model	a) Annexin I antibody b) Annexin I peptide, ac2-26	1. Abolish effects of glucocorticoids on adherent leukocytes 2. Prolongs the time to complete diapedesis process	Mancuso et al., 1995.

1b) Yes, the peptides work via a cell surface receptor. The concept that annexin I is exported from target cells enabling annexin I to bind to a cell surface receptor to exert its biological function originated from the identification of specific annexin I binding sites on peripheral blood leukocytes (Goulding et al., 1996). In later studies using murine models of acute inflammation, blockade of cell surface binding sites by inactivated annexin I prevented the biological activity of annexin I N-terminal peptide 2-26 (Perretti et al., 1993). This finding that inactivated annexin I binds to the same high affinity binding sites as the biologically active annexin I peptide is consistent with a requirement for a peptide binding site interaction for its biological activity.
1c) Studies presented in section 1.5.4 of the candidate's thesis examine how annexin I peptides 2-26 and 1-188 differ in their anti-inflammatory activities, both *in vivo* and *in vitro*.
1d) A summary of known effects of MIF and its interactions with glucocorticoids.

MIF Effects With Regard To GC Interactions	Reference
GCs induce MIF secretion in macrophages	Calandra et al., 1995.
Dose-dependent, biphasic secretion of MIF ie. Low GC concentrations induced MIF, high concentrations inhibited MIF secretion in mice	Calandra et al., 1995.
MIF able to override GC inhibition of macrophage cytokines →GC counter-regulatory actions of MIF	Calandra et al., 1995.
Mouse model of endotoxemia: MIF overcame protective GC effects	Calandra et al., 1995.
Biphasic regulation of MIF observed in rheumatoid FLS	Leech et al., 1999.
1. In antigen specific T & B cell responses, low GC concentrations induced MIF secretion from human or mouse T cells 2. MIF override GC-mediated suppression of T cell proliferation and production of IL-2 and IFNγ	Bacher et al., 1996.
In adrenalectomised rats, downregulation of tissue MIF	Leech et al., 2000.
In alveolar cells, MIF overrides the anti-inflammatory effects of GCs on cytokine release	Donnelley et al,. 1997.

1e) Findings by Honma et al (2000) suggest that endogenous MIF is not involved in the formation of TNFα which plays an essential role in LPS-induced shock. This conclusion differs from that obtained from previous experiments which utilised anti-MIF antibodies. One explanation for this is that other cytokines may compensate for the defect. Previous reports indicated that not only TNFα but also IL-1, IL-6, IL-8 and IFNγ participate in the host response to LPS (Honma et al., 2000). These mediators may act alone or in combination to activate macrophages and lymphocytes. Another explanation is that the polyclonal antibodies used by Bernhagen et al (1993) or Calandra et al (1995), might have cross-reacted with molecule(s) other than MIF. It is also conceivable that the immune complex itself, consisting of MIF plus antibody, has a protective role in endotoxic shock. In recent studies, Bozza et al (1999) analysed the role of MIF in sepsis using MIF-deficient mice following LPS administration. In contrast to studies by Honma et al (2000), they observed a significant difference in the survival rate between normal and MIF-deficient mice after LPS administration. At present, the reason for this discrepancy is unclear. In addition, experimental systems between Honma and Bozza appear to be very similar. It has been suggested that the response to LPS is highly influenced by pre-exposure to LPS.
1f) Studies by Lan et al suggest that TNFα or TNFα-mediated inflammatory events upregulate MIF production in immunologically-induced renal disease (Lan et al., 1997). In contrast to these

findings, Leech et al observed RA FLS-conditioned medium induced peripheral blood mononuclear cell TNFα release, and this induction was significantly inhibited by anti-MIF mAb, suggesting that MIF is an upstream regulator of TNFα release in RA. Interestingly, FLS MIF was not increased by exposure to IL-1β, TNFα, or IFNγ (Leech et al, 1999). Together, these studies suggest that MIF acts upstream in cell systems and that MIF effects are indirect in *in vivo* systems.

2a) The importance of annexin I binding sites from a biological standpoint is still unknown. Moreover, the lack of an annexin I receptor knock-out model makes exploration of the biological function of annexin I binding sites difficult. One can only propose the biological effect on the basis of peptide known to bind to receptor.

2b) The concept that differences between RA and OA could be due to differences in membrane or some other non-specific factor is considered unlikely.

2c) This is the subject of future studies in the candidate's former laboratory.

2d) This is the subject of future studies in the candidate's former laboratory.

2e) At present, there is no source of biologically active full length annexin I commercially available.

2f) The examiner is correct to point out that the functional association between reduced annexin I binding sites and the inflammatory phenomena of RA FLS has not been shown. The fact that both phenomena have been observed in cells therefore cannot be construed as related until further experiments are undertaken.

3a) Results of COX2 protein analysis by FACS are consistent with mRNA and enzyme activity studies. The candidate does not believe it necessary to verify the FACS method with an older, less reliable and less quantitative technique.

3b) The effects of annexin I on PLA_2 were lost in the presence of IL-1β, suggesting, for reasons not presently understood, that IL-1β induced PLA_2 activity through mechanisms not sensitive to inhibition by annexin I peptide 2-26.

3c) The examiner's comments are noted.

3d) Yes, the effects of annexin I antibodies on FLS activity are the subject of ongoing studies in the candidate's former laboratory.

3e) The examiner's explanation of the biphasic production of PGE_2 may well explain the candidate's results and it is noted with thanks.

4a) Studies presented in Table 1 were performed using 4 different RA FLS. Future experiments need to be undertaken with a much larger sample number.

4b) Other effects of IL-1β are the subject of current investigation at Monash University.

4c) Studies examining the effects of MIF as a co-factor with IL-1β are continued at Monash University.

5a) See comment for 5b.

5b) The experiment where MIF and IL-1β are used concurrently has been performed in the candidate's former laboratory. IL-1β is observed to be a consistently more potent stimulus of COX2 than MIF, although the difference in COX2 induction is at most moderate.

6a) The addition of extra positive controls and the combination of MIF and other cytokines is of interest but does not devalue the significant effects of MIF on proliferation that the candidate has demonstrated in these studies.

6b) The examiner's comments are noted with interest.

6c) Further studies on NFκB are continued at Monash University.

6d) Angiogenesis and dysregulated cell proliferation are pivotal elements of rheumatoid synovial pathology. The candidate notes that the discussion could have been better linked with the data.

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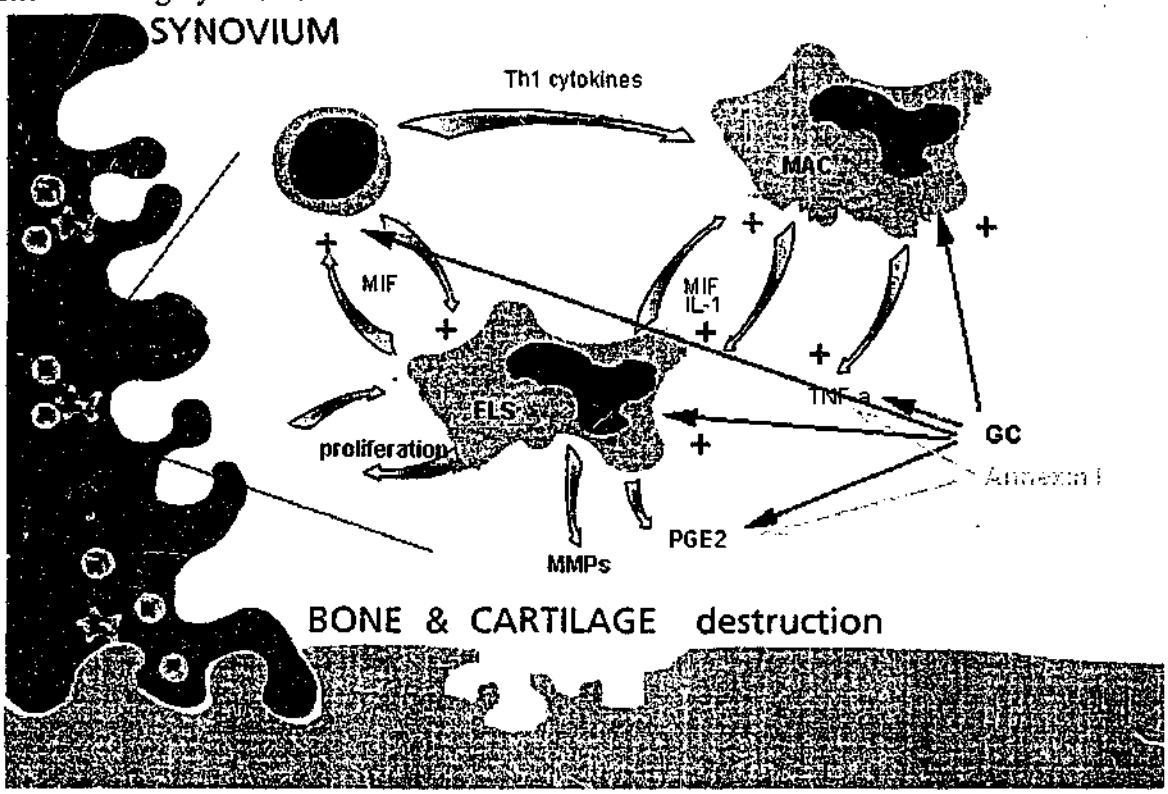
Examiner 2

Chapter 1

Paragraph 1, comment1) Diagrams such as prostanoid synthetic pathway referred to are readily available in general biological and medical textbooks and are so well described the candidate thought it not imperative to include them.

Paragraph 1, comment2) The proposed role of human FLS in synovial inflammation. Soluble mediators that contribute to inflammatory events and which are generated in response to FLS activation in inflammatory responses are illustrated. The stimulatory effect of glucocorticoids (GC) on MIF in cell types present in synovium is designated (+), and regulated by annexin I are shown with grey arrows.



Paragraph 2) The pro- and anti-inflammatory effects of COX2 like that of NO remain unresolved. In the case of MIF and annexin I, pro- and anti-inflammatory *in vivo* effects respectively, are associated with increased and decreased effects on prostaglandins.

Paragraph 4) The non-genomic effects of steroids and their structural aspects are considered beyond the scope of this thesis.

Paragraph 6) The examiner's comments are noted.

Chapter 2

Paragraph 1) Higher concentrations of annexin I than 10 μM are not achievable using the candidate's reagent and are unlikely to be biologically relevant. Lower concentrations could be interesting. However in all cases, the experimental size is dependent on the limiting resource of the population of primary cultured human synovial cells.

Paragraph 2) No data exists on the nature of the binding molecule on human FLS.

Paragraph 3) In section 2.4.5, FLS were incubated with 10^{-7} M of dexamethasone for 24h.

Paragraph 4, comment1) The 2-26 peptide was checked and was negative for LPS contamination.

Paragraph 4, comment1) A limited supply of annexin I peptide prevented a fuller dose response curve being undertaken. The Giga-Hama protein was LPS positive and was therefore not used in cell culture experiments.

Chapter 3
Paragraph 1) The examiner's comments are noted and increase basal activity is a feature of cultured synovial cells
Paragraph 2) Experiments examining the effects of neutralising anti-annexin I antibodies on synoviocyte PGE_2 production are ongoing in the candidate's former laboratory
Paragraph 2) The correlation between enzyme activity and expression is not taken for granted. As a result, both methods were undertaken in Chapter 3.
Paragraph 2) There is no zero baseline for COX2 protein analysis by FACS. The candidate disagrees with the examiner's comments that the FACS graph is misleading.

Chapter 4
Paragraph 2) Synergistic effects between MIF and IL-1 would be interesting. The absence of these experiments, however, should not change the conclusion of this study.
Paragraph 4) The examiner's comments are noted.

Chapter 5
Paragraph 1, comment 1) In addition to the experiments presented, work by collaborators have shown a significant increase in COX2 to be induced by higher concentrations of MIF. Effects were specific to COX2 since no effect on COX1 was observed (data in press).
Paragraph 1, comment 2) Selective COX inhibitors were not used.
Paragraph 2) Possible effects of MIF on constitutive COX expression are the subject of ongoing investigation.

Chapter 6
Paragraph 1) The second bar had a p value of 0.06. ie p=0.06.
Paragraph 2, comment 1) Figure 6.6.4
Nuclear translocation of p50 and p65 subunits of NFκB in IL-1β-stimulated FLS co-treated with MIF mAb or negative control mAb.
FLS were treated with (a-d) IL-1β (0.1 ng/ml) and (c-d) control, IgG_1 (50 μg/ml); or (e-f) IL-1β (0.1 ng/ml) and MIF mAb (50 μg/ml), for 30 minutes and immunostained.
Paragraph 2, comment 1) To ensure that the biotinylated swine anti-goat antibody did not cross react with neutralising mouse antibodies, the sections were incubated for 1 hour with blocking agent that consisted of 10% fetal calf serum/10% swine serum/0.05% azide in tris-buffered saline solution.
Paragraph 3) In Figure 6.6.6, the 500 ng/ml of MIF dose was chosen to be a supramaximal dose. Further experiments are continuing in the candidate's former laboratory.
Paragraph 4) Further experiments are continuing in the candidate's former laboratory.

EFFECT OF TWO GLUCOCORTICOID-INDUCIBLE PROTEINS ON HUMAN FIBROBLAST-LIKE SYNOVIOCYTES.

A thesis submitted for the degree of Doctor of Philosophy

To the

Faculty of Medicine

Monash University

By

Annaleise Sampey, BSc(Hons) (Monash Univeristy Australia)

February 2001.

This thesis is dedicated to the memory of my brother Paul Vincent Sampey
(25/10/1977-12/4/1997)

MONASH UNIVERSITY
THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
ON.....21November 2001.....
.....[redacted].....
for Sec. Research Graduate School Committee

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Summary

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease whose manifestations are characterised by synovial hyperplasia, inflammation and altered immune responses. Despite unknown aetiology, it is widely believed that overactivity of pro-inflammatory systems and underactivity of anti-inflammatory mechanisms contribute to immune-inflammatory dysfunction operant in RA. The hypothalamic-pituitary-adrenal axis (HPA) is a pivotal modulatory system associated with the inflammatory response. Under the regulation of the HPA axis, glucocorticoids mediate important anti-inflammatory and immunosuppressive actions. Evidence suggests that disturbances in HPA function may contribute significantly to the pathogenesis of inflammatory diseases such as RA. Since the discovery of two glucocorticoid-inducible proteins, annexin I and macrophage migration inhibitory factor (MIF), increased understanding in the clinical use of glucocorticoids have been matched by scientific studies investigating the mechanisms of glucocorticoid actions in inflammation.

The glucocorticoid-induced anti-inflammatory mediator annexin I has important effects in animal models of arthritis and is expressed in human rheumatoid synovium. The findings presented in Chapter 2 represent the first study to demonstrate annexin I binding sites on human fibroblast-like synoviocytes (FLS), with notably reduced annexin I binding sites observed on RA FLS compared with osteoarthritic (OA) FLS. The results indicate that there is regulation of annexin I binding sites on human FLS in response to pro-inflammatory cytokine and enzyme treatment. These data suggest that annexin I binding sites are altered at sites of inflammation. In addition, the finding of inhibition of FLS PLA_2 activity, in response to annexin I N-terminal peptide provides evidence of direct anti-inflammatory effects of extracellular

annexin I on FLS activation. It is likely that reduced annexin I binding may impair sensitivity of certain pro-inflammatory processes to glucocorticoids.

Prostaglandins (PG), such as PGE_2, mediate the pain and inflammation associated with RA. In eicosanoid generation, phospholipase A_2 (PLA_2) and cyclooxygenase-2 (COX2) are pivotal regulatory enzymes, whereby non-steroidal anti-inflammatory drugs (NSAIDS) act primarily by inhibiting COX and subsequently PGE_2 synthesis. Annexin I has been described as a mediator of the anti-inflammatory actions of glucocorticoids, both *in vivo* and *in vitro*. Despite FLS being implicated as an important source of PGE_2, the regulatory activity of annexin-I on human FLS has not previously been explored. In Chapter 3, I demonstrate the effects of annexin-I on phospholipase A_2 and cyclooxygenase activities and prostaglandin E_2 release in cultured human fibroblast-like synoviocytes. In this study, uncoupling of the effects of annexin-I and glucocorticoid on constitutive and cytokine-stimulated arachidonic acid production implicates glucocorticoid-independent regulatory activities for annexin I in inflammatory processes. These findings are consistent with the conclusion that COX2 is the rate-limiting enzyme in FLS PGE_2 generation. Moreover, the lack of effect of annexin I on FLS PGE_2 synthesis, despite anti-inflammatory effects observed *in vivo*, suggests that annexin I modulates inflammation via eicosanoid-independent mechanisms. This possibility requires further investigation, and studies examining the role of annexin I in such mechanisms may significantly impact on the current understanding of glucocorticoid inflammatory regulation.

Another glucocorticoid-inducible protein, macrophage migration inhibitory factor (MIF), has importance as an endogenous pro-inflammatory protein, with the unique capacity to reverse the inhibitory effects of glucocorticoids. The expression of MIF in human RA synovium and FLS, and the ability of FLS-derived MIF to induce

monocyte TNFα production, suggests an important role for MIF in RA inflammatory processes. MIF has also recently shown by our group to have a crucial role in animal models of RA. The functional effects of MIF upon these cells have not previously been reported. The data presented in Chapters 4 and 5 implicate MIF as an inducer of synoviocyte activation in RA. MIF is shown to induce FLS PLA_2 and COX2 in RA FLS at the levels of their expression and activity. An essential role for MIF in the activation of these phenomena in FLS by IL-1β is also seen. Moreover, the finding that MIF antagonises the inhibitory actions of glucocorticoids in cytokine-activated FLS provides evidence that this counter-regulatory system is operative in RA. These studies establish MIF as a potential therapeutic target in human RA.

In keeping with its description as a delayed-early response cytokine, considerable evidence supports the involvement of MIF in cell proliferation. Nuclear factor kappa B (NFκB) is a transcription factor reported to be involved in cytokine-induced FLS activation. Glucocorticoids are thought to act through prevention of cytokine signalling via NFκB. However, the effects of MIF on FLS proliferation, and the potential for MIF to activate NFκB in RA FLS, have not been previously examined. Studies presented in Chapter 6 show an additional and critically important effect of MIF on synoviocyte proliferation, and provide evidence that MIF-induced PLA_2 and COX2 activities, in addition to FLS proliferation, are not mediated via NFκB. Moreover, I demonstrate that the role of MIF in IL-1β-induced activation of these three aspects of synoviocyte activation is independent of NFκB. These data further establish MIF as a key therapeutic target in human RA with potential anti-inflammatory and disease-modifying effects.

Evidence from these studies extend the current knowledge of the endogenous, glucocorticoid-inducible proteins, annexin I and MIF, in glucocorticoid-regulated inflammatory processes operative in RA FLS.

Statement

To the best of my knowledge:

No material in this thesis has been accepted for award of any degree or diploma in any university or other institution, except where it is clearly acknowledged;

No material in this thesis has been previously published or written by another author, except where due reference is made herein;

All the material presented in this thesis represents the work of the candidate, except where noted herein.

(signed)

Annaleise V. Sampey

Publications arising from work presented in this thesis

Articles in refereed journals

1. **Sampey AV**, Hutchinson P & Morand EF. Annexin I surface binding sites and their regulation on human fibroblast-like synoviocytes. Arthritis Rheum 2000:43(11):2537-42.
2. **Sampey AV**, Hutchinson P & Morand EF. Annexin I and dexamethasone effects on phospholipase and cyclooxygenase activity in human synoviocytes. Mediators Inflamm 2000:9(3-4):125-32.
3. **Sampey AV**, Hall PH, Mitchell RA, Metz CN & Morand EF. Rheumatoid synoviocyte activation by macrophage migration inhibitory factor. Arthritis Rheum (in press).
4. **Sampey AV** & Morand EF. A role for macrophage migration inhibitory factor in rheumatoid synovial fibroblast proliferation (manuscript in preparation).

Presentations at the meetings of learned societies, published as abstracts

1. Morand EF, **Sampey AV**, Yang Y-H, Hutchinson P & Hall PH. Annexin I surface binding sites and their regulation on human fibroblast-like synoviocytes. Arthritis Rheum 43(Suppl): S67 2000.
2. Morand EF, **Sampey AV**, Lacey D, Hall PH & Bucala R. NF-κB-independent activation of rheumatoid arthritis synovial cells by MIF. Arthritis Rheum 43(Suppl): S407 2000.
3. Morand EF, **Sampey AV**, Lacey D, Hall PH & Bucala R. NF-κB-independent activation of rheumatoid arthritis synovial cells by MIF. Inflammation Research (Suppl) 2000.
4. **Sampey AV**, Hutchinson P & Morand EF. Annexin I N-terminal peptide effects on PLA_2 and COX2 activity in human fibroblast-like synoviocytes. Arthritis Rheum 42(Suppl): S249 1999.
5. **Sampey AV**, Hall PH, Bucala R & Morand EF. Macrophage migration inhibitory factor (MIF) activation of rheumatoid synoviocytes. Arthritis Rheum 42(Suppl): S283 1999.
6. **Sampey AV**, Hall PH & Morand EF. Effect of macrophage migration inhibitory factor on eicosanoid generation in synovial fibroblasts. Australian Rheumatology Association 45 1999.
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Abbreviations

AA	Arachidonic acid
ACTH	Pituitary adrenocorticotrophic hormone
ADX	Adrenalectomy
AP-1	Transcription factor activator protein 1
AVP	Arginine vasopresin
CIA	Collagen-induced arthritis
COX	Cyclooxygenase
CRH	Corticotropin-releasing hormone
DTH	Delayed type hypersensitivity
EGF	Epidermal growth factor
FLS	Fibroblast-like synoviocytes
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
HLA	Histocompatibility antigen
HPA	hypothalamo-pituitary-adrenal
HRE	Hormone response elements
ICAM-1	intracellular adhesion molecule-1
iNOS	inducible nitric oxide synthase
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
NFκB	Nuclear factor kappa B
NO	Nitric oxide

NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
PB	Peripheral blood
PCNA	Proliferating cell nuclear antigen
PG	Prostaglandin
PLA_2	Phospholipase A_2
PGE_2	Prostaglandin E_2
PMN	Polymorphonuclear neutrophils
POMC	Pituitary pro-oplomelanocortin
PVN	Hypothalamic paraventricular nucleus
RA	Rheumatoid arthritis
ROS	Reactive oxide species
SCID	Severe combined immunodeficient
SCW	Streptococcal cell walls
TIMP	Tissue inhibitors of metalloproteinase
UDPGD	Uridine diphosphoglucose dehydrogenase
VCAM-1	Vascular cell adhesion molecule-1

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CHAPTER ONE: LITERATURE REVIEW

1.1 Overview

This thesis concerns studies of the effects of certain key pro- and anti-inflammatory mediators on synovial cell function. In particular, the effect of these mediators on eicosanoid generation will be addressed. I will review literature associated with the pathogenesis of rheumatoid arthritis (RA), and the ability of anti-inflammatory and pro-inflammatory mediators to regulate mechanisms relevant to RA eicosanoid generation. The anti-inflammatory actions of annexin I and glucocorticoids (GC) and the pro-inflammatory actions of macrophage migration inhibitory factor (MIF) will be elaborated and examined in detail. The structure, function and distribution of annexin I, the glucocorticoids and MIF and their roles in health and disease will also be described. I will detail the *in vitro* experimental models that have been utilised in this thesis. Signal transduction pathways influenced by these mediators will also be discussed. Prior to this, I will outline the structure and function of synovium and the involvement of cellular and soluble mediators in the pathogenesis of RA.

1.2 Rheumatoid arthritis: pathology and pathogenesis

Rheumatoid arthritis (RA) is a chronic systemic disease whose aetiology remains unknown. RA affects about 1% of the population worldwide, with females affected approximately 3 times more commonly. Synovial hyperplasia, inflammation, and altered systemic immune responses are key features of RA pathology. Proliferation of resident synovial lining cells and infiltration of activated bone marrow-derived cells dominate the chronic inflammatory synovitis. Invasive synovial tissue and its activated products may result in the progressive destruction of articular cartilage and subchondral bone. The disease results in multi-joint involvement, with joint erosions

producing deformities that preferentially involve the metacarpo- and interphalangeal joints of the hands and feet.

The following sections will briefly outline the structure and function of synovium and the involvement of cellular and soluble components of the immune system in the pathogenesis of RA. In view of the wide range and complexity of immune phenomena reported to RA, the review will focus on phenomena relevant to studies presented in this thesis.

1.2.1 Normal synovium

In normal joints, synovium is defined as the soft tissue that lines diarthrodial joints, tendon sheaths and bursae (Edwards, 1994). Synovium covers all intra-articular structures except the articular surfaces of cartilage. The synovium produces a thin film of synovial fluid, rich in hyaluronic acid, which covers the synovium and cartilage. Synovial fluid is required for its viscosity and lubricating properties (Krane et al., 1990) and is responsible for the delivery of nutrients to articular cartilage (Edwards, 1994).

The cells are predominantly separated by interstitium such that there is no direct cell to cell contact. Synovium consists of two distinct layers. The intima or cellular layer consists of cells designated Type A (macrophage-like) and Type B (fibroblast-like) synoviocytes. Normal synovium lining is between one to three cells thick.

In common with resident macrophages in other tissues, macrophage-like synoviocytes (MLS) have a high content of cytoplasmic organelles and non-specific esterase activity. MLS express macrophage surface differentiation markers including CD14, CD68, class II major histocompatibility complex molecules such as HLA-DR, and Fc receptors (Edwards, 1995).

Fibroblast-like synoviocytes (FLS) account for the majority of cells and have fewer organelles and more extensive endoplasmic reticulum. Consistent with their presumed mesenchymal origin, these cells exhibit bipolar fibroblast morphology when cultured *in vitro* on plastic (Wilkinson et al., 1992). FLS do not express leukocyte phenotype surface antigens and express only low levels of MHC class II molecules. FLS express a number of surface adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), CD44 and β_1 integrins (Firestein, 1996). To date, lack of agreement on the existence of definitive markers of FLS has prevented consistent identification of these cells. Unlike stromal fibroblasts however, synovial fibroblasts are distinguished by their expression of uridine diphosphoglucose dehydrogenase enzymatic activity (Firestein, 1994). This enzyme is usually involved in the synthesis of hyaluronic acid. Although it remains obscure whether FLS represent a terminally differentiated tissue-specific phenotype, evidence in favour of this hypothesis derives from the observation that these cells secrete hyaluronic acid in culture.

In normal synovium, the sublining or subintimal layer is relatively acellular, consisting mainly of fat cells, fibroblasts and occasional lymphocytes and macrophages. Close to the under surface of the synovial lining lies a rich bed of fenestrated microvessels. Lymphatic vessels and nerve fibres are also identifiable in synovial tissue. The acellular extracellular matrix contains a variety of macromolecules, including collagen, fibronectin and proteoglycans (Firestein, 1994). Lymphatic vessels are involved in the clearance of synovial fluid proteins, which have been delivered to synovial tissue via microvasculature. Microvascular endothelial cells are an important component of normal synovial tissue. The synovial microvasculature is therefore well positioned to actively contribute to synovial

inflammatory processes, given its ability to interact with surrounding tissue cells and blood-derived immune cells.

1.2.2 Inflammatory synovium in rheumatoid arthritis

In RA, the synovium undergoes dramatic changes. The earliest described changes in RA involve prominent tissue oedema and increased vascularity (Zvaifler, 1983; Lydyard and Edwards, 1994). Both FLS and MLS numbers become markedly expanded, leading to hyperplasia of the synovial intima (Firestein, 1996). The inflamed synovial intima that results is approximately six to eight cells thick. The sublining layer also contains dramatically increased numbers of macrophages, T and B lymphocytes, plasma cells, dendritic cells, activated fibroblasts and mast cells (Harris, 1990; Firestein, 1996). Initially, the lymphocyte infiltrate consists predominantly of small aggregates of T-cells with a more diffuse infiltrate in between (Firestein, 1994). True lymphoid follicles with germinal centres may soon develop, although pseudofollicles and diffuse lymphocytic infiltrates are more common (Firestein, 1994).

In the sublining region, the expansion of blood vessels, especially capillaries and post-capillary venules, is especially prominent. One feature relatively specific to RA is the development of pannus, the highly vascularised inflamed synovium that resembles granulation tissue, found at the synovial interface with cartilage and bone. Pannus tissue is comprised primarily of fibroblasts and macrophages (Zvaifler and Firestein, 1994), most of which are thought to be invasive synovial lining cells (Chu et al., 1992). The relative lack of T cells in the pannus suggests that RA synoviocytes possess some features of autonomy from T cell directed immunity. The pannus is responsible for the active invasion and eventual destruction of cartilage, periarticular and subchondral bone. The activated cells of the pannus release cytokines and

prostaglandins that perpetuate the inflammatory process, as well as matrix metalloproteinases (MMPs) that perpetrate tissue damage. The contribution of synovial cells and soluble mediators present in synovium and pannus, in the rheumatoid synovial inflammatory process will be elaborated further in sections 1.2.3-1.2.5.

1.2.3 Synovial cells

1.2.3.1 Fibroblast-like synoviocytes

The participation of FLS in the initiation of RA remains unproven. Nevertheless, their involvement in the perpetuation of disease is now acknowledged. Increased understanding of their role in diseased synovium, their capacity to recruit immune cells and to deviate immune responses has led investigators to reassess their role in RA pathogenesis.

FLS are non-phagocytic and lack demonstrable surface leukocyte or macrophage differentiation antigens. FLS in the intimal lining express a variety of surface adhesion receptors including VCAM-1, ICAM-1, CD44 (the hyaluronic acid receptor) in addition to β1 and β3 integrins (Bresnihan, 1999; Edwards, 1995; Firestein, 1996). Sublining fibroblasts also express these proteins. A stellate or dendritic morphology is characteristic of FLS freshly isolated from rheumatoid synovium. FLS are readily distinguishable from antigen presenting cells such as dendritic cells that are smaller and exhibit prominent surface class II expression. The observed stellate morphology is believed to reflect a 'transformed' phenotype that results from exposure to synovial inflammatory products. For example, quiescent cultured FLS develop this morphology following IL-1β treatment (Baker and Dayer, 1983).

There has been considerable controversy regarding mechanisms of synovial lining hyperplasia. In original studies, the lack of mitotic figures in synovial lining cells, in addition to the absence of immunostaining for some proliferation markers suggested that local cell division may not contribute to the increased cell numbers. However, evidence of the contribution of local FLS division to synovial hyperplasia has emerged from recent studies demonstrating the expression of PCNA/cyclin, c-Myc and nucleolar organiser region by lining synoviocytes (Qu et al., 1994; Aicher et al., 1994). Studies also report that RA FLS express other proto-oncogenes and transcription factors such as c-fos, jun-b, myc/c-myc, myb, ras, and the zinc factor Z-225/egr-1 (Trabandt et al., 1990; Ritchlin and Winchester, 1989; Case et al., 1989; Kinne et al., 1993; Trabandt et al., 1992; Aicher et al., 1994). These 'immediate-early' response genes are induced by a variety of activating stimuli and can therefore be implicated as markers of cell activation (Kinne et al., 1995). *In vivo*, studies suggest that loss of growth control in synovial fibroblasts may contribute to synovial lining hyperplasia and local invasive properties of pannus (Muller-Ladner et al., 1998). Firestein et al have observed that long-term cultured RA FLS migrate *in vitro* into artificial matrices about four-fold more efficiently than do control ligament fibroblasts (Firestein, 1996).

Considerable evidence also suggests that FLS exhibit some characteristics of transformed cells, which have acquired amplified proliferative and invasive properties. In culture, RA FLS proliferate at a faster rate than cells derived from normal joints. Unlike normal fibroblasts which require adherence to a surface for growth, cultured RA FLS are not contact inhibited, and their growth is anchorage independent (Gay et al., 1989; Lafayatis et al., 1989a).

Recent studies show that the tumor suppressor protein, p53, is overexpressed in RA synovial intimal lining and in cultured RA FLS (Firestein et al., 1996). In subsequent studies, the somatic mutations of the p53 gene observed in RA synovium and cultured FLS (Firestein et al., 1997) have been previously identified in human neoplastic tissue (Beroud et al., 1996). It is therefore of great interest that that the proinflammatory cytokine macrophage migration inhibitory factor has the ability to functionally inactivate p53, which may provide a link between inflammation and tumorigenesis (Hudson et al., 1999; Chapter 6).

Induction or enhancement of FLS proliferation *in vitro* has been reported to be associated with IL-1β, IL-7, TNFα, and IFN-γ treatment. Conversely, exposure to IL-2, IL-3, IL-6, IL-8, GMCSF and granulocyte stimulating factor (GCSF) have not been observed to exert this effect (Alvaro-Garcia et al., 1990; Nishiya, 1994).

Cultured FLS from RA joints retain a profile of pro-inflammatory mediator production similar to those of freshly isolated specimens for multiple passages, an observation that argues in favour of the hypothesis these cells represent a differentiated disease-specific phenotype (Ritchlin et al., 1994). Activation of fibroblasts *in vitro* is known to generate several functional responses, such as production of prostanoids, cytokines and membrane metalloproteinases that could contribute significantly to joint pathology in RA. For example, high levels of prostaglandin E_2 (PGE_2) and inducible cyclooxygenase-2 (COX2) protein and gene expression are observed in IL-1β-stimulated cultured FLS from RA patients (Crofford et al., 1994). A more detailed discussion of FLS eicosanoid generation and its regulation by proinflammatory cytokines will be discussed at length in sections 1.2.4 and 1.2.5.

RA FLS release a number of cytokines and growth factors including IL-1β, IL-6, IL-16, GM-CSF, bFGF and TGF-β and MIF. IL-1 amplifies joint inflammation by inducing FLS to secrete IL-1, IL-6, IL-8 and monocyte chemoattractant protein-1 (Dalton et al., 1989; Guerne et al., 1989; Koch et al., 1991; Koch et al., 1992a). The naturally occurring and specific competitive inhibitor of IL-1, IL-1 receptor antagonist, inhibits IL-1-induced PGE_2 and collagenase production by cultured RA FLS (Arend et al., 1990; Seckinger et al., 1990). Long term constitutive IL-1β production by FLS may result in continuous autocrine stimulation of fibroblast mitogenesis, in addition to recruiting and activating infiltrating immune cells. Unlike TNFα or IL-1, IL-6 does not stimulate FLS production of metalloproteinase-1 (MMP-1) or PGE_2. It may be that IL-6 does not directly induce FLS production of factors induced by IL-1 but amplifies FLS responses to other stimuli. For example, IL-6 enhances the IL-1-induced FLS production of pro-stromelysin. FLS secrete GM-CSF on stimulation by IL-1 or TNFα (Alvaro-Garcia et al., 1989). The induction of basic fibroblast growth factor by rheumatoid synoviocytes may result in the neovacularisation and contribute to the proliferative and invasive properties of synovial tissue (Melnyk et al., 1990). Exogenous TGF-β also stimulates collagen transcription and inhibits collagenase mRNA levels in cultured FLS (Lafayatis et al., 1989b).

FLS are an important source of MMPs such as collagenase, stromelysin and cathepsins, which are believed to be major contributors of cartilage destruction in RA. Low level constitutive MMP expression has been described in resting FLS (MacNaul et al., 1990). Exposure to IL-1 or TNFα rapidly induces MMP gene expression and protein production, and activation (MacNaul et al., 1990; Kumkumian et al., 1989; Dayer et al., 1985).

The down regulation of MMPs, cytokines, prostaglandins and class II expression observed in long term culture, may reflect withdrawal from the cytokine inflammatory milieu. In the absence of other co-stimuli or cells, however, the ability of FLS to constitutively produce cytokines and growth factors for more than 12 weeks in culture highlights an important contribution of FLS to synovial inflammation (Bucala et al., 1991).

RA FLS also produce proteins that serve to downregulate the synovial inflammatory response, including TNFα soluble receptors and IL-1 receptor antagonist (IL-1RA) (Alvaro-Garcia et al., 1993; Taylor, 1994; Firestein et al., 1994).

A distinctive cell type was recently identified in the invasive pannus of RA synovium in the region of erosions. Similar cells were not found in OA lesions. These cells, designated as pannocytes, are believed to be either derived from FLS or have mesenchymal origin (Firestein, 1996). Pannocytes have a distinctive rhomboid morphology with the capacity to grow in culture for a prolonged time period without becoming senescent. Similarly to FLS, pannocytes express the major integrins and release metalloproteinases, with significantly higher levels of VCAM-1. Interestingly, like FLS, pannocytes produce type I collagen and do not express nitric oxide (Firestein, 1996; Zijlstra et al., 1997). It remains unclear whether pannocytes are derived from FLS or chondrocytes or whether they represent a truly separate cell lineage.

Studies in this thesis focus primarily on the function of FLS. The following sections will provide a brief overview of other cell types identified in RA synovium.

1.2.3.2 Macrophages

The predominance of macrophages and macrophage cytokine products within inflammatory synovium supports a central role for macrophages in RA pathogenesis.

Approximately 20% of RA synovial cells express macrophage surface markers including CD14, CD68 and CD11b (Firestein and Zvaifler, 1990). This population comprises a mixture of both macrophage-like synoviocytes and tissue macrophages present in the sublining layer. In RA, the mechanism underlying macrophage expansion is uncertain and may reflect migration from peripheral blood. Although studies in rat adjuvant arthritis have identified markers of synovial macrophage proliferation including PCNA in synovium (Santos et al., 1997b), studies in human RA have not produced evidence of *in situ* macrophage proliferation.

The ability of synovial macrophages to recognise and present antigen is facilitated by their expression of CD40, CD54, CD80, CD86, ICAM-3 and MHC class II molecules. Macrophages in RA synovial tissue are also characterised by increased expression of IL-1β and TNFα (Burmester et al., 1997). Activated synovial macrophages also exhibit increased transcription of many other cytokine genes including IL-6, IL-8, G-CSF and GM-CSF (Firestein and Zvaifler, 1990; Hahn et al., 1993; Seitz and Hunstein, 1985).

In RA, macrophages accumulate in the synovial intimal and subintimal layers and especially at the cartilage-pannus junction. At the cartilage-pannus junction in RA, macrophages produce matrix-degrading enzymes including collagenase, elastase, stromelysin and gelatinase (Bresnihan, 1999). However, the potential of macrophages to directly degrade cartilage may be modest in comparison to that of synovial fibroblasts (Tetlow et al., 1995; Tetlow et al., 1993; Jensen et al., 1991). Burmester et al suggest that the predominant role of synovial macrophages may be in amplifying the pathogenetic process as a result of cytokine production and that the fibroblast, by secreting proteolytic enzymes, may be the primary effector cell in bone and cartilage destruction (Burmester et al., 1997). In *in vitro* model systems, cartilage degradation

by co-cultures of mouse fibroblasts and macrophages greatly exceeded the degradation observed with each culture alone, suggesting that macrophage-derived cytokines drive the release of matrix metalloproteinases by synovial fibroblasts (Janusz and Hare, 1993).

Activated synovial macrophages are also an important source of reactive oxygen species and plasminogen activators (Fujii et al., 1990; Hamilton et al., 1991) and chemokines such as monocyte chemoattractant protein-1 (MCP-1) (Kunkel et al., 1996; Akahoshi et al., 1993). It is believed that expression of macrophage migration inhibitory factor (section 1.6) may act to prevent egress of synovial macrophages. Macrophages release platelet-derived growth factor that stimulates fibroblast growth factor and may contribute to the 'transformed' phenotype of synovial fibroblasts (Lafayatis et al., 1989a). IL-8 derived from synovial macrophages is a very potent promoter of angiogenesis (Koch et al., 1992b), as are the adhesion molecules VCAM-1 and E-selectin, both of which are upregulated by macrophage-derived TNFα (Koch et al., 1995).

An important role for macrophages in sustaining synovial inflammatory processes has emerged from several animal models of arthritis. For example, large numbers of macrophages are observed in synovial tissue derived from adjuvant, collagen, antigen-induced and SCW arthritis. The overexpression of TNFα in a transgenic mouse model leads to a chronic inflammatory and erosive arthritis that can be prevented by TNFα neutralisation (Keffer et al., 1991). Moreover, the administration of TNFα monoclonal antibodies either prior to or after the onset of animal arthritis led to an attenuation of the severity of inflammation and joint destruction (Thorbecke et al., 1992; Piquet et al., 1992; Williams et al., 1992). Cytokines such as IL-10 and IL-4 are potential inhibitors of IL-1 and TNFα production by macrophages in the synovial

tissue, and *in vivo* data suggest that these cytokines also reduce joint inflammation in animal models of arthritis (van den Berg W.B., 1997). Selective depletion of macrophages by local treatment with clodronate-containing liposomes prevents the onset of inflammation in animal models of disease (Van Lent et al., 1996). Evidence therefore supports a crucial role for macrophages and macrophage-derived products that are able to both perpetuate and amplify inflammatory processes in RA.

1.2.3.4 T cells

The synovial lining in RA is usually devoid of T cells. T cell infiltrates in the sublining rheumatoid synovium may exist as discrete lymphoid aggregates, or a diffuse infiltrate without organisation. T cells are also abundant in synovial fluid. In contrast the excess of CD4 T cells in synovial tissue, the number of CD4 and CD8 T cells in synovial fluid are approximately equal (Firestein, 1994).

The contribution of T cells to RA pathogenesis is still controversial. T cells constitute approximately 40% of synovial tissue cells (Firestein, 1994). In the sublining tissue of RA patients, the CD4 helper/inducer subset predominates, with a CD4/CD8 ratio between 4:1 and 14:1 (Firestein, 1994). The majority of these CD4 cells express CD45RO+, representing the mature memory cell phenotype (Panayi et al., 1992). A significant percentage of synovial T cells express CD25 (IL-2 receptor) and most express MHC class II, which suggests that these cells are activated (Forre et al., 1992). T cells in the RA synovium have been reported to mainly present a Th1 pattern of cytokine production (IL-2 and IFN-γ), and this profile has been confirmed in synovial fluid T cells of patients with (Dolhain et al., 1996). These findings are consistent with studies showing that Th1 cytokines are involved in the pathogenesis of autoimmune diseases, such as experimental autoimmune encephalomyelitis, whilst Th2 cytokines are thought to be protective. Other reports, however, suggest the

lymphokine profile in RA synovial tissue to be a combination of Th1 and Th2 (van der Lubbe et al., 1995).

The involvement of T cells in the initiation of RA remains controversial, despite evidence supporting T cell hyporeactivity in chronic disease. Synovial T cells exhibit only low level production of Th1 lymphokines such as IL-2 and IFN-γ (Hasler and Dayer, 1988; Howell et al., 1991; Chen et al., 1993). Mitotic figures are rare in rheumatoid synovial lymphoid aggregates and there is a lack of proliferation in the synovial lymphoid population (Verwilghen et al., 1990). The cause of this hyporeactive state is not yet fully elucidated (Panayi, 1997). In a T cell-independent model of synovitis, the severe combined immunodeficient (SCID) mouse develops an aggressive destructive arthritis after instillation of RA FLS into the joint (Muller-Ladner et al., 1991). Moreover, the induction of synovitis in the absence of T cells has been described in the MRL/l mouse model as well as the c-fos transgenic mouse model (O'Sullivan et al., 1985; Shiozawa et al., 1992). Therapies that specifically target T cells include total lymphoid irradiation and thoracic duct drainage. Interestingly, these therapies do not have the profound effects that would be anticipated, given the previously ascribed central role of T cells in RA pathogenesis. Conversely, quiescent RA can flare if the patient is administered human recombinant IL-2 for the treatment of malignant disease (Lavelle-Jones et al., 1990; Aarden et al., 1987) and cyclosporin does exert a therapeutic effect in RA, suggesting that activation of T cells can perpetuate disease. In contrast to this, invasive pannus can mediate tissue injury in an apparently autonomous fashion whilst T cells in close proximity appear relatively inactive. More recently, studies have shown that T cells activated by IL-15 induce macrophage TNFα release via a cell contact dependent mechanism involving T cell membrane bound molecules CD69 and ICAM (McInnes et al., 1997;

McInnes and Liew, 1998). These findings may explain how synovial T cells, despite their relative quiescence, may participate in the inflammatory processes.

1.2.3.5 Neutrophils

The correlation between neutrophil activity and the development of disease has suggested a critical role for these cells in RA (Leirisalo-Repo et al., 1993). Compared to their abundance in synovial fluid, there are relatively few neutrophils in chronically inflamed synovium (Leirisalo-Repo, 1994). Neutrophils in synovial fluid may also contribute significantly to cartilage damage observed in RA. Neutrophils are activated by synovial fluid GM-CSF, IL-8 and immune complexes, to release myeloperoxidase, MMPs and oxygen radicals. Moreover, the neutrophil chemotactic response and generation of oxygen radicals are associated with erosions in early RA (Leirisalo-Repo et al., 1993). Adhesion of human RA FLS to cartilage is notably enhanced by pre-treatment with neutrophil proteases (McCurdy et al., 1995). This finding suggests that neutrophils contribute both directly and indirectly to pannus invasion and chondrocyte damage. Recent studies show that PMN are capable of contributing to immune and inflammatory response via the production of cytokines including IL-1β, IL-6 and TNFα, the chemokines IL-8 and MIP-1α, and growth factors such as M-CSF and G-CSF (Cassatella, 1995). They may also act as accessory cells in the immune response by upregulation of surface MHC class II molecules in response to IFN-γ and G-CSF (Gosselin et al., 1993).

Animal models of disease, such as carageenan-induced arthritis, also exemplify the capacity of neutrophils to initiate joint inflammation (Yang et al., 1997). In adjuvant arthritic rats, the relative lack of neutrophils in synovial tissue compared with the abundance in synovial fluid closely reflects human disease. The observation that monoclonal antibody-mediated depletion of neutrophils significantly inhibits rat

adjuvant arthritis despite the relative lack of neutrophils in synovial tissue in established disease (Santos et al., 1997a) supports their importance in disease pathogenesis.

1.2.3.6 Other cells

Dendritic cells are readily identifiable in synovial effusions of patients with RA (Zvaifler et al., 1985). Dendritic cells are characterised by the presence of multiple, long, dendritic, cytoplasmic processes. These professional antigen-presenting cells express MHC class II antigen. They are non-phagocytic and are distinct from macrophages in their lack of Fc receptors and surface CD14 (Tsai and Zvaifler, 1988). Dendritic cells lack conventional cytoplasmic enzymes such as myeloperoxidase and non-specific esterase (Firestein et al., 1987). Surface markers such as RFD1 are thought to be specific for dendritic cells (Poulter et al., 1986). Dendritic cells are likely to be derived from peripheral blood monocytes, although cells expressing a dendritic cell profile of surface antigen expression can be identified in peripheral blood.

Thomas et al, have recently characterised antigen-presenting CD14/CD33 positive dendritic cells in rheumatoid synovium (Thomas et al., 1994). Their contribution in the perpetuation of RA synovial inflammation is still under investigation.

B cells and plasma cells infiltrate the subintimal lining region of RA synovial tissue. B cells synthesising type II collagen antibodies and rheumatoid factors are readily detected (Lydyard and Edwards, 1994; Firestein, 1994). Distinct from other B cells, a significant percentage of RA derived B cells express the surface antigen CD5. Circulating CD5 positive cells are elevated in a variety of autoimmune diseases and are associated with the generation of autoantibodies, including rheumatoid factors (Firestein, 1994). The expression of surface MHC class II on B cells indicates that

these cells are also capable of antigen presentation (Lydyard and Edwards, 1994; Firestein, 1994). The contribution of B cells in antigen presentation in RA, however, remains controversial.

Mast cells are able to secrete eicosanoids, MMPs and vasoactive and chemotactic molecules (Firestein, 1994). The presence of mast cell growth factors in synovial fluid may relate to the local accumulation of small numbers of mast cells in rheumatoid synovium (Firestein et al., 1988). Despite small numbers of mast cells in RA synovium, there are significantly more mast cells in joint samples of RA patients compared to controls (Godfrey et al., 1984; Okada, 1973). Whether the increased numbers of mast cells in RA contributes to the cause or an effect of the inflammatory process remains to be elucidated.

Natural killer cells are present in very small numbers in rheumatoid synovial fluid and are distinct from the conventional NK cells found in blood (Goto and Zvaifler, 1985). Despite the relative lack of natural killer cells in synovial inflammation (Fox et al., 1984), the observation that natural killer cells mediate antibody-dependent cellular cytotoxicity of certain immune responses may be of significance to synovial inflammation.

Having described aspects of cellular constituents relevant to RA pathology, the following section will review the involvement of cell-derived products to RA pathogenesis.

1.2.4 Eicosanoids

1.2.4.1 Prostaglandins and RA

Eicosanoids are potent, biologically active compounds of the cellular microenvironment. In general, eicosanoids have significant net proinflammatory effects and are therefore known as lipid mediators of inflammation.

Oxidative metabolism of polyunsaturated fatty acids generates the major lipid mediators of inflammation, including arachidonic acid metabolites, prostaglandins (prostaglandin E_2: PGE_2, prostaglandin I_2: PGI_2, prostaglandin $F_{1\alpha}$: $PGF_{1\alpha}$, prostaglandin $F_{2\alpha}$: $PGF_{2\alpha}$), leukotrienes (leukotriene B_4: LTB_4, leukotriene C_4: LTC_4, leukotriene D_4: LTD_4, leukotriene E_4: LTE_4), thromboxane A_2 (TxA_2) and platelet-activating factor (PAF). Many inflammatory stimuli can activate cells to produce PGE_2, as will be discussed below.

Prostaglandin E_2 is found in biologically significant concentrations in patients with RA and is the principle eicosanoid produced by RA FLS. In synovial tissue from patients with RA, PGE_2 release is significantly increased compared to levels from cartilage or bone (Wittenberg et al., 1993). Prostaglandin E_2 is believed to exert predominantly proinflammatory effects in RA, including the evocation of pain and stimulation of bone resorption in osteoclasts, and is a required cofactor for the induction of vaso-permeability. PGE_2 also exhibits anti-inflammatory effects in that it inhibits many of the proinflammatory activities of neutrophils and suppresses macrophage and monocyte production of IL-1 and TNFα. NSAIDs act primarily by inhibiting COX, thereby preventing the formation of prostaglandin in normal and inflamed tissues (Needleman and Isakson, 1997).

Prostaglandins (PGs) are synthesized in a broad range of tissue types and serve as autocrine or paracrine mediators to signal changes within the immediate environment. Prostaglandin receptors, which transduce signals following ligand binding, consist of two classes: a G-coupled cytoplasmic receptor class and nuclear PPAR receptor class

(Forman et al., 1996). Prostaglandins exert a variety of physiological effects that are involved in inflammatory pain and symptoms. Prostaglandins cause vasodilation, increase vascular permeability, and sensitise pain fibres to algesic stimuli (Davies et al., 1984; Vane and Botting, 1994).

Recent studies suggest that PGE_2 is the arachidonic acid (AA) metabolite necessary and sufficient for inflammation. Investigations utilising a specific neutralising mAb to PGE_2, 2B5, supports a role for PGE_2 in inflammation and inflammatory pain (Munich et al., 1995; Portanova et al., 1996). In the rat, anti-PGE_2 mAb inhibited carrageenan-induced edema as effectively as the NSAID indomethacin (Portanova et al., 1996). In the same study, the effect of indomethacin was completely reversed by co-administration of PGE_2 and carrageenan.

Animal models of disease further exemplify a critical role for PGE_2 in chronic inflammation (Portanova et al., 1996). In a rat model of adjuvant arthritis, administration of complete Freund's adjuvant into the paws elicits chronic inflammatory arthritis, accompanied by significant increase in local PG production (Anderson et al., 1996). Anti-PGE_2 mAbs were observed to inhibit arthritis as effectively as NSAID (Portanova et al., 1996). These findings suggest a critical role for PGE_2 in the development of arthritis, and identify PGE_2 as the predominant eicosanoid involved in inflammatory processes. The results also infer that the analgesic and anti-inflammatory effects of NSAIDs are likely to result from the capacity of these agents to suppress the production of PGE_2.

Proinflammatory mediators associated with inflammation, including IL-1 and LPS, have been found to increase PG production *in vitro* (Roshak et al., 1997; Mino et al., 1998; Wilborn et al., 1995; Smeets et al., 2000). Moreover, stimulation with these mediators, in RA FLS and other cell types, are observed to induce the expression of

COX2 and subsequently produce large amounts of PGE_2 at the inflammatory site (Wilborn et al., 1995; Hulkower et al., 1994; Xie et al., 1992; Lee et al., 1992; Maier et al., 1990). In contrast, IL-4, which inhibits the production of pro-inflammatory cytokines such as IL-1, was seen to inhibit the production of PGE_2 in RA synovial cells via suppression of COX2 mRNA synthesis. In this study, the effect of IL-4 on COX2 mRNA was restricted to IL-1β-stimulated macrophages.

Conversely, evidence from a number of *in vitro* studies report the anti-inflammatory activity of annexin I to be eicosanoid-mediated. For example, several studies have implicated annexin I in glucocorticoid-suppression of PGE_2 release from macrophages (Flower, 1988) and the A549 human adenocarcinoma cell line (Croxtall and Flower, 1992; Croxtall and Flower, 1994). Moreover, transfection of A549 cells with anti-sense DNA for an N-terminal region of annexin I not only blocks glucocorticoid suppression of PGE_2 release, but also prevents the glucocorticoid-induced cell surface expression of newly synthesised annexin I (Croxtall and Flower, 1994). The results of these studies will be discussed further in Chapter 3.

1.2.4.2 The prostaglandin synthetic pathway: phospholipases (PLA_2s)

Arachidonic acid (AA; 5,8,11,14-*cis*-eicosatetraenoic acid) is a polyunsaturated fatty acid found abundantly in phospholipids of cell membranes (Davies et al., 1984; Bonventre, 1992; Murakami et al., 1997). Free AA may be liberated either directly by phospholipase A_2 (PLA_2) or indirectly through the sequential action of phospholipase C and diacylglycerol lipase (Davies et al., 1984; Bonventre, 1992). Metabolism of AA via the COX pathway involves a bis-dioxygenation reaction to produce the unstable endoperoxide intermediate prostaglandin G_2 (PGG_2). The subsequent reduction of PGG_2 by a hydroperoxidase then forms a second unstable endoperoxide intermediate, prostaglandin H_2 (PGH_2). PGH_2 breaks down non-enzymatically, resulting in the

production of prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2). Additionally, prostaglandin endoperoxides may be metabolised by prostaglandin I_2 (PGI_2) synthase or by thromboxane A_2 (TXA_2) synthase to form PGI_2 or TXA_2. Via non-enzymatic reactions, these unstable intermediates break down rapidly to produce 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2, respectively. Alternatively, lipoxygenase enzymes may metabolise AA to produce hydroperoxyeicosatetraenoic acids (HPETEs) or leukotrienes (Bonventre, 1992; Murakami et al., 1997).

Phospholipase A_2 can be classified into several groups, including cytosolic ($cPLA_2$), secretory ($sPLA_2$), Ca^{2+}-independent ($iPLA_2$) and platelet activating factor-acetylhydrolases (PAF-AH). In accordance with their primary structures, $sPLA_2$ have been further subdivided into two main groups, type I and type II. Cytosolic PLA_2 (or type IV PLA_2) is an important PLA_2 isoenzyme that regulates lipid mediator generation, resulting from cell activation (Dennis, 1994; Kudo et al., 1993; Mayer and Marshall, 1993; Clark et al., 1995). In most cell types $cPLA_2$ is constitutively expressed, and it is also noteworthy that Type II $sPLA_2$ has been detected in a variety of inflammatory conditions and sites, such as human rheumatoid arthritis (Kramer et al., 1989; Seilhamer et al., 1989, Hara et al., 1989, Hara et al., 1988). Investigations that explore the role of group II $sPLA_2$ in inflammatory processes will be discussed below.

In 1991, the cDNA for human $cPLA_2$ was cloned (Clark et al., 1991; Sharp et al., 1991). Currently, $cPLA_2$ is the only cloned isoenzyme of PLA_2 identified that exhibits marked selectivity to arachidonate-containing phospholipids. Cytoplasmic PLA_2 encodes an 85 kDa protein that lacks a signal sequence, and homology with $sPLA_2$s. Moreover, the deduced sequence is encoded by a 3.4 kb mRNA that does not appear to be a member of any closely related gene family. More recently, the $cPLA_2$ cDNAs

in a variety of animals including the mouse, rat, chicken and zebrafish, were cloned (Nalefski et al., 1994). Approximately 94% sequence homology exists between human and mouse $cPLA_2$. The structure of $cPLA_2$ consists of an NH_2-terminal domain that contains a Ca^{2+}-dependent lipid-binding (CaLB) domain that is responsible for the translocation of $cPLA_2$ from the cytosol to the membrane. Several proteins show significant homology with this domain, such as protein kinase C (PKC), GTPase-activating protein, synaptotagmin and phospholipase C. Despite the lack of sequence homology between $cPLA_2$ and $sPLA_2$s, a four amino acid motif (Gly-Thr-Leu-Tyr) exists in the catalytic domains of $cPLA_2$ and $sPLA_2$. Moreover, the domain between the CaLB and catalytic domain contains a MAP kinase phosphorylation consensus sequence around Ser^{505}. Phosphorylation of this serine residue increases the intrinsic activity of $cPLA_2$ severalfold and may change the conformation of $cPLA_2$ (Lin et al., 1993; Nemenoff et al., 1993).

The human $cPLA_2$ gene is located on the q arm of chromosome 1 (Miyashita et al., 1995). The 5' flanking region has properties consistent with housekeeping genes in that it lacks a TATA or CAAT box, but is atypical given that it is not GC-rich, has no SP1 sites, and contains a long stretch of CA repeats. In the promoter region of the $cPLA_2$ gene, three potential AP-1 binding sites, two PEA3 motifs, a putative glucocorticoid response element, and an asymmetric NFκB site are found (Miyashita et al., 1995; Tay et al., 1994b). The 5' flanking region of Hep-2 cells, however, failed to respond to TNFα or glucocorticoids with increased expression of the reporter gene (Tay et al., 1994b), suggesting that additional regulatory elements are required for effective upregulation of the $cPLA_2$ transcript. In the 3' untranslated region of $cPLA_2$ cDNA, the presence of three ATTTA motifs, suggests that the turnover of $cPLA_2$ is rapid (Tay et al., 1994b).

Moreover, in spite of $cPLA_2$ transcript levels subject to modulation in some cell lines (Tay et al., 1994a; Nakamura et al., 1992; Hoeck et al., 1993; Chepenik et al., 1994; Schalkwijk et al., 1993; Wu et al., 1994; Roshak et al., 1994; Angel et al., 1994; Maxwell et al., 1993), the effects observed are relatively small and cell-type specific. Extensive data also indicates that the $cPLA_2$ protein is subject to post-transcriptional regulation of expression and post-translational regulation of enzyme activity (see below). Collectively, these findings suggest that the $cPLA_2$ promoter provides constitutive expression of $cPLA_2$ protein that is also capable of rapidly responding to physiological stimuli and various second messengers.

Unlike the Ca^{2+}-independent hydrolysis of monomeric phospholipids by $cPLA_2$ (Nalefski et al., 1994; Huang et al., 1994), submicromolar concentrations of Ca^{2+} are required for $cPLA_2$ activity toward phospholipid vesicles, given that the affinity of the CaLB domain for the membrane surface increases when the Ca^{2+} concentration is increased within the physiological range (100 nM to 1 μM) (Clark et al., 1991). It is suggested that the increased Ca^{2+} concentration acts to colocalize both the membrane substrate and catalytic site of $cPLA_2$. For instance, a $cPLA_2$ mutant lacking a CaLB domain failed to exhibit catalytic activity toward phospholipid liposomes, but demonstrated full enzyme activity when monomeric phospholipid was used as the substrate (Nalefski et al., 1994).

As mentioned earlier, $cPLA_2$ is constitutively expressed in most cell types. Increased PLA_2 activity with arachidonic acid selectivity, in response to IL-1β stimulation, was originally described in fibroblasts (Hulkower et al., 1992) and Hep-2 cells (Goppelt-Struebe and Rehfeldt, 1992). Following molecular cloning of the $cPLA_2$ gene, Lin et al observed a marked increase in $cPLA_2$ protein levels in IL-1α stimulated WI-38 fibroblasts (Lin et al., 1992). Similarly in rat mesangial cells, $cPLA_2$ levels were

increased by IL-1 (Schalkwijk et al., 1993; Schalkwijk et al., 1992). In human synovial cells, increased $cPLA_2$ expression almost completely paralleled the *de novo* induction of COX2 (Angel et al., 1994). Moreover, studies by Roshak et al confirm reports that stimulation of FLS with IL-1β upregulates both $cPLA_2$ and COX2 and results in the accumulation of PGE_2 (Roshak et al., 1996). In this study, exposure of RA FLS to $cPLA_2$ anti-sense oligonucleotides decreased IL-1β upregulation of $cPLA_2$ activity and protein. In addition, the effects on $cPLA_2$ were observed to correlate with reduced PGE_2 formation, in a concentration-dependent manner. The anti-sense effect was specific, in that it had no effect on $sPLA_2$ activity or IL-1β upregulation of COX2 protein levels.

In human monocytes, exposure to LPS also increases $cPLA_2$ expression. In these cells, the half-life of $cPLA_2$ was approximately 4 hours, indicating a rapid turnover of the $cPLA_2$ protein. The expression of $cPLA_2$ induced by pro-inflammatory stimuli was believed to depend on the presence of a NFκB-regulatory element in the 5'-flanking region of the $cPLA_2$ gene (Moskowitz et al., 1982; Tay et al., 1994b).

In cells induced by pro-inflammatory stimuli, glucocorticoids are potent suppressors of the upregulation of $cPLA_2$. Hep-2 cells provided the first evidence of the inhibitory effect of dexamethasone on $cPLA_2$ activity (Goppelt-Struebe and Rehfeldt, 1992). Subsequent investigations observed the capacity of dexamethasone to suppress $cPLA_2$ accumulation in a variety of cytokine-induced cells (Lin et al., 1992; Hoeck et al., 1993; Schalkwijk et al., 1993; Nakatani et al., 1994). In these studies, dexamethasone exerted no effect on basal $cPLA_2$ expression levels, but attenuated its increased expression after cytokine induction. Sensitivity to glucocorticoids is consistent with the presence of a glucocorticoid response element in the promoter region of the

cPLA$_2$ gene (Miyashita et al., 1995; Tay et al., 1994b) and to the involvement of NFκB.

To date, few investigations have explored the role of glucocorticoids on cPLA$_2$ in animal models of arthritis. Studies presented in Chapters 3,4 and 6 examine the effects of glucocorticoids, and two glucocorticoid-inducible proteins, annexin I and MIF on cPLA$_2$ enzyme activation and expression in human arthritic synoviocytes. The role of type II sPLA$_2$ in local processes, however, has been studied by injecting purified or recombinant type II sPLA$_2$ into sites, including the skin (Pruzanski et al., 1986), subcutaneous air pouch (Cirino et al., 1994), lung (Edelson et al., 1991), and joints (Vadas et al., 1989; Bomalaski et al., 1991; Vishwanath et al., 1988) of experimental animals. For example, injection of recombinant human type II sPLA$_2$ into a subcutaneous air pouch, where the lining resembles the synovial membrane, in the rat induces an inflammatory process resembling rheumatoid arthritis in humans (Cirino et al., 1994). Injection of purified native type II sPLA$_2$ into the joints of rats (Vadas et al., 1989) and rabbits (Bomalaski et al., 1991) induces an inflammation that is exudative in the early stages and proliferative in the later stages. Injection of type II sPLA$_2$ into the hind paws of healthy rats does not cause inflammation, whereas this treatment exacerbates oedema of inflamed paws of rats with adjuvant arthritis (Murakami et al., 1990). Moreover, sPLA$_2$ deficient rats do not show impaired or altered inflammatory responses and overexpression of sPLA$_2$ in transgenic mice is not accompanied by increased inflammation. In contrast with TNFα transgenic mice, however, a co-transgenic mouse with dysregulated expression of TNFα and sPLA$_2$ results in increased synovial inflammation (Bomalaski and Clark, 1993).

An important mechanism for the anti-inflammatory effects of glucocorticoids involves the inhibition of arachidonic acid release by sPLA$_2$. In adrenalectomised

(ADX) rats, for example, group II $sPLA_2$ mRNA was significantly increased in lung tissue of ADX rats, compared to group I $sPLA_2$, and was found to correlate with increased group II $sPLA_2$ protein in lung, spleen, liver, and kidney. This increase was reversed by the administration of exogenous corticosterone. Following ADX, total $sPLA_2$ activity was significantly greater. In addition, the concentration of annexin I strongly correlated with the $sPLA_2$ activity in the lung, spleen, liver, and kidney. Moreover, the concentrations of annexin I in all these tissues declined following ADX. The results from this study suggest that the upregulation of $sPLA_2$ and downregulation of annexin might account for the enhanced inflammatory response in hypoglucocorticoid states (Vishwanath et al., 1993). Further evidence implicating annexin I in the inhibition of PLA_2 activity in a variety of *in vitro* systems will be presented in section 1.5.5 and Chapter 3.

1.2.4.3 The prostaglandin synthetic pathway: cyclooxygenases (COX)

In the last decade, one of the most important advances in prostaglandin biochemistry has been the discovery that cyclooxygenase (COX) exixts as two isoforms. The isoforms COX1 and COX2 have been cloned and characterised. Human COX1 cDNA encodes a 599 amino acid protein, whereas COX2 cDNA encodes a 604 amino acid protein. Sequence analysis reports approximately 60% identity between these COX isoforms. COX1 and COX2 genes are located on chromosomes 9 and 1, respectively (Kraemer et al., 1992; Fletcher et al., 1992). The COX isoforms exhibit identical intron/exon arrangements. However, the translational start site and signal peptide that are contained in a single exon for COX2 are located on exons 1 and 2 in COX1. Characteristic of rapidly degraded RNA, the COX2 gene is relatively smaller in size (8 kb) compared to the COX1 gene (22 kb). In common with other 'housekeeping' genes, the COX1 gene is continuously transcribed, stably expressed and lacks both

TATA and CAAT elements in its promoter region (Smith and DeWitt, 1995). Morevoer, COX1 mRNA and protein are expressed in most tissues under basal conditions (Wang et al., 1993). In some cell types, increased COX1 levels are found to be associated with differentiation (Smith et al., 1993). In differentiated tissues however, the levels of COX1 mRNA and protein do not vary considerably in response to external stimuli. COX1 is therefore responsible for the generation of prostaglandins that regulate important physiologic processes such as gastrointestinal cytoprotection, vascular homeostasis and renal function.

Consistent with highly regulated genes involved in inflammation, the COX2 promoter contains TAAT and CAAT elements, in addition to a number of transcriptional elements such as nuclear factor kappa B (NFκB) and the cyclic AMP response element binding protein (Appleby et al., 1994). COX2 mRNA contains an extensive 3' untranslated region that consists of multiple 'AUUUA' instability sequences and several different polyadenylation signals involved in the rapid degradation of the transcript following induction (Ristimaki et al., 1994). Moreover, the notion that COX2 is an 'immediate-early' gene product that is rapidly upregulated during inflammatory processes appears to be consistent with its gene structure.

Despite differences in the structure and regulation of the COX genes at the level of DNA and RNA, the protein structure and enzymatic functions of the COX isoforms are highly conserved. The major differences in sequence are at the N-terminal where COX2 has 17 less amino acids in the signal peptide and at the C-terminal where COX2 has 18 more residues than COX1. Overall, the structures of COX1 and COX2 exhibit high sequence identity and the residues crucial for catalytic function are highly conserved (Smith et al., 1995).

Both COX isoforms have been crystallised and the crystal structures are highly homologous (Picot et al., 1994). Although the enzyme structure and function of the COX1 and COX2 are highly conserved, it has been suggested that these enzymes may participate in different enzyme systems. It has been reported that both COX isoforms are present within the phospholipid bilayers of the endoplasmic reticulum and nuclear envelope (Morita et al., 1995; Spencer et al., 1998), which may be of relevance given that COX is one of the key enzymes involved in PGE_2 production.

Another major difference between COX1 and COX2 appears to be in their ability to utilise alternative arachidonic acid substrate pools that become mobilised in response to inflammatory stimuli (Reddy and Herschman, 1994; Murakami et al., 1994). For example, in both fibroblasts and immune cells COX2 was able to utilise endogenous AA whereas COX1 was not. In these systems, COX1 requires endogenous AA (Herschman, 1996).

In most rodent tissues, COX1 expression has been detected under basal conditions, using Northern blot analysis, immunohistochemical localisation and *in situ* hybridisation. The presence of COX1 in the gastroinstestinal tract, platelets, endothelial cells, in the renal medullary collecting ducts and interstitium may be relevant to its physiological functions (Smith and DeWitt, 1995; Seibert et al., 1994). Under basal conditions, COX2 exhibits restricted expression, with highest levels observed in the brain, vas deferens and renal cortex (DuBois, et al., 1998).

Upregulation of COX2 expression is observed in response to a number of proinflammatory cytokines, mitogens and endotoxins (Crofford et al., 1994, Hulkower et al., 1994; Angel et al., 1994; Fletcher et al., 1992; O'Sullivan et al., 1992; Chapter 5). Studies also report the ability of glucocorticoids to inhibit cytokine-

induced COX2 expression in a variety of cells and tissues (Crofford et al., 1994; Kujubu et al., 1991; Xie et al., 1991; O'Banion et al., 1991; Chapter 5).

Prior to molecular cloning of COX2, Masferrer et al observed upregulation of COX activity by endotoxin (Masferrer et al., 1990) and inhibition by glucocorticoids both *in vivo* and *in vitro* (Masferrer et al., 1992; Masferrer et al., 1994). Increased understanding of COX2 expression has emerged from animal models of acute and chronic inflammation. For example, increased COX expression was observed in the inflamed paw tissue of Lewis rats with adjuvant induced- or streptococcal cell wall arthritis (Sano et al., 1992). Within the joint and surrounding tissue, COX expression was observed in synovial lining cells, sublining FLS, vascular endothelial cells, chondrocytes, osteoblasts, infiltrating mononuclear inflammatory cells and adjacent bone marrow. COX expression was notably reduced following treatment with dexamethasone. In spite of the marked increase in COX expression, no increase in COX1 mRNA was observed. These findings, in addition to the observed glucocorticoid inhibition of COX immunostaining suggests that COX2 expression largely accounts for the increased COX expression *in vivo*.

In other studies, the contribution of specific COX isoforms to inflammation was evaluated using rats with adjuvant-induced arthritis (Anderson et al., 1996). Anderson et al observed that COX2 mRNA, rather than COX1 mRNA, increased with or just prior to paw swelling. In the arthritic paws, upregulation of COX2 mRNA was associated with increased COX2 protein and PGE_2 production. Adminstration of a selective COX2 inhibitor significantly suppressed paw oedema in the arthritic animals, and reduced tissue PGE_2 to baseline levels. The anti-inflammatory effects of the COX2 inhibitor were indistinguishable from the observed effects of indomethacin.

This level of suppression was markedly enhanced in adjuvant arthritic animals treated with dexamethasone.

In early studies, examination of synovial tissue in RA patients revealed intense COX expression, compared to synovial tissue of patients with osteoarthritis or trauma injury. In RA synovial tissue, COX immunostaining was localised to the synovial lining layer, subsynovial synoviocytes, vascular endothelial cells, and infiltrating mononuclear cells. In this study, the level of COX expression was found to parallel the development of clinical disease and correlate with synovial mononuclear cell infiltration in experimentally induced arthritis. In osteoarthritic synovial tissues, COX immunostaining was less intense than RA tissues. In synovial tissues of patients with traumatic injury, low levels of COX staining were detected (Sano et al., 1992). Greater variability in immunohistochemical staining was observed when utilising an antibody that recognised the unique carboxy terminal peptide of human COX2, compared to using the polyclonal anti-COX antisera (Sano et al., 1992; Crofford et al., 1994). Little staining was detected in the synovial lining layer and sublining synoviocytes, although staining was detected in infiltrating mononuclear cells and vascular endothelial cells. Moreover, COX1 and COX2 mRNA was detected in synovial tissue of RA patients, with lower levels of COX2 mRNA observed (Crofford et al., 1994).

Increased understanding of mediators that regulate COX expression has been provided by *in vitro* systems that utilise cultured human synovial tissues.

COX1 and COX2 were expressed in synovial explant tissues including FLS, MLS, endothelial cells and mononuclear cells. Expression of COX2 was markedly increased in cells exposed to IL-1β or phorbol ester (PMA). Moreover, pretreatment with dexamethasone eliminated basal and induced COX2 expression without modulating

the level of COX1 expression (Crofford et al., 1994). In cultured primary RA FLS, COX2 was expressed at low baseline levels, but was significantly increased following exposure to IL-1β or PMA. Moreover, complete inhibition of COX2 expression was observed in IL-1 induced cells that were pre-treated with dexamethasone (Crofford et al., 1994; Hulkower et al., 1994). In other studies, IL-4 inhibited the production of PGE_2 in freshly isolated synovial cells and antagonised IL-1β and LPS stimulated increases in COX2 but not COX1 levels (Sugiyama et al., 1995). In the synovial joint, COX2 expression and regulation is also observed in cell types including macrophages, endothelial cells, chondrocytes and osteoblasts. *In vitro* evidence suggests that COX2 regulation is similar in most cell types (Ristimaki et al., 1994; O'Sullivan et al., 1992; Wilborn et al., 1995; Geng et al., 1995; Picot et al., 1994). *In vitro* studies also confirm the involvement of anti-inflammatory cytokines in the supression of COX2 expression. In cultured murine osteoblasts, for example, both IL-4 and IL-13 inhibited IL-1 induced COX2, but not COX1, mRNA expression and PGE_2 production (Onoe et al., 1996). Taken together, the available data suggest that COX2 is a critically important enzyme in PGE_2 production in RA.

1.2.5 Other soluble mediators

1.2.5.1 Cytokines

In RA synovium, analysis of cytokine mRNA and protein reveals proinflammatory cytokines, including TNFα, IL-1, IL-6, GM-CSF, and chemokines such as IL-8 to be abundant in all patients regardless of therapy (Szekanecz et al., 1998; Feldmann et al., 1996b). Cytokines derived from T-cells, including IL-2, IL-3, IL-4 and IFN-γ are less readily detectable at the mRNA and protein level (Firestein et al., 1990). The local

and systemic production of these cytokines may account for many of the pathologic and clinical manifestations of RA.

Interleukin-1β and TNFα are among the major proinflammatory cytokines. They exert overlapping effects in RA by inducing other cytokines, as well as PGE_2 generation, cartilage destruction, bone resorption and angiogenesis. Elevated levels of IL-1β, TNFα and IL-6 have been detected in serum and synovial fluid of patients with RA (Arend and Dayer, 1995; Eastgate et al., 1988; Kahle et al., 1992; Tetta et al., 1990; Saxne et al., 1988; Yocum et al., 1989; Manicourt et al., 1993; Houssiau et al., 1988; Arvidson et al., 1994). In animal models of arthritis, intra-articular injection of IL-1β or TNFα induces synovitis leading to eventual cartilage destruction (Chandrasekhar et al., 1990; Staite et al., 1990; Brahn et al., 1992; Cooper et al., 1992).

The ability of IL-1 to initiate synthesis of PGE_2 is perhaps one of the most important biological properties of IL-1, accounting for many local and systemic effects, including bone resorption in which PGE_2 is implicated (Klein and Raisz, 1970). Furthermore, IL-1 may enhance the RA inflammatory process by stimulating the production of the proinflammatory mediator of angiogenesis, IL-8, from synovial fibroblasts and macrophages (Postlethwaite et al., 1988). In antigen-induced arthritis in mice, IL-1 blockade prevented both inflammation and cartilage destruction (Tyler and Benton, 1988). In streptococcal cell wall-induced arthritis in rats, IL-1RA ameliorated the inflammatory effects of IL-1 (Schwab et al., 1991). Also, glucocorticoids inhibit IL-1 production by monocytes and macrophages at the transcriptional and post-translational levels (Beutler et al., 1986; Wicks et al., 1994). The effect of glucocorticoids on RA FLS will be examined in more detail in Chapter 3.

Tumor necrosis factorα also plays a pivotal role in RA pathogenesis. Tumor necrosis factorα stimulates fibroblast proliferation and the production of PGE_2 and collagenase (Dayer et al., 1985). It is believed that TNFα may also enhance neovascularization via its abiltiy to increase bFGF from synovial cells (Takahashi et al., 1991). This cytokine may also lead to the production of other cytokines such as IL-1, and there is evidence that TNFα is the dominant signal regulating IL-1 production (Brennan et al., 1991). Indeed, blockade of TNFα with specific neutralising antibodies inhibited the spontaneous release of IL-1 (Brennan et al., 1989) and GM-CSF (Haworth et al., 1991) in cultured RA synovial cells. Tumor necrosis factorα transgenic mice spontaneously develop a destructive arthritis. Disease in these animals is preventable upon treatment with anti-TNFα (Keffer et al., 1991). Similarly, collagen induced arthritis in mice is ameliorated when these animals are given anti-TNFα either before or after the onset of disease (Thorbecke et al., 1992; Piquet et al., 1992; Williams et al., 1992). Lastly, collagen-induced arthritis was also reduced in frequency and severity by the infusion of recombinant TNFα receptors, given either before the onset of or during the course of the disease (Wooley et al., 1993). This finding of TNFα dominance in the RA cytokine profile led to the initiation of successful clinical trials of anti-TNFα Ab in human RA (Feldmann et al., 1996a). Tumor necrosis factorα is a major stimulator of bone resorption (Lorenzo et al., 1987).

Both IL-1 and TNFα induce the synthesis and secretion of IL-6 and there is evidence from experimental models of arthritis that the production of TNFα precedes the production of IL-6 (Wong and Clark, 1988). In addition, IL-6 induction may contribute to damage to the cartilage matrix (Bender et al., 1990), may amplify the effects of IL-1 and TNFα and induce the synthesis of acute phase reactants and

rheumatoid factors (Arend and Dayer, 1990). Treatment of RA patients with anti-TNFα antibodies has recently been shown to reduce significantly serum levels of IL-6, which were elevated before treatment in most patients (Elliot et al., 1994).

MIF is a proinflammatory cytokine identified in a range of cell types. Its broad range of actions, its relationship with TNFα, its contribution to animal arthritis and potential role in human RA and RA FLS inflammatory processes will be discussed in detail in this thesis (sections 1.6.4, 1.6.5, 1.6.4.3 and 1.6.5.1, Chapters 4-6).

The experimental focus of this thesis primarily concerns IL-1 and MIF. A complete exposition of the data arising from the study of other cytokines relevant to RA is beyond the scope of this thesis.

1.2.5.2 Matrix metalloproteinases

The matrix metalloproteinases (MMPs), particularly collagenase, stromelysin and gelatinase are believed to be among the agents responsible for cartilage breakdown and bone erosion in RA. In response to cytokines, growth factors and reactive oxygen species, these proteolytic enzymes are released in the inflamed joint by synovial fibroblasts, chondrocytes and leukocytes (Walakovits et al., 1992; Oleksyszyn and Augustine, 1996). Collagenase and stromelysin are highly expressed in the synovium and cartilage of RA and OA patients, and in these tissues in animal models of arthritis (Wolfe et al., 1993). Moreover, stromelysin mRNA and proteins levels are higher in RA synovium compared with osteoarthritis synovium (Wolfe et al., 1993). Increased stromelysin and collagenase have been described in rheumatoid synovial fluid and in rheumatoid synoviocyte supernatants, and their enzyme activity was observed to correlate with disease severity. Furthermore, explants from rheumatoid synovial tissue have been found to release large amounts of metalloproteinases (Walakovits et al., 1992; Clark et al., 1993; Manicourt et al., 1995). The importance of RA FLS in

cartilage degradation is highlighted in animal models of arthritis (Geiler et al., 1994). In one study, RA FLS and macrophages were co-cultured with radiolabelled human cartilage. Intensive cartilage degradation by RA FLS was observed after co-cultivation with macrophages, presumably mediated by proinflammatory cytokines such as IL-1β and TNFα. Interestingly, direct contact of FLS with cartilage was essential for cartilage degradation (Scott et al., 1997).

The extent of cartilage turnover and damage is determined by the balance between tissue inhibitors of metalloproteinase (TIMP) and MMPs. MMPs contribute to the normal turnover of cartilage matrix components in addition to joint destruction in inflammatory diseases. The significance of these mediators in joint destruction highlights their potential as therapeutic targets in RA (Odeh, 1997).

1.2.5.3 Inducible nitric oxide synthase (iNOS) and nitric oxide

Nitric oxide exists as a gaseous free radical that is generated by the oxidation of L-arginine by nitric oxide synthase (NOS). L-arginine analogues, including N-nitro-arginine-methylester (L-NAME) and N-iminoethyl-L-ornithine (L-NIO), can inhibit the activity of this enzyme. Three distinct isomeric forms of NOS have been identified and cloned. The calcium dependent form (cNOS) is constitutively expressed by neuronal and endothelial cells. The low level release of NO that results is believed to be involved in intracellular signalling processes. cNOS-derived NO production may exert a protective, anti-inflammatory role, including inhibition of cyclooxygenase activity (Lyons, 1995; Flower, 1999; Di Rosa et al., 1996). Conversely, the calcium-independent form is regulated in many cell types including macrophages and neutrophils. Lipopolysaccaride- or cytokine-mediated stimulation results in the expression of inducible NOS (iNOS), leading to excessive and sustained production of NO which is believed to be predominantly proinflammatory. More recently, NO

has been shown to inhibit collagen and proteoglycan synthesis (Taskiran et al., 1994), activate MMPs (Murrell et al., 1995), increase susceptibility to injury by other oxidants such as H_2O_2 (Clancy et al., 1997), and decrease expression of IL-1 receptor antagonist (Pelletier et al., 1996).

In animal models, including adjuvant arthritis and carageenan arthritis, studies support a role for NO in the development of arthritis. Elevated levels of NO production and amelioration of disease by NOS inhibitors have been observed in collagen-induced arthritis, streptococcal cell wall arthritis and adjuvant arthritis (Cannon et al., 1996; McCartney-Francis et al., 1993; Stefanovic-Racic et al., 1993; Stefanovic-Racic et al., 1992; Santos et al., 1997b).

Increased levels of NO have been observed in synovial fluid and serum of patients with RA and OA, compared to normal individuals (Farrell et al., 1992; Sakurai et al., 1995; Ueki et al., 1996). Moreover, elevated NO production, iNOS protein and mRNA expression have been identified in synovial tissue derived from RA (Sakurai et al., 1995). In peripheral blood monocyte and serum, nitrite concentrations in RA patients were observed to correlate with disease severity (St.Clair et al., 1996). These findings in humans, taken with the requirement for NO in animal models of human disease, implicate a role for NO in the pathogenesis of RA. Of note, however, FLS are not believed to contribute significantly to NO production in RA.

1.2.5.4 Reactive oxygen species (ROS)

Several lines of evidence implicate reactive oxygen species (ROS) as mediators of inflammation and tissue destruction in RA. Reactive oxygen species may originate from activated neutrophils, lymphocytes and endothelial cells present in the inflamed joint. Reactive oxygen species are generated by activated neutrophils and macrophages in the inflamed joint and include the superoxide anion, hydrogen

peroxide and hydroxyl radicals (Halliwell, 1995; Odeh, 1997). The generation of ROS in the inflamed joint is in part though a mechanism termed hypoxia-perfusion injury (Shingu et al., 1994; Blake et al., 1989). It is believed that the ROS that develops may lead to the degradation of collagen and proteoglycans in RA (Greenwald et al., 1976; Dean et al., 1984; Halliwell and Gutteridge, 1985; Bates et al., 1985). Tumor necrosis factorα, which is present in high concentrations in serum and synovial fluid of patients with RA, can also induce chondrocyte and neutrophil generation of ROS, thereby potentially amplifying the destructive process in the rheumatoid joint (Klebanoff et al., 1986; Tsujimoto et al., 1986; Tiku et al., 1988). Conversely, low level generation of ROS by neutrophils is associated with lack of erosions during early events of RA.

Reactive oxygen species have also been implicated as intracellular signalling molecules, capable of mediating the biological effects of cytokines (Lo et al., 1998; Lo and Cruz, 1995). Increased understanding of ROS as mediators of tissue damage and the capacity to influence cells relevant to RA pathology is still required.

1.2.5 Other soluble mediators

Soluble mediators examined in the experimental sections of this thesis have been given particular attention. Other soluble mediators involved in RA pathogenesis include chemokines, complement proteins and neuropeptides. Notwithstanding their importance, a complete exposition of the data arising from soluble mediator research in RA is beyond the scope of this thesis.

1.3 Glucocorticoids

Glucocorticoids are potent anti-inflammatory and immunosuppressive drugs commonly used in the treatment of a wide variety of chronic inflammatory diseases,

including RA. Glucocorticoids have been widely used therapeutically for the last 50 years, and are unparalleled in the extent of their effects on immune function. However, the mechanisms by which glucocorticoids exert their immunosuppressive and anti-inflammatory actions remain incompletely understood.

The following section will review the anti-inflammatory effects of glucocorticoids relevant to RA inflammatory processes. In addition, I will describe intracellular signalling mechanisms influenced by glucocorticoids.

1.3.1 Molecular basis of glucocorticoid action

Glucocorticoids exert their effects by binding to glucocorticoid receptors (GCR) in the cytoplasm, which then dimerizes and translocate to the nucleus. The hormone-receptor complex then binds to glucocorticoid response elements (GRE) on glucocorticoid-responsive genes, resulting in the transcription of specific target genes. Binding of the liganded GCR to DNA usually results in activation of transcription through positive GREs (pGRE), but binding to negative GREs (nGRE) can result in repression of transcription. Recently, it has been shown that the hormone-receptor complex can modulate transcription in a non-GRE dependent manner by interfering with the activity of transcription factors including CREB, AP-1 and NFκB (Cato and Wade, 1996; Didonato et al., 1996; Barnes, 1998). The activity of these factors is regulated in response to extracellular signals received by cell surface receptors. Interaction with transcription factors is likely to be an important determinant of glucocorticoid responsiveness and is a key mechanism whereby glucocorticoids exert their anti-inflammatory actions (Barnes and Adcock, 1993). For example, transcriptional interference with AP-1 transcriptional activity leads to decreased expression of the metalloproteinases, collagenase and stromelysin, as well as decreased activity of IL-2 gene promoters (Saatcioglu et al., 1994).

Identification of genes that are regulated by glucocorticoids is necessary to explore the mechanisms of steroid actions. For example, glucocorticoids may suppress inflammation by increasing the synthesis of several anti-inflammatory proteins. Glucocorticoids increase the synthesis of annexin I (lipocortin1), an important anti-inflammatory protein that plays a prominent role in many of the anti-inflammatory actions of glucocorticoids and in addition to the regulatory actions of steroids on the HPA axis itself (Goulding and Guyre, 1993a; section 1.5). Another protein upregulated by corticosteroids *in vivo* in humans is the anti-inflammatory cytokine IL-10. Glucocorticoids also inhibit the transcription of several cytokines relevant in inflammatory processes, including IL-1β, IL-2, IL-3, IL-6, IL-11, TNFα, GM-CSF and chemokines including IL-8, RANTES, MCP-1, MCP-3, MCP-4, MIP-1α and eotaxin (Siebenlist et al., 1994; Scheinman et al., 1995a). Interestingly, there is no negative GRE consensus sequence in the upstream promoter region of any of these genes, suggesting that glucocorticoids inhibit transcription indirectly. Glucocorticoids also inhibit genes such as COX2 and iNOS, despite these genes lacking GRE binding sites in their promoter region. These responses may be modulated by NFκB/IκB interactions.

1.3.2 Nuclear factor kappa B (NFκB) and inhibition of its activation by glucocorticoids

NFκB plays a critical role in regulating the expression of many inflammatory and immune genes (Barnes and Karin, 1997). NFκB is a heterodimeric protein composed of subunits that belong to the *rel* protooncogene family. NFκB usually consists of a p65 (RelA) and a p50 subunit. Other subunits, including rel, relB, v-rel and p52 may also be part of the activated NFκB complex, which suggests that alternative forms of

NFκB may activate different sets of target genes (Finco and Baldwin, 1995; Barnes and Karin, 1997; Sha, 1998). Unlike AP-1, which is constitutively nuclear, NFκB is retained in the cytoplasm of nonstimulated cells though the interaction with the inhibitors IκBα and IκBβ. Upon cell stimulation, for example, with inflammatory mediators such as IL-1 and TNFα, IκB is rapidly phosphorylated, ubiquitinated, and consequently protelyzed. This results in liberation of NFκB dimers that translocate to the nucleus, where they activate target genes by binding to specific sites in gene promoters.

Many stimuli, including cytokines (IL-1, TNFα, IL-17), activators of protein kinase C (phorbol esters, PAF), viruses and oxidants, may activate NFκB. These stimuli act by means of protein kinases that phosphorylate IκB, however several signal transduction pathways may be involved.

Similar to AP-1, NFκB is known to activate the transcription of a wide variety of immunoregulatory genes including those coding for cytokines (IL-1β, TNFα, IL-2, IL-6, IL-8, GM-CSF, M-CSF, G-CSF), chemokines (IL-8, MIP-1α, MCP-1), receptors (IL-2 receptor, TCR) and adhesion molecules (ICAM-1, VCAM-1, E-selectin). Inflammatory enzymes regulated by NFκB include $cPLA_2$ and COX2, in addition to iNOS and 5-lipoxygenase (Barnes and Karin, 1997). NFκB is not the only transcription factor involved in the regulation of these genes, however, and it frequently functions with other transcription factors such as AP-1 and nuclear factor IL-6 (Stein and Baldwin Jr., 1993a; Stein et al., 1993b). Since several cytokines exert their proinflammatory effects by activating transcription factors including AP-1 and NFκB. Glucocorticoid interference with these same transcription factors impacts both the synthesis as well as the action of proinflammatory cytokines.

Increasing evidence suggests that glucocorticoids inhibit the action of transcription factors such as NFκB and AP-1 (Barnes and Adcock, 1993; Saatcioglu et al., 1994). Interaction of the GCR with these transcription factors results in negative regulation of target genes. A direct interaction may exist between the GCR and AP-1 (Yang-Yen et al., 1990) and between the receptor and NFκB (Haddad et al., 1996; Ray and Prefontaine, 1994; Adcock et al., 1994; Scheinman et al., 1995b). Thus, glucocorticoids activate GCR that may then bind to activated NFκB and prevent it from binding to κB sites on genes that contribute to inflammatory processes. This interaction may occur in the cytoplasm or the nucleus.

Although still controversial, GC have been reported to increase the synthesis of IκBα, which binds to activated NFκB in the nucleus (Barnes and Karin, 1997). Inhibition of gene transcription by glucocorticoids may result either from the interaction between the GCR-ligand complex and NFκB, preventing binding of the latter to DNA, or from the induction of IκBα, thus inhibiting translocation to the nucleus (Auphan, et al., 1995; Scheinman, et al., 1995). These findings suggest that GC inhibit key stages of NFκB activation, and thereby inhibit the transcription of a range of genes whose products are involved in the immune response.

1.4 The control of inflammation by the hypothalamo-pituitary-adrenal axis

1.4.1 The HPA axis

The relevance of glucocorticoid-mediated regulation of inflammation extends beyond the effects of exogenous glucocorticoids administered therapeutically. The hypothalamic-pituitary adrenal (HPA) axis provides an essential interface between the internal and external environment and enables the organism to adapt to diverse

noxious stimuli. Adrenal glucocorticoid hormones, cortisol in humans and corticosterone in rats, act to restore homeostasis through multiple mechanisms that include modulation of immune/inflammatory processes.

The early observations of Seyle (1936) noted that experimental stress was associated with hypertrophy of the adrenal glands, and it was also shown in the 1930s that adrenal deficient animals are extremely sensitive to trauma and infection. These findings led to the concept that adrenal glucocorticoids were crucial in the stress response. The original concept that adrenal glucocorticoids contributed to the injurious effects of stress (Seyle, 1946) has been largely replaced by the concept that glucocorticoids act primarily to limit, or inhibit the effects of a stressor (Munck et al., 1984; Chrousos, 1995).

Activation of the HPA axis by diverse physiological stimuli and stressors results in substantial quantities of glucocorticoids being released into the circulation (Buckingham et al., 1996). Activation of the HPA axis is initiated by stimulation of hypothalamic neuronal pathways in the paraventricular nuclei by circulating cytokines, glucose and hormones, as well as to centrally produced neurotransmitters and peptides.

The subsequent release of corticotrophin releasing hormone-41 (CRH-41) and arginine vasopressin (AVP) into the hypophysial-portal circulation results in the individual or synergistic stimulation of corticotrophin (ACTH) release from the pituitary. ACTH stimulates the synthesis and release of glucocorticoids from the zona fasciculata of the adrenal cortex. The pituitary gland also releases prolactin, β-endorphin, melanocyte stimulating hormones (α-MSH) and other neuropeptides that act on the gonadal and thyroid glands (Chikanza and Grossman, 1996; Van de Kar et al., 1985).

Glucocorticoids are the principal effectors of the HPA axis and participate in the control of whole body homeostasis and the body's response to stress. In addition glucocorticoids negatively regulate the HPA axis, via specific receptors in the anterior pituitary gland, hypothalamus and other brain areas, to inhibit the secretion of ACTH and its hypothalamic releasing factors. Over-secretion of glucocorticoids is thus prevented by a complex negative feedback system. Increased circulating levels of glucocorticoids can also suppress the release of other pituitary hormones such as growth hormone, prolactin, thyrotrophin and gonadotrophins (Buckingham and Flower, 1997).

1.4.2 Modulation of the HPA-Immune axis

The mechanisms by which immune insults activate the HPA axis are the focus of much current research. Several investigations have drawn attention to evidence that the neuroendocrine and immune systems produce and use common mediators. It is believed that these factors fulfil local regulatory functions and may thereby contribute to the manifestation of the reciprocal communication between the neuroendocrine and immune systems. These factors include cytokines, interferons, phospholipid metabolites (eicosanoids, PAF) as well as amines (eg., histamine, 5-HT), peptides (eg., substance P, bradykinin, angiotensin II, thymic peptides) and enzymes (eg., PLA_2).

Inflammatory cytokines including IL-1, TNFα and IL-6 are major factors mediating interactions between the activated immune system and the HPA axis. They activate the HPA axis individually or synergistically to stimulate CRH release from the hypothalamus. Passive immunisation of CRH with specific antisera abolishes the acute effect of IL-1, IL-6 and TNFα to stimulate ACTH release (Uehara et al., 1987; Naitoh et al., 1988; Bernardini et al., 1990). *In vitro*, IL-1, TNFα and IL-6 increase

CRH release from hypothalamic explants (Tsagarakis et al., 1989; Cambronero et al., 1992; Navarra et al., 1990; Gisslinger et al., 1993), an effect that glucocorticoids and prostanoid synthesis inhibitors antagonise (Perlstein et al., 1993).

It is also noteworthy that the proinflammatory cytokine macrophage migration inhibitory factor (section 1.6) has been shown to be released by corticotrophs and thyrotrophs of the anterior pituitary gland in response to CRH (Nishino et al., 1995).

Interest in the role of eicosanoids in the regulation of the secretion of ACTH and its hypothalamic releasing factors has gained considerable momentum, and it is now apparent that these inflammatory mediators are important in the cascade of events that triggers the HPA responses to immune insults. At the pituitary level, prostanoids (prostaglandins, thromboxane A2 and prostacyclin) released locally are inhibitory of ACTH release. At the hypothalamic level, by contrast, prostanoids exert a powerful stimulatory influence on the release of CRH-41 *in vivo* and *in vitro*.

The cellular origin and the processes that initiate the release of eicosanoids that activate the HPA axis remains controversial. Particular attention has focused on the interrelationships of cytokines and eicosanoids in the hypothalamus. Considerable evidence now suggests that the release of CRH-41 and AVP initiated by IL-1, IL-6 and TNFα is dependent on the local generation of prostanoids, possibly via activation of $cPLA_2$. For example, increases in CRH-41 and AVP released *in vitro* by these cytokines are readily blocked by cyclooxygenase inhibitors such as indomethacin and ibuprofen. Furthermore, IL-1β and IL-6 produce increases in the release of PGE_2 from hypothalamic tissue *in vitro* and *in vivo* that, on a temporal basis, parallel the release of CRH-41 and AVP.

1.4.3 HPA dysfunction and inflammatory arthritis

Failure to mount an appropriate adrenocortical response in conditions of stress is potentially hazardous, and evidence now suggests that disturbances in HPA function may represent a significant contribution to the aetiology of chronic autoimmune disorders. Strong evidence for the contribution of the HPA axis dysfunction to inflammation is shown by studies in Fischer (F344) and Lewis (LEW/N) rats, two highly inbred strains selected for their resistance or vulnerability to inflammatory disease. Sternberg et al have shown that increased susceptibility to streptococcal cell wall (SCW) arthritis in LEW/N rats is related to a defect in the regulation of CRH biosynthesis and secretion (Calogero et al., 1992; Sternberg et al., 1989b), resulting in blunted corticosterone responses to challenge with SCW, IL-1 and other physical and psychological stressors (Sternberg et al., 1989a). Fischer rats develop severe inflammatory disease if administered the glucocorticoid receptor antagonist RU 486. Conversely, concurrent treatment of Lewis rats with replacement doses of glucocorticoid reverses the susceptibility to SCW arthritis (Sternberg et al., 1989a). Studies of HPA axis function in human RA have shown conflicting results, the discussion of which is beyond the scope of this thesis. Nonetheless, the concept that endogenous glucocorticoids contribute to the regulation of inflammation is now widely accepted.

1.5 Annexin I

1.5.1 The annexins and annexin I

Annexins constitute a family of structurally related proteins that exhibit Ca^{2+}-dependent binding to membrane phospholipids. During the 1980s, annexins were identified by several different names, including lipocortin, calpactin, endonexin, synexin and chromobindin (Swairjo and Seaton, 1994; Raynal and Pollard, 1994). The

term annexin, which derives from these proteins' property of 'annexing' phospholipid membranes, was suggested in 1990 as a basis for a common nomenclature (Crumpton and Dedman, 1990). Following cDNA cloning, at least thirteen different genes of the annexin family have been found. Annexins have been implicated in a broad range of cellular functions including inhibition of PLA_2, transduction of mitogenic signals, regulation of membrane trafficking, transmembrane channel activity, inhibition of coagulation and cell-matrix interactions (Swairjo and Seaton, 1994; Raynal and Pollard, 1994).

Unlike other annexin family members, annexin I has both properties of lipid interaction and steroid-inducibility, and as a result, the original nomenclature of lipocortin 1 has been retained by some investigators. Annexin I is a 37 kDa protein that was first identified in extracts of conditioned media from glucocorticoid-stimulated peritoneal macrophages (Blackwell et al., 1980). Subsequently, annexin I was shown to possess anti-inflammatory effects on eicosanoid synthesis, in addition to other glucocorticoid-like properties (Blackwell et al., 1980; Blackwell et al., 1982). The ability of annexin I to inhibit PLA_2 appears to be of major importance for the understanding of its anti-inflammatory functions and putative role as a 'second-messenger' of glucocorticoid effects (Flower and Blackwell, 1979). Other groups have reported similar findings, although alternative names for this protein were given (Blackwell et al., 1980; Cliox et al., 1983; Hirata et al., 1980; Russo-Marie and Duval, 1982; Russo-Marie et al., 1979).

1.5.2 Structure, distribution and function

The ability of annexins to display similar properties regarding Ca^{2+} and phospholipids derives from their common primary structure. Each annexin consists of two distinct regions, namely the unique N-terminal domain, and the C-terminal domain or 'core'.

The 34 kDa C-terminal domain is the conserved region of the molecule and strictly defines the annexin family. With one exception, it is composed of 4 repeats of a 70 amino acid sequence containing an increased homology region called the 'endonexin fold'. The exception is annexin VI that has 8 repeats instead of 4 (Geisow, 1986a; Geisow et al., 1986b). Due to this conserved primary structure, annexins have a high degree of identity with each other. Within mammals, any annexin has between 40 and 60% identity with each another (Hauptmann et al., 1989), and for one given annexin, the interspecies variation is low. The overall sequence of the annexin core domain is fairly hydrophilic, due to an abundance of polar and charged amino acids. Moreover, there is no significant stretch of hydrophobic amino acids.

Unlike the core domain, the sequence of the N-terminal domain is highly variable, albeit with a few exceptions. The length varies from a few amino acids (such as annexin IV and V), to more than 160 amino acids (such as annexin VII and XI) (Raynal and Pollard, 1994). The N-terminus of annexins I and II also display some similarity, except for an intervening 11 residue sequence (Klee, 1988). Moreover, it is widely believed that the N-terminal domain confers the biological specificity of individual annexins, and thus enables the proteins to exert distinct and specific actions on their respective target cells.

Genomic analysis performed on annexins I, II, III and VII have shown that the locations of the exon-intron boundaries are very well conserved in the core domain and varies in the N-terminal domain. It is also noteworthy that there is no obvious relationship between the exon-intron organisation of annexin genes and the primary structure of the proteins. Within the family of annexin I genes, the locations of the exon-intron boundaries are completely identical in human, mouse and rat (Horlick et al., 1991; Kovacic et al., 1991).

Since the 3D crystal structure of human annexin V was the first solved by Huber et al. (1990b), crystal structures have been determined for many annexins, including human annexin I, II, III, VI, VII and XII (Weng et al., 1993; Burger et al., 1996; Favier-Perron et al., 1996; Sutton and Sprang, 1996; Liemann et al., 1996; Luecke et al., 1995). These crystal structures have confirmed that all annexins have the same topology and each of the highly conserved repeats corresponds to a compact folded α-helical domain of similar structure. As deduced from the primary structure analysis, they are composed of 4 domains corresponding to the 4 repeats. Each domain consists of five amphiphilic α-helices organised in a right-handed superhelix. The axes of the four helices A, B, D and E are orientated approximately anti-parallel, and helix C lies almost perpendicular to the others. These domains which form the conserved core, are folded into an almost planar, cyclic array. In the centre of the cyclic arrangement lies a putative calcium channel that is mostly hydrophilic and forms a funnel-shaped pore (Huber et al., 1990a). The calcium binding sites of the annexins are located on the loops of convex surface between the helices A and B within the consensus sequence in domains I, II, and VI and have been shown to be the membrane binding site of the molecule. A second type of calcium binding site has been found in all annexin structures, where the calcium is bound in the D-E loop. This binding site displays a lower affinity to calcium ions and a higher affinity to lanthanides (Huber et al., 1992). Evidence supports the view that in the membrane bound state the convex surface of the annexin molecule lies along the plane of the phospholipid membrane, with the loops in direct contact with membrane components. The concave surface includes the capping helices, an extended, non-helical connection between domains 2 and 3, and the N-terminus, which also has an extended conformation. The N-terminus thus faces the cytosol, where its variable sequence can be accommodated without perturbing

calcium-dependent membrane binding. In several annexins, this region is also the site of *in vitro* phosphorylation by tyrosine kinases or protein kinase C (Haigler and Schlaepfer, 1992). The orientation of the N-terminus also allows it to participate in interactions with other intracellular proteins or membrane proteins.

Human annexin I was cloned by Wallner et al in 1986 (Wallner et al., 1986). The cloning for rat and guinea pig annexin I sequences, shortly thereafter, demonstrated approximately 80% sequence homology between these species (Pepinsky et al., 1986; Sato et al., 1989). The annexin I gene is located on chromosome 9 (Huebner et al., 1988) and consists of 13 exons that extend across 18.5 kb (Kovacic et al., 1991). In the annexin I sequence, at least one putative glucocorticoid response element is present (Browning et al., 1990). Moreover, the presence of an AP-1 site in the annexin I promoter region is consistent with the effects of phorbol esters on the induction of the annexin I gene and its protein (Browning et al., 1990). In addition, the elucidation of the annexin I sequence confirmed that annexin I was identical to the previously identified p35 substrate of the EDGF-receptor kinases (Brugge, 1988; Northup et al., 1988; Haigler et al., 1987).

The release of annexin I from certain cell types is well described. Studies in the human prostate gland have provided the most compelling evidence in favour of the secretion of annexin I, where high concentrations of annexin I are detected in seminal plasma. Whereas both annexin I and IV are expressed at similar levels and co-localise in the ductal epithelium of the prostate, only traces of annexin IV were detected in the seminal plasma. This finding suggests that the prostate cells selectively export annexin I, which is especially interesting, given that annexin I lacks a hydrophobic signal sequence which should otherwise be involved in targeting the protein to the endoplasmic reticulum (Christmas et al., 1991; Chapter 2). Conversely, there are also

reports in which cells expressing high intracellular levels of annexin I failed to release the protein into the culture medium (Northup et al., 1988; Bienkowski et al., 1989). With evidence that annexin I is released from certain cell types, the concept that it mediates its biological effects via specific cell surface receptors arises. This is consistent with the identification of annexin I binding sites in human and murine leukocytes (Goulding et al., 1996; Perretti et al., 1993). Data from these studies, in addition to the first description of annexin I binding sites on human FLS will be discussed in Chapter 2.

In vivo studies also report the presence of annexin I in a wide range of cells and tissues. High levels of annexin I are observed in a variety of specific cell types including ductal epithelium, epithelia of airways, cytoreticulum of the thymus, stroma of the spleen and syncytial trophoblasts in addition to neutrophils and macrophages. In human term placenta, very high annexin I expression was found in the syncytial trophoblast (Fava et al., 1989). Studies have also identified a covalently linked homodimer form of annexin I that is particularly abundant in preparations obtained from human placenta, and accounts for approximately 20% of the protein. Interestingly, the site of cross-linking of the homodimer is localised within the N-terminal region of annexin I (Pepinsky et al., 1989). Of particular relevance to studies presented in this thesis, annexin I is observed in the synovial lining layer of RA and non-rheumatoid synovial tissue, predominantly found in cells of the macrophage cell lineage (Goulding et al., 1995).

Other studies observe annexin I immunoreactivity in the pars distalis of the mature human hypophyses, in scattered or moderate numbers of cells. The selective distribution of annexin I immunoreactivity is found to overlap that of some corticotrophs and is also found in elongated processes of S100-containing

folliculostellate cells. In addition, annexin I is observed in epithelial cells lining colloid cysts of the residual pars intermedia in pituitaries of varying age. Studies of the neurohypophysis, however, failed to detect any annexin I immunoreactivity (Johnson et al., 1990). Subsequent investigations by these authors also examined the distribution of annexin I in the central nervous system. Annexin I immunoreactivity was detected in ependymocytes, choroid plexus epithelia, and scattered subependymal astrocytes throughout the ventricular system in the human central nervous system. In this study, the low amounts of annexin I identifiable in brain is consistent with the limited distribution of annexin I in central nervous system and suggests that annexin I may have a role in the normal function of ependymocytes (Johnson et al., 1989). In epidermal cells freshly isolated from normal human skin, expression of annexin I is seen mainly in basal and suprabasal keratinocytes. Compared with keratinocytes, low levels of annexin I were detected in Langerhans cells. High expression of annexin I was also observed in cultured keratinocytes, with annexin I mainly localized in the cytoplasm (Serres et al., 1994). Annexin I was readily detected in the basal keratinocytes of normal human skin (Fava et al., 1989).

In addition to the intracellular localisation of annexin I in alveolar type II cells and macrophages, annexin I has been found in bronchoalveolar lavage fluids from humans (Ambrose and Hunninghake, 1990; Smith et al., 1990).

Constitutive annexin I expression is observed in all leukocyte subsets in human peripheral blood, with the exception of B cells. Annexin I content is maximal in monocytes and polymorphonuclear neutrophils and least in lymphocytes. Investigations using non-B cell subsets observed variations in annexin I content, with greatest annexin I content found in natural killer and CD57+ cells (Morand et al., 1995c). Studies examining unstimulated and thrombin-activated human platelets were

also found to contain annexins I and IV, but not annexins II and VI (Eldering et al., 1993).

Studies by Goulding et al originally detected the presence and amount of annexin I in human plasma and peripheral blood leukocytes (Goulding et al., 1990b). In addition, levels of annexin I measured in circulating murine neutrophils and monocytes are higher than levels observed in lymphocytes. The finding that mouse peritoneal macrophages exhibit higher annexin I levels compared to circulating neutrophils and monocytes (Perretti and Flower, 1996), confirms reports by Peers et al that peritoneal macrophages are a major source of annexin I (Peers et al., 1993).

1.5.3 Regulation of annexin I synthesis

Interest in annexin I biology gained considerable momentum with the early discovery that glucocorticoids increase annexin I synthesis and alter its subcellular disposition. Subsequent studies demonstrated increased expression of annexin I mRNA and protein following glucocorticoid exposure, in a variety of cell types, including human monocytes (Goulding et al., 1990a; Browning et al., 1990), human skin epidermal cells and cultured keratinocytes (Serres et al., 1994), primary cultures of osteoblasts (Suarez et al., 1993), a fibroblast cell line (Newman et al., 1994), lung epithelial cell line (Wong et al., 1991), rat macrophages and thymus (Fuller and Verity, 1989; Wallner et al., 1986) and hepatocytes (Karasik et al., 1988). Collectively, these studies infer that annexin I is under the regulatory control of glucocorticoids, at least in certain tissues.

In vivo studies also provide evidence that endogenous and exogenous glucocorticoids regulate annexin I. For example, a modest, but transient increase in annexin I expression is observed in mononuclear leukocytes from patients receiving hydrocortisone (Goulding et al., 1990b). Similarly, the levels of annexin I in human

bronchoalveolar lavage fluid were found to be associated with chronic steroid treatment (De Caterina et al., 1993). Predictably, levels of annexin I mRNA and protein are substantially reduced in adrenalectomised animals (Vishwanath et al., 1992), but are readily restored by maintenance doses of glucocorticoids. It is also noteworthy that expression of PLA_2 has been observed to increase in adrenalectomised rats (Vishwanath et al., 1993). Therefore, both downregulation of annexin I and upregulation of PLA_2 may be associated with the increase of inflammatory response in hypoglucocorticoid states.

The complexity of annexin I regulation is illustrated by other studies which report no glucocorticoid-induced change in annexin I occurring in either primary cultures or cell lines (Bienkowski et al., 1989; Browning et al., 1990; Gebicke-Haerter et al., 1991; Hullin et al., 1989). In these studies, the difficulties associated with the induction of the annexin I gene suggested a requirement for additional factors, other than glucocorticoid, or for the presence of a critical differentiation state in the tissue under investigation. Negative results of some early studies may also be associated with the total cell annexin I observed, rather than more subtle changes in separate cell fractions.

It is of interest that glucocorticoids regulate the subcellular distribution of annexin I in addition to synthesis of the protein (Browning et al., 1990; Solito et al., 1994). Glucocorticoids cause the rapid exportation of annexin I from the cytoplasm to the cell surface, and promptly replenish the depleted intracellular stores with newly synthesised protein (Croxtall and Flower, 1994; Perretti and Flower, 1996; Taylor et al., 1993; Taylor et al., 1995). This process of externalisation occurs relatively rapidly, usually 0.5 to 2 hours after glucocorticoid administration. Although the mechanisms whereby annexin I crosses the membrane are still undefined, it is widely

believed that the exportation of annexin I is an important facet of annexin I biology, as it enables the protein to gain access to cell surface binding sites where it is believed to exert its biological actions (Buckingham and Flower, 1997; Christian et al., 1997; Harbuz and Lightman, 1992; Latocha et al., 1989; Loxley et al., 1993).

It is now well accepted that several factors other than glucocorticoids also regulate the biosynthesis of annexin I. Studies on the promoter region of the gene encoding annexin I have shown that IL-6 and phorbol esters may also positively influence annexin I synthesis. For example, in the A549 epithelial cell line, PMA-induced differentiation is associated with increased expression of annexin I (Solito et al., 1998). From these studies, it is suggested that a two-tier regulatory system exists for the synthesis of annexin I, and the implications of this system are yet to be resolved. Moreover, despite the controversy concerning the glucocorticoid induced synthesis of annexin I, the debate concerning the role of annexin I as a mediator of anti-inflammatory glucocorticoid effects is still very active.

1.5.4 Anti-inflammatory effects of annexin I and annexin I peptides

Annexin I was first identified in extracts of conditioned medium from glucocorticoid-stimulated peritoneal macrophages (Blackwell et al., 1980). Studies subsequently showed that an injection of partly purified annexins significantly reduced the volume of exudate and leukocyte infiltration in rat carrageenan-induced pleurisy (Blackwell et al., 1982). These findings lead to the identification of annexin I as an anti-inflammatory mediator with glucocorticoid-like properties, and thus heralded annexin I as a potential second messenger protein for glucocorticoids (Blackwell et al., 1980; Blackwell et al., 1982). Studies thereafter by Cirino et al reported that infusion of recombinant human annexin I in the isolated guinea-pig lung inhibited agonist-induced production of eicosanoids, similarly to the effect during treatment with

glucocorticoids (Cirino et al., 1987). This anti-inflammatory activity of annexin I has also been reported in other models of inflammation. For example, release of arachidonic acid from isolated macrophages was inhibited by treatment of purified annexins (Errasfa et al., 1988).

In whole animals, annexin I exhibits some striking glucocorticoid-like properties. Human recombinant annexin I locally administered inhibits paw oedema induced by injections of carrageenan. In the same study, annexin I exerted no inhibitory action when animals were pre-treated with a cyclooxygenase/lipoxygenase inhibitor, suggesting a PLA_2-dependent mechanism of action for annexin I (Cirino et al., 1989). Conversely, Northup et al observed no inhibition of the rat paw oedema model by recombinant human annexin I, in addition to no addition in annexin I synthesis with glucocorticoid treatment, despite inhibition of PLA_2 *in vitro* (Northup et al., 1988). Difficulties with the source, purity and especially tertiary refolding of recombinant annexin I may explain some negative results. An annexin I peptide, ac 2-26, has been observed to inhibit carrageenan paw oedema and air pouch neutrophil migration models, suggesting that it shares the actions of annexin I in these acute models of inflammation (Cirino et al., 1993). Other anti-inflammatory actions of annexin I and related peptides have been documented *in vivo*. Consistent with findings by Cirino et al, an anti-serum raised against the N-terminal 1-188 amino acid sequence of annexin I was reported to reverse the inhibitory effects of dexamethasone in the rat carrageenan oedema model (Duncan et al., 1993).

In rats, intracerebroventricular and intravenous injections of human recombinant annexin I was observed to inhibit pyrogenic actions of cytokines and other agents (Davidson et al., 1991). In mice, body temperature of young animals increased upon injection of IL-1β. However, the pyrogenic effect of the cytokine was significantly

reduced in aging animals. The impaired febrile responses to murine IL-1β in the aged mice were normalized by intravenous injection of the glucocorticoid receptor antagonist RU-38486 or of an antiserum to annexin I (Strijbos et al., 1993). Moreover, it has been shown that injection of annexin I peptide 1-188 is sufficient to inhibit pyrogenic effects of cytokines (Carey et al., 1990; Strijbos et al., 1992a). In conscious rats, intracerebroventricular injection of an annexin I 188 amino acid peptide was observed to dose-dependently reduce the acute increases in colonic temperature and oxygen consumption, which resulted from central injections of recombinant IL-1β and IFN-γ. Conversely, the annexin I 188 peptide did not affect the response to PGE_2, and its activity was prevented by heat treatment or by pretreatment of animals with polyclonal antiserum raised to the fragment. Moreover, antiserum treatment significantly enhanced the thermogenic responses to IL-1β in rats treated with dexamethasone without affecting the responses in normal animals (Carey et al., 1990). Intracerebroventricular injection of CRF, or recombinant IL-1α, IL-1β, IL-6, IL-8, or TNF-α was found to significantly increase resting oxygen consumption and colonic temperature in conscious rats within 2 hours postinjection. Administration of recombinant annexin I 188 peptide produced small increases in the thermogenic effects. Pretreatment with annexin I significantly attenuated the thermogenic and pyrogenic effects of centrally injected IL-1β, IL-6, IL-8, or CRF, but failed to modify the actions of IL-1α or TNF-α (Strijbos et al., 1992b).

Much experimental evidence implicates a role for annexin I in neutrophil extravasation, especially that derived from a murine 6 day old pouch model of acute inflammation. Injection of human recombinant IL-1 into a murine air-pouch induced the migration of neutrophils that was inhibited by annexin I. In studies of this model that are not associated with endogenous eicosanoids, intravenous administration of

annexin I inhibits PMN infiltration in response to IL-1β and zymozan, and its effect is mimicked by annexin I peptides. In addition, monoclonal antibodies raised against annexin I inhibit the action of glucocorticoids in the murine air pouch model of IL-1 induced neutrophil migration (Perretti and Flower, 1993b; Getting et al., 1997). Moreover, studies by this group and others have reported the effect of annexin I on neutrophil migration following induction by stimulating agents such as IL-1, IL-8, substance P, glycogen and zymosan (Getting et al., 1997; Perretti, 1994; Perretti et al., 1993a; Perretti and Flower, 1993b; Teixeira et al., 1998).

In the hamster cheek pouch model, dexamethasone treatment had no effect on the inflammatory stimuli-induced leukocyte rolling or adhesion, but greatly increased the rate of detachment of the adherent leukocytes. In the same study, an anti-annexin I antibody was found to abolish these effects of glucocorticoids on adherent leukocytes, and annexin I peptide ac 2-26 prolongs the time required to complete the diapedesis process, suggesting that this specific effect upon leukocyte diapedesis is mediated by endogenous lipocortin 1 (Mancuso et al., 1995).

In other studies, intracerebroventricular injection of the annexin I peptide 1-188 appeared to protect, to some extent, rat brain from neuronal death and odema induced by focal cerebral ischemia. In the same study, injection of a neutralising annexin I antiserum was observed to significantly exacerbate damage (Relton et al., 1991). Annexin I 1-188 peptide has also been shown to inhibit neuronal damage induced by activation of excitatory amino acid receptors (Black et al., 1992). The mechanism of these neuroprotective effects is thought to involve a putative inhibition of eicosanoid production by annexin I.

Other activities of annexin I have been reported, although the mechanisms of these effects remain unclear. For example, annexin I has been reported to inhibit superoxide

generation by NADPH oxide in alveolar macrophages, thus mimicking an effect of glucocorticoids (Maridonneau-Parini et al., 1989). In a recent study, annexin I peptide ac 2-26 was found to suppress both the initial rate and maximal response of the FcγR-mediated superoxide release and phagocytosis in human neutrophils obtained from peripheral blood (Goulding et al., 1998). Annexin I also inhibits the formation *in vitro* of aggregates between erythrocytes sensitised with anti-erythrocyte IgG and leukocytes expressing Fc receptor for IgG, thus reproducing to some extent, glucocorticoid activity *in vivo*. However, it is not known how annexin I produced this effect (Goulding and Guyre, 1993b).

The involvement of annexin I in cell differentiation was first provided Violette et al, who reported that both human recombinant and placental annexin I and glucocorticoids were found to induce differentiation of a human squamous carcinoma cell line SqCC/Y1. In this study, glucocorticoid-induced maturation of the SqCC/Y1 cells was antagonized by an annexin I mAb, PLA_2 and arachidonic acid, whereas annexin I-induced differentiation of SqCC/Y1 cells was inhibited by arachidonic acid. These findings support a role for annexin I in mediating the effects of glucocorticoids on epidermal cell differentiation (Violette et al., 1990). In the A549 human adenocarcinoma cell line, human recombinant annexin I produced cell growth arrest and abolished the generation of PGE_2. In addition, the neutralising anti-annexin I antibody 1A substantially reversed the inhibitory activity of dexamethasone on growth arrest and PGE_2 synthesis, suggesting that dexamethasone-induced suppression of proliferation in A549 cells is attributed to eicosanoid inhibition mediated by annexin I (Croxtall and Flower, 1992). In studies using polymorphonuclear neutrophils, a role for annexin I in the inhibition of chemotaxis has also been implicated (Fradin et al., 1988).

Several lines of evidence implicate annexin I in glucocorticoid suppression of pro-inflammatory mediator release. In J774.2 macrophages LPS induced the expression of iNOS and significantly increased nitrite generation. This effect was prevented by dexamethasone, which also increased cell surface annexin I. Pretreatment of J774.2 cells with anti-annexin I also abolished the inhibitory effect of dexamethasone on iNOS expression and nitrite accumulation. An annexin I peptide 1-188 also inhibited iNOS in J774.2 macrophages activated by LPS, and this too was prevented by anti-annexin I. These findings suggest that the extracellular release of endogenous annexin I mediates the inhibition by dexamethasone of the expression of iNOS (Wu et al., 1995). Moreover, in rat primary microglial cells annexin I peptide 2-26 inhibited the LPS-stimulated release of PGE_2 and NO and these effects were partially abrogated by a specific antiserum raised against the annexin I peptide 2-26 (Minghetti et al., 1999). Other studies also have investigated the contribution of annexin I to cytokine regulation. Sudlow et al examined the ability of annexin I to suppress the release of pro-inflammatory cytokines from human peripheral blood mononuclear cells. In this study, glucocorticoid suppression of LPS-stimulated TNFα and PGE_2 secretion from peripheral blood mononuclear cells was inhibited by a polyclonal antibody to annexin I 1-188, but not mimicked by annexin I peptide 1-188. Annexin I has been reported to inhibit recombinant human IL-1β-stimulated TNFα and PGE_2 secretion from peripheral blood mononuclear cells. The results of this study implicate endogenous annexin I in glucocorticoid suppression of TNFα and PGE_2 release and suggest an extracellular site of action for annexin I. (Sudlow et al., 1996). Moreover, the finding that the annexin I peptide failed to mimic the inhibitory action of recombinant human annexin I is consistent with studies investigating cytokine-stimulated leukocyte activation and accumulation. In the mouse air-pouch model, the annexin I 2-26

peptide exhibited low potency inhibitory effects on leukocyte migration elicited by IL-1, compared to recombinant human annexin I (Perretti et al., 1993a). Morand et al also observed that a neutralising monoclonal annexin I antibody did not inhibit LPS-stimulated IL-1β release from human monocytes and an annexin I peptide 1-188 also exhibited no effect (Morand et al., 1993). In contrast, a recent study by Yang et al indicated that exacerbation of adjuvant arthritis severity by anti-annexin I treatment is accompanied by increases in synovial production of TNFα and PGE_2 (Yang et al., 1999). Evidence implicating a role for annexin I in cytokine regulation is therefore somewhat conflicting, and the mechanisms involved remain unknown.

As noted previously, the inhibitory actions of annexin I are believed to occur in parallel with its externalisation, suggesting an extracellular site of action for annexin I protein (Croxtall and Flower, 1994; Wu et al., 1995). Given that the binding site capacity of annexin I is altered at sites of inflammation, further investigations are required to determine both the physiological relevance and nature of the annexin I binding sites (see section 1.5.6, Chapter 2).

It is of interest that glucocorticoids are effective in most experimental models of inflammation, whilst annexin I only partially mimics the anti-inflammatory actions of glucocorticoids. For example, glucocorticoids inhibit the development of carrageenan- and histamine-induced paw oedema in rats, whilst annexin I inhibits carrageenan-induced oedema, but is unable to suppress the responses to histamine. More recently, the modulation of intracellular adhesion molecule-1 expression by glucocorticoids has been observed to be independent of endogenous annexin I (Perretti et al., 1996). These findings suggest the existence of at least two distinct mechanisms of glucocorticoid action, both independent of and dependent on annexin I.

1.5.5 Anti-inflammatory effects of annexin I involving PLA_2

In early reports, the possibility that glucocorticoids directly inhibited PLA_2 activity appeared unlikely, given that glucocorticoids had no action on PLA_2 in a cell free homogenate (Blackwell et al., 1978). Moreover, the effect of glucocorticoids on intact cells was abolished by blockers of gene transcription or translation, suggesting that glucocorticoids which were widely known as activators of gene transcription, might induce the transcription of a PLA_2 inhibitor (Flower and Blackwell, 1979; Blackwell et al., 1982; Hirata et al., 1980). This inhibitor of PLA_2 activity was subsequently identified and named annexin I. This hypothesis seemed validated by studies which reported annexin I inhibition of PLA_2 activity in a range of tissues including kidney, neutrophils, lung, cultured fibroblasts, endothelial cells and placenta. In support of these findings, studies utilising adrenalectomy observed an association between physiological control of tissue PLA_2 activity and annexin I glucocorticoid by endogenous glucocorticoids (Vishwanath et al., 1993).

Early studies of the inhibitory activity of annexin I demonstrated that the inhibition of pancreatic PLA_2 by annexin I could be abolished when the ratio of annexin/phospholipid in the reaction mixture was decreased (Davidson et al., 1987; Aarsman et al., 1987). From these observations, it was proposed that the mechanism could be due not to a specific interaction of the annexins with PLA_2, but to depletion of its substrate by the proteins bound to the phospholipids in the presence of Ca^{2+}. However, conflicting evidence by Hayashi et al reported that a monoclonal antibody to annexin I inhibited the PLA_2-inhibitory activity of the protein, but did not alter its binding to phospholipid (Hayashi et al., 1990). Moreover, the possibility that annexin I is not involved in glucocorticoid actions on PLA_2 is also suggested by studies which report PLA_2-inhibitory activity of cellular annexin I but does not demonstrate

glucocorticoid induction of annexin I protein (Hullin et al., 1989; Bienkowski et al., 1989; Northup et al., 1988; Fujimoto et al., 1991; Buckingham et al., 1994).

In spite of the negative results of some studies, the involvement of annexin I in the regulation of PLA_2 activity is increasingly accepted, and studies that support this hypothesis are presented in Chapter 3.

1.5.6 Rheumatic diseases and annexin I

Several lines of evidence support a role for annexin I in the regulation of human rheumatic diseases. Abundant expression of annexin I is observed in RA synovial tissue, predominantly in the synovial lining associated with CD14/CD68+ cells of the monocyte/macrophage lineage. Increased staining for annexin I was observed in RA tissue compared to OA or control (Goulding et al., 1995). Given that other studies have failed to detect significant levels of annexin I in serum or plasma, these findings suggest that annexin I is produced locally (Goulding et al., 1990b; Christmas et al., 1991). Studies in my department demonstrate glucocorticoid-inducible expression of annexin I mRNA in cultured human RA FLS (Yang et al., unpublished observations).

Annexin I has recently been extensively investigated for its potential involvement in the modulation of joint inflammation, particularly in PhD studies in my laboratory by Dr Yuanhang Yang. In carrageenan-induced arthritis, administration of anti-annexin I mAb was associated with exacerbation of synovial fluid neutrophilia, and with prevention of the therapeutic effect of dexamethasone (Yang et al., 1997). In this same study, administration of annexin I N terminal ac2-26 peptide significantly inhibited arthritis severity. In the more complex rat adjuvant arthritis model, Yang et al also demonstrated the expression of annexin I in blood leukocytes and synovial leukocytes and resident cells, and reported that increases in synovial and blood cellular annexin I in the presence of arthritis were dependent upon adrenal

glucocorticoids (Morand et al., 1996). Moreover, an inverse correlation was demonstrated between synovial annexin I and inflammation in this model. Functionally, the expression of annexin I by rat adjuvant arthritis synovial macrophages, and upregulation of cell-surface translocation by glucocortioids, was also reported by Yang et al (Yang et al., 1998). That study also demonstrated the involvement of annexin I in inhibition of nitric oxide production by synovial macrophages. Finally, an extensive study in rat adjuvant arthritis revealed that anti-annexin I mAb administration caused exacerbation of disease severity, prevented the effects of exogenous glucocorticoids, and caused upregulation of synovial TNFα and PGE_2, with a trend to upregulate NO (Yang et al., 1999). Taken with the earlier description of the expression of annexin I in RA synovium, these studies supported the investigation of annexin I biology in human synovial cells *in vitro* which forms a substantial part of this thesis.

The presence of annexin I autoantibodies has been described in patients with rheumatic diseases, including RA, systemic lupus erythematosus (SLE), and psoriasis. Hirata et al first described the existence of IgM and IgG antibodies to annexin I in patients with RA and SLE (Hirata et al., 1981). Studies thereafter by Goulding et al confirmed and extended these findings, demonstrating a strong correlation between IgM and less dramatically IgG anti-annexin I autoantibody levels, and prolonged high dose glucocorticoid treatment. In the same study, high titres of annexin I autoantibodies were observed in RA patients receiving higher glucocorticoid doses (Goulding et al., 1989). Moreover, Podgorski et al observed high titres of annexin I autoantibodies in association with the relative *in vivo* resistance to glucocorticoid therapy in patients with RA (Podgorski et al., 1992). Glucocorticoid resistance may alternatively relate to impaired induction of annexin I by glucocorticoids (Morand et

al., 1994). Other autoimmune diseases including Felty's syndrome, and inflammatory bowel diseases such as Crohn's disease or ulcerative colitis (Stevens et al., 1993), also exhibit elevated anti-annexin antibody levels in the absence of glucocorticoid therapy. In other studies, significantly higher levels of anti-annexin IgM and IgG antibodies were observed in SLE patients, despite no correlation between anti-annexin antibody titres and serum PLA_2 levels or activity (Pruzanski et al., 1994). Impairment of annexin I responses is reported to correlate with inflammatory rheumatic diseases. Morand et al reported impaired blood leukocyte annexin I synthesis in response to exogenous glucocorticoids in subjects with RA (Morand et al., 1994). As noted previously, specific, saturable binding sites for annexin I have been detected on human and murine leukocytes (Goulding et al., 1990; Perretti et al., 1993a). A detailed discussion of annexin I binding sites is provided in Chapter 2. Reduced binding site numbers on cells in sites of inflammation have also been reported (Perretti et al., 1993a; Goulding et al., 1992).

Confirmation of the functional nature of these binding sites is provided by Goulding et al, who reported that recombinant human annexin I inhibits IgG binding to leukocyte Fc receptors only occurred in cells with annexin I-binding capacity (Goulding and Guyre, 1993b). Moreover, although N-terminal-deleted annexin I bound to cells, it did not interfere with IgG binding to Fc receptors. Collectively, these findings indicate that specific binding of annexin I to human leukocytes by regions independent of the N-terminus, in addition to some interactions requiring the N-terminus, are necessary for inhibition of leukocyte function. Further characterisation of the nature of the annexin I binding sites, together with studies of signal transduction and cell function following annexin binding will provide greater understanding of the mechanisms of action and physiological importance of annexin I

in rheumatic immune and inflammatory processes. The presence of annexin I binding sites on synovial cells has not been previously reported.

1.6 Macrophage migration inhibitor factor (MIF)

1.6.1 Structure, distribution and function

1.6.1.1 MIF: History

In 1966, experiments by Bloom and Bennett first identified macrophage migration inhibitory factor (MIF) as a non-dialysable substance that inhibited the random migration of cultured macrophages and was involved in delayed-type hypersensitivity (Bloom and Bennet, 1966; David, 1966). This then unidentified substance represented the first lymphokine activity to be discovered and was called macrophage migration inhibitory factor. Based on its early discovery, Martin has recently suggested it could be known as IL-(0) (Martin, 2000). Preceding the identification of the MIF molecule, T-cell supernatants were additionally observed to induce tumoricidal activity in cultured macrophages (Churchill et al., 1975). The elucidation of the precise role of the MIF molecule was confounded by the description of two other cytokines, IFN-γ and IL-4, which exhibit macrophage migration inhibition activity (Thurman et al., 1985; McInnes and Rennick, 1988), and by the lack of availability of both purified, mitogen-free MIF protein and of specific, neutralising anti-MIF antibodies (David, 1993b; David, 1993a).

1.6.1.2 MIF: structure and biochemical properties

MIF is a 12.5 kDa protein. Mouse MIF was first successfully cloned from the cDNA of the AtT-20 anterior pituitary cell line and human MIF from the Jurkat T-cell line. Mouse and rat MIF were found to exhibit 90% homology over 115 amino acids with

human MIF. This is the highest known homology for a rodent-human cytokine pair (Bernhagen et al., 1994). MIF protein in both mice and humans lack an N-terminal leader sequence and is released from cells by as yet unidentified granular secretion or by a non-conventional pathway. Although two potential N-glycosylation sites exist in the primary sequence, mass spectroscopic analysis has shown that MIF is not post-translationally modified (Bernhagen et al., 1994; Zeng et al., 1993).

The mouse and human genes are less than 1 kB in size and exhibit a high degree of homology with similar intron exon structure (Kozak et al., 1995; Mitchell et al., 1995; Paralkar and Wistow, 1994). The mouse MIF gene is located on chromosome 10. Several consensus sequences implicated in the transcriptional regulation of the MIF gene have been identified (Mitchell et al., 1995; Paralkar and Wistow,1994). The murine MIF promoter region includes regulatory sequences implicated in the expression of the protooncogene c-fos, an Sp-1 site, a cAMP responsive element (CRE), an AP-2 site, and a negative glucocorticoid responsive element (nGRE), all located in close proximity to the RNA transcription start site. A cytokine-1 (CK-1) site and a nuclear factor kappa B (NFκB) site were also identified. The MIF promoter therefore contains regulatory sequences that are characteristic of both cytokine (NFκB) and endocrine hormone (CRE and nGRE) genes (Mitchell et al., 1995; Paralkar and Wistow, 1994). Mouse MIF mRNA is 0.6 kB (Mitchell et al., 1995), and MIF mRNA is constitutively expressed in most mouse tissues examined (Nishino et al., 1995; Meinhardt et al., 1996).

The promoter region of the human MIF gene contains multiple Sp-1 sites and a CRE, and is located on chromosome 19 (Paralkar and Wistow, 1994). Human MIF mRNA is 0.8 kB (Paralkar and Wistow, 1994) and is constitutively expressed at high baseline

levels in many organs including the kidney, brain and liver (Paralkar and Wistow, 1994).

To date, very little is known about signal transduction mechanisms involved in the regulation of MIF. Hirokawa et al were first to describe MIF expression in association with signal transduction mechanisms (Hirokawa et al., 1998). In this study, TNFα induced MIF mRNA expression in 3T3-L1 adipocytes, and tyrosine kinase inhibition resulted in suppression of MIF mRNA induction by TNFα. This finding suggests that upregulation of MIF mRNA expression by TNFα is mediated by a tyrosine kinase-dependent pathway. Investigations which have explored potential intracellular signalling pathways utilised by MIF will be detailed in section 1.6.5.

1.6.1.3 MIF: crystal structure and biochemistry

The X-ray crystal structure of human MIF has been solved at 2.6 A resolution (Sun et al., 1996). MIF exists as a trimer of three identical subunits. Each monomer consists of two antiparallel α helices and 6 β strands. Thus, six α helices and three β sheets completely wrap around to form a barrel containing a solvent accessible channel. This channel is lined by hydrophilic molecules and is positively charged, suggesting that it may interact with negatively charged moieties (Calandra and Bucala, 1997). These structural data suggest that MIF participates in novel ligand-receptor interactions.

In conjunction with studies investigating the enzymatic pathways responsible for melanin biosynthesis, MIF was shown to have enzymatic activity (Rosengren et al., 1996). A cytoplasmic activity, later identified as MIF was responsible for catalysing a tautomerisation reaction which involved the conversion of the non-naturally occurring D-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The enzymatic activity of MIF was confirmed by studies showing that recombinant human MIF catalysed the same tautomerisation reaction (Rosengren et al., 1996).

1.6.1.4 MIF: gene knockout mouse

Using targeted disruption in embryonic stem cells, Bozza et al generated a mouse strain lacking the MIF gene. Newborn MIF -/- mice developed normally in size, behaviour and fertility. Northern blot analysis and ELISA confirmed the absence of MIF mRNA and protein in homozygotes. Organs including kidney, liver, spleen, thymus, adrenal, lungs, brain, heart and intestine were histopathologically normal. Splenic and thymus-derived lymphocyte populations were normal as demonstrated using flow cytometry. Normal neutrophil accumulation was observed in thioglycollate elicited peritoneal exudates. Macrophages derived from MIF -/- mice stimulated with LPS and IFN-γ had diminished production of TNFα but interestingly exhibited an increased production of NO. Given that MIF has been reported to enhance NO production by IFN-γ-stimulated macrophages in vitro, this increased NO production observed in MIF -/- mice suggests that MIF either dampens NO production by these cells, or increases its overall NO turnover (Bozza et al., 1999). More detailed characterisation of these mice is not yet available. The response of MIF -/- mice to endotoxin challenge will be further discussed in section 1.6.4.2.

1.6.2 Cell and tissue sources of MIF

1.6.2.1 T-cells

From an historical perspective, MIF was originally considered to be a T cell cytokine released upon lymphocyte-specific stimulus (Bernhagen et al., 1998). This description was consistent with its association with delayed-type hypersensitivity. Since the cloning and identification of the MIF molecule, the presence of pre-formed MIF has been confirmed in resting T cells (Calandra et al., 1994). Human peripheral blood and mouse splenic T cells are known to express MIF mRNA and release MIF protein

when stimulated by specific antigen, mitogen and anti-CD3 antibody. In further experiments, MIF release from mouse splenocytes was also induced by concentrations of staphylococcal exotoxin that were lower than those required to induce IL-2 or IFN-γ. Anti-MIF monoclonal Ab treatment inhibits T-cell proliferation and IL-2 production *in vivo* (Bacher et al., 1996). Investigations using Th1 and Th2 clones observed that MIF is produced predominantly by the Th2 subset of T helper cells. This finding is supported by the observation that anti-MIF mAb treatment decreases antibody production in mice. In collagen-induced arthritis in mice, anti-MIF treatment was associated with decreased levels of IgG_{2a}, compared to IgG_1 antibody subclasses, which is associated with decreased Th1 responsiveness (Mikulowska et al., 1997). In contrast, the demonstrated role of MIF in mechanisms of cell mediated immunity, such as delayed-type hypersensitivity and resistance to *Leishmania*, suggests an important contributory role of MIF in Th1 responses.

MIF secretion by T cells is also induced by glucocorticoids in a dose-dependent manner and has subsequently been shown to override glucocorticoid-mediated suppression of T cell proliferation (Bacher et al., 1996). The unique ability of MIF to override the inhibitory actions of glucocorticoids will be discussed further in Chapter 6.

1.6.2.2 Monocytes/Macrophages

The original description of MIF considered it a product of the activated T cell and its target of action the monocyte/macrophage. Recent studies, however, have identified the macrophage as an important source of MIF (Calandra et al., 1995; Calandra et al., 1994). High levels of MIF mRNA and protein are constitutively expressed in unstimulated macrophages. Macrophage MIF is released upon stimulation with various proinflammatory stimuli, including exotoxins, endotoxins and cytokines such

as IFN-γ and TNFα. Of note, the LPS concentration required to induce MIF mRNA levels in macrophages were at least two orders of magnitude lower than that required to induce TNFα. The release of MIF from cytoplasmic stores, prior to the initiation of new synthesis, suggests that MIF may be an 'early response' cytokine. Furthermore, the production of MIF in response to increasing concentrations of LPS follows a bell-shaped curve, decreasing at higher levels. This finding may be a protective defence mechanism of the host to prevent the detrimental effects of excessive amounts of MIF release.

Several lines of evidence suggest an important role for MIF in macrophage pro-inflammatory functions. At high levels, MIF is observed to induce TNFα secretion by macrophages and to synergise with IFN-γ to promote NO production. MIF has been observed to induce phagocytosis of foreign particles by macrophages in an autocrine and paracrine fashion (Onodera et al., 1997) and to enhance intracellular killing and H_2O_2 generation by macrophages. MIF also activates murine macrophages to kill *Leishmania major*, and this MIF-mediated activation is believed to be dependent on TNFα produced endogenously by macrophages, as well as iNOS (Juttner et al., 1998). The capacity for T cell supernatants to induce tumoricidal activity in cultured macrophages was reproduced in studies where recombinant MIF activated human macrophages to kill tumor cells (Churchill et al., 1975).

1.6.2.3 Eosinophils

Unstimulated human circulating eosinophils contain pre-formed MIF. Stimulation of eosinophils with PMA released significant quantities of MIF protein in a concentration and time-dependent manner. This stimulated release was blocked by the protein synthesis inhibitor cyclohexamide, and by protein kinase C inhibition, thereby implicating protein kinase C in the regulation of MIF secretion. Physiologic stimuli

including C5a and IL-5 were also observed to stimulate MIF release from eosinophils (Rossi et al., 1998). The potential importance of MIF as a modulator of eosinophil-dependent inflammatory disorders remains unresolved, although overexpression in subjects with asthma has been reported.

1.6.2.4 Other cells

MIF has also been identified in a variety of other cell types including vascular endothelial cells (Goebeler et al., 1991) and a transformed human B cell line (Wymann et al., 1999). Notwithstanding evidence demonstrating MIF mRNA and protein expression in various cell types, MIF expression is not ubiquitous. For example, granulocytes which are a prominent component of the host inflammatory response to invasion, lack MIF expression (Calandra et al., 1994). In spite of its widespread expression, MIF release is also tightly regulated, as demonstrated by its stimulus- and cell-specific secretion from monocytes/macrophages, pituitary cells and T cells (Bernhagen et al., 1993; Calandra et al., 1994; Bacher et al., 1996).

1.6.2.5 Neural and related tissues

In the rat brain, abundant expression of MIF has been detected in neurons of the cortex, hypothalamus, hippocampus, cerebellum and pons. MIF is prominently expressed in the hippocampus, which may provide an important link between MIF and glucocorticoid action. Intracisternal injection of LPS was observed to increase MIF mRNA and protein expression in a distribution that co-localised with other cytokines such as TNFα, IL-1β and IL-6 (Bacher et al., 1998). The presence of MIF within the hypothalamus also complements prior studies that identify MIF as an important constituent of the HPA axis. In addition to neurones and their processes,

MIF expression was also detected diffusely throughout the brain, implicating glial cells as a source of MIF expression (Bacher et al., 1998).

Studies have also reported MIF expression in cornea and retina, and that MIF is immunolocalised to the basal cells of corneal epithelium and endothelial cells (Matsuda et al., 1996; Matsuda et al., 1997b). Increased expression of MIF mRNA was observed during corneal wound healing after penetrating injury, concomitant with keratinocytes and lens epithelial cell differentiation. Immunohistochemical analysis identified MIF in the basal cells of corneal epithelium and endothelial cells before injury. At 24 hours following injury, positive MIF staining reappeared on the basal cells of the injured area, reaching its maximum at 48 hours and subsequently returning to the preinjured expression level. Moreover, levels of MIF in the contralateral eye remained unchanged. In the aqueous humor, however, the concentration of MIF was elevated in both the injured and contralateral eyes, suggesting an important role for MIF in sympathetic opthalmia (Matsuda et al., 1997a).

Recently, the expression of MIF mRNA and protein was detected in myelinated peripheral nerves. MIF mRNA and protein were detected in rat sciatic nerves, and immunohistochemical analysis identified Schwann cells as sources of MIF protein. Days following nerve transection, MIF expression was detected in axons as well as non-neuronal cells in proximal segments. The dramatic increase in MIF mRNA levels observed 12 hours after nerve transection, and the diminished levels several days after injury, suggests the involvement of MIF in nerve regeneration (Nishio et al., 1999).

1.6.2.6 MIF and the HPA axis

Studies by Bernhagen et al identified MIF as an abundant, pre-formed protein that is released by the anterior pituitary gland in response to pro-inflammatory stimulation (Bernhagen et al., 1993). In the anterior pituitary gland, MIF was found to localise to

corticotrophs and thyrotrophs. Within each cell type, a subset of granules was observed to contain both MIF and ACTH, or MIF and TSH. Following experimentally induced endotoxaemia, the pituitary content of MIF-containing granules decreased significantly. In addition, CRH was observed to be a potent MIF secretagogue *in vitro*, inducing the release of MIF from corticotrophic cells at concentrations lower than that required for ACTH release (Nishino et al., 1995). The joint release by CRH of MIF and ACTH, although at different concentrations of CRH, indicated that initiation of the stress response via the HPA axis involves the concomitant release of MIF. In addition, MIF protein was found to account for approximately 0.05% of the total pituitary protein content, in comparison to other pituitary hormones, such as ACTH and prolactin, being 0.2% and 0.08%, respectively. The observations that MIF is a proinflammatory cytokine produced by immune cells and is released, together with the glucocorticoid-stimulating hormone ACTH, by the corticotropic cells of the anterior pituitary gland, may appear contradictory. The subsequent finding, however, that glucocorticoids directly induce MIF release from T cells and macrophages resolves this paradox and implicates MIF as a glucocorticoid-induced counter regulator that acts to limit or fine-tune the potent immunosuppressive effects of glucocorticoids (Calandra et al., 1995).

1.6.2.7 Other tissues

MIF mRNA and protein expression have also been reported in cultured human granulosa cells. The concentration of MIF in follicular fluid significantly decreased as the follicular size increased, suggesting that MIF may regulate oocyte development (Wada et al., 1997). Expression of MIF mRNA and protein has been reported in the Leydig cells of the normal adult rat testis (Meinhardt et al., 1996; Meinhardt et al., 2000). Addition of recombinant MIF to cultured adult rat seminiferous tubules and

purified Leydig cells resulted in a dose-dependent decrease in the secretion of inhibin by the seminiferous tubules, indicating an important regulatory role for MIF in testicular function (Meinhardt et al., 1996). MIF has been detected in murine early embryos and reproductive organs. MIF mRNA was expressed in the ovary, oviduct and uterus during the preimplantation period and all stages of the estrus cycle (Suzuki et al., 1996). Morevoer, MIF mRNA was expressed in ovulated oocytes, zygotes, 2-cell embyros, 8-cell embryos and blastocytes. Although MIF is ubiquitously expressed in reproductive tissues, studies have established that the MIF knockout mouse have normal fertility, in addition to predictable litter sizes for heterozygous and homozygous matings.

MIF expression has been identified in human kidney, with predominant expression in renal tubular epithelial cells and to a lesser extent in Bowman's capsular epithelial cells (Imamura et al., 1996). The capacity of MIF to mediate immune and inflammatory processes in the kidney will be described in 1.6.4.4. The expression of MIF has been reported in primary cultured human keratinocytes and surgically obtained human epidermis, especially in the epidermal basal layer (Shimizu et al., 1996; Shimizu et al., 1999b). The involvement of MIF in immune responses involving the skin, namely DTH response will be further discussed in section 1.6.4.1.

Expression of MIF mRNA in murine osteoblasts and an osteoblastic cell line suggests the involvement of MIF in the regulation of bone metabolism (Onodera et al., 1996). MIF is detectable in normal human lung parenchymal tissue (Kayser et al., 1993). Until recently, knowledge of MIF expression in normal synovial tissue has been lacking, with the exception of one report of MIF expression in pseudosynovial tissues associated with loosening of prosthetic joints (Suzuki et al., 1996). The expression of MIF in human RA synovial tissue will be discussed in detail in section 1.6.5.1.

Moreover, the detection of MIF in diseases involving these tissues and organs will be discussed in sections 1.6.4-1.6.5.

1.6.3 MIF and glucocorticoids

The novel finding that glucocorticoids induce rather than inhibit MIF secretion was first described in macrophages (Calandra et al., 1995). The dose-response curve to glucocorticoids, however, was bell-shaped, where low concentrations of glucocorticoids induced MIF release, and high concentrations inhibited the secretion of MIF in mice, thereby reducing the counter-regulatory effect of MIF (Calandra et al., 1995). The capacity of glucocorticoids to both induce and suppress the production of molecules in a concentration dependent manner is consistent with the concept that physiologic glucocorticoids exert both permissive and inhibitory regulation of the immune-inflammatory response. Following from this concept, the capacity for MIF to override in a dose-dependent manner glucocorticoid inhibition of macrophage cytokines such as TNFα, IL-1β, IL-8 and IL-6, constituted the first evidence of the glucocorticoid counter-regulatory actions of MIF (Calandra et al., 1995). Further work revealed that MIF can completely overcome the protective effects of glucocorticoids in a mouse model of endotoxemia. Similar biphasic regulation of MIF expression has recently been shown in human RA FLS and is discussed in detail in section 1.6.5.1 (Leech et al., 1999). These findings prompted the study of the ability of MIF to override the inhibitory actions of glucocorticoids in human RA FLS, as presented in Chapter 6.

In antigen-specific T cell and B cell responses, low concentrations of glucocorticoids were found to induce MIF secretion from human or mouse T cells. When primed cells were incubated with antigen, MIF was found to override the glucocorticoid-mediated suppression of T cell proliferation and production of IL-2 and IFN-γ. The importance

of this observation was highlighted by the fact that in mice, the administration of neutralising anti-MIF antibodies at the time of immunisation with a soluble antigen, resulted in significant inhibition of the development of both antigen-specific T cells and a primary antibody response (Bacher et al., 1996).

Taken together, the findings that MIF is induced by low level glucocorticoids, while counteracting their action, suggests that MIF is an endogenous counterregulator of the inhibition of inflammation by glucocorticoids. This physiological role is supported by a recent study by Leech et al. demonstrating downregulation of tissue MIF in adrenalectomised rats (Leech et al., 2000).

1.6.4 MIF in animal models of disease

1.6.4.1 Delayed-type hypersensitivity (DTH)

Whilst MIF has had a long standing association with DTH, its role in this cell-mediated immune response has only recently been elucidated. MIF mRNA and protein are expressed prominently in DTH skin lesions in mice (Bernhagen et al., 1996). The predominant cellular origin was observed to be macrophages, and not T cells as originally hypothesized. The administration of anti-MIF Abs to mice significantly inhibited the development of DTH, in terms of cellular infiltration, vascular dilatation and dermal expansion. This study confirmed the essential role for MIF in the tuberculin DTH reaction, and established macrophages as an important source of MIF *in vivo*.

1.6.4.2 Endotoxemia and sepsis

Interperitoneal injection of endotoxin induces a rise in plasma MIF levels, accompanied by a fall in MIF pituitary content of MIF protein paralleled by a slower, time-dependent increase in the expression in the pituitary of MIF-encoding mRNA

(Calandra et al., 1995; Bernhagen et al., 1993). It is presumed that this response may reflect the replenishment of intracellular MIF stores by pituitary cells. The adminstration of recombinant MIF in this mouse model of septic shock significantly potentiated the lethal effects of endotoxin. Moreover, neutralising anti-MIF Ab was found to be fully protective to mice given an otherwise lethal injection of LPS. In response to LPS administration, significant quantities of pre-formed MIF were detected in various cells and tissues of the rats. Hours following LPS administration, loss of intracelluar MIF protein is accompanied by the induction of MIF mRNA and the restoration of intracellular MIF by 24 hours (Bacher et al., 1997).

Experiments in the recently reported murine MIF knockout model have confirmed that MIF -/- mice are resistant to the lethal effects of high dose bacterial LPS as well as to *Staphylococcus aureus* enterotoxin B (SEB), and had lower plasma levels of TNFα, but normal levels of IL-6 and IL-10. This reduction in TNFα observed in MIF deficient mice suggests that MIF may act upstream from TNFα. MIF deficient mice, however, were rendered susceptible to low doses of LPS by sensitising mice to the effects of TNFα using galactosamine, indicating that MIF is not required for the lethal effects of TNFα. Further studies showed that MIF -/- mice were resistant to the lethal effects of SEB with galactosamine (Bozza et al., 1999).

More recently, high concentrations of MIF were detected in the peritoneal exudate fluid and in the systemic circulation of mice with bacterial peritonitis (REF). Anti-MIF antibody protected TNFα knockout mice from lethal peritonitis induced by cecal ligation and puncture (CLP) (Calandra et al., 2000). Anti-MIF antibody also protected normal mice from lethal peritonitis induced by CLP as well as *Escherichia coli*, even when treatment commenced 8 hours following CLP. Conversely, co-injection of recombinant MIF and *E.coli* increased the lethality of peritonitis. In the plasma of

patients with severe sepsis or septic shock high concentrations of MIF were detected (Calandra et al., 2000). These studies, together, implicate a critical role for MIF in the pathogenesis of septic shock. A recent study, however, has reported that MIF is not crucial for LPS-induced endotoxaemia. In this study, administration of LPS into MIF-deficient mice caused shock (Honma et al., 2000). Upon LPS injection, no significant difference in LPS susceptibility or TNFα formation was detected between the MIF-deficient mutant and normal mice. In addition, the LPS-induced TNFα response of macrophages was suppressed by dexamethasone, although no difference was observed between the macrophages of the two strains. These findings suggest that endogenous MIF does not exert any significant effect on LPS-induced TNFα production or on glucocorticoid-mediated suppression.

1.6.4.3 Arthritis

Given that macrophages are implicated as the initiating antigen-presenting cell in collagen-induced arthritis (CIA)(Michaelsson et al., 1995), Mikulowska et al examined the role of MIF in a murine model of CIA. Treatment with neutralising anti-MIF Ab prior to immunisation led to delayed onset and lowered frequency of arthritis. This was associated with decreased IgG_{2a} responses to type II collagen with no differences observed in the production of IgG_1. This finding suggested that anti-MIF treatment may be associated with a decrease in Th1 responsiveness. Surprisingly however, anti-MIF treatment increased the overall T cell proliferative response to type II collagen (Mikulowska et al., 1997).

In adjuvant arthritis (AA) in rats, Leech et al reported that MIF was detected in AA synovial tissue, predominantly in synovial lining cells, including macrophages and fibroblast-like synoviocytes. Levels of MIF were increased in established AA sera and cultured synovial macrophages. Treatment with anti-MIF Ab led to a profound, dose-

dependent inhibition of AA in terms of clinical score, paw swelling, and synovial lavage leukocyte numbers, and also resulted in reduced synovial macrophage and T cell accumulation (Leech et al., 1998).

1.6.4.4 Glomerulonephritis

In the normal kidney, MIF is constitutively expressed in both glomerular and tubular epithelial cells. During the development of rat crescentic anti-glomerular basement membrane glomerularnephritis, MIF expression by glomerular and tubular epithelial cells is markedly upregulated. Upregulation of MIF expression correlated with glomerular macrophage accumulation. MIF expression by *in situ* hybridisation co-localised to macrophages, T cells and fibroblast-like cells within the glomerulus (Lan et al., 1996).

MIF has been reported to have a pathogenic role in immunologically-induced kidney disease in the rat. In contrast to control Ab-treated rats, anti-MIF treatment substantially reduced proteinuria, prevented the loss of renal function, significantly reduced histological damage including glomerular crescent formation, and significantly inhibited renal leukocytic infiltration and activation. Importantly, anti-MIF administration did not effect the secondary antibody response or immune deposition within the kidney (Lan et al., 1997). Additional studies by the same group observed that anti-MIF Ab treatment partially reversed rat crescentic glomerulonephritis by restoring normal renal function and reducing histological damage compared with untreated animals. Importantly, anti-MIF Ab treatment was associated with increased serum corticosterone levels (Yang et al., 1998).

Lan et al also sought to determine whether TNFα upregulates renal MIF expression in rat crescentic glomerulonephritis, using soluble TNFα receptors to antagonise TNFα. TNFα receptor treatment almost completely abrogated MIF mRNA and protein

expression in established rat crescentic glomerulonephritis, and also inhibited glomerular and interstitial macrophage infiltration, suppressed renal injury and histological damage. These findings suggest that TNFα or TNFα-mediated inflammatory events upregulate MIF production in immunologically induced renal disease (Lan et al., 1997).

MIF has also been detected in non-immunologically induced kidney disease in the rat. In a model of lipid-induced glomerular injury, upregulation of MIF mRNA expression by intrinsic glomerular cells is detected prior to macrophage infiltration. Consistent with immunologically induced kidney disease, this study provides evidence that local synthesis of MIF influences pathogenic processes in the kidney (Hattori et al., 1999).

1.6.4.5 Lung injury in the rat

In a rat model of acute lung injury, anti-MIF Ab significantly attenuated LPS-induced migration of neutrophils in alveoli. MIF immunostaining was detected in bronchial epithelial cells and alveolar macrophages even in the absence of LPS. Pre-treatment with anti-MIF Ab significantly attenuated neutrophil accumulation, which may correlate with the marked reduction in levels of macrophage inflammatory protein-α (MIP-1α) (Makita et al., 1998).

1.6.4.6 Models of neoplasia

In a murine model of B cell lymphoma, anti-MIF mAb significantly reduced the growth and the vascularization of the lymphoma, implicating a key role for MIF in tissue angiogenesis (Chesney et al., 1999).

The ability of MIF to influence cellular proliferation was indirectly examined in a rat model of galactose cataract induction. Lens epithelial mRNA expression was increased 12 fold in galactosemic rats compared to controls and associated with

enhanced lens epithelial cell proliferation (Wen et al., 1996). In a murine colon carcinoma cell line, MIF protein and mRNA were highly expressed. Transfecting an antisense MIF plasmid into these cells significantly suppressed cell proliferation (Takahashi et al., 1998). Furthermore, administration of anti-MIF Ab into tumor bearing mice was found to significantly suppress tumor-induced angiogenesis, suggesting that MIF is involved in the growth and invasion of melanoma, in association with neovascularisation (Shimizu et al., 1999a).

With considerable evidence involving MIF in animal models of inflammatory and immune disease, corresponding evidence of its contribution to human disease is now being sought.

1.6.5 MIF in human disease

1.6.5.1 Rheumatoid arthritis

Leech et al were first to report MIF expression in RA synovial tissue (Leech et al., 1999). In this study, MIF was detected in RA synovial cells, including macrophages and fibroblast-like synoviocytes. Constitutive MIF mRNA expression was detected in unstimulated cultured RA FLS, which were observed to release abundant MIF. Serum, synovial fluid and FLS intracellular MIF were significantly elevated in RA patients compared to controls. Interestingly, FLS MIF was not increased by exposure to IL-1β. TNFα, or IFN-γ. Biphasic regulation of MIF by glucocorticoids was also noted in cultured RA FLS in that low concentrations induced, while high concentrations inhibited, synoviocyte MIF expression. RA FLS-conditioned medium induced peripheral blood mononuclear cell TNFα release, and this induction was significantly inhibited by anti-MIF mAb, suggesting that MIF is an upstream regulator of TNFα release in RA.

In accordance with Leech et al (Leech et al., 1999), other groups have subsequently observed high expression of MIF in the synovial tissues of RA joints, demonstrating increased MIF protein expression in RA synovial fluids, compared to OA and controls (Onodera et al., 1999). Together, the existing data suggest a key role for MIF in RA pathogenic processes.

The novel finding that peripheral blood mononuclear cell TNFα release was induced by culture in RA FLS conditioned medium, and that this induction was significantly inhibited by anti-MIF mAb, suggests that MIF is an upstream regulator of TNFα release in RA. These findings of MIF expression in RA FLS, and the reported ability of MIF to counter-regulate the effects of glucocorticoids in cytokine-treated cells, provided the inspiration to undertake studies investigating the effects of MIF on RA FLS eicosanoid generation (chapters 4-6).

More recently, MIF has been shown to upregulate expression of MMPs in RA FLS (Onodera et al., 2000). In this study, mRNA levels of interstitial collagenase, stromelysin and IL-1β were increased following stimulation by MIF. Similarly, a slight upregulation of TIMP by MIF was observed. Interestingly, the upregulation of collagenase and stromelysin mRNA observed in OA FLS were much lower compared to RA FLS. These findings suggest that MIF plays an important role in tissue destruction of RA joints via induction of proteinases.

1.6.5.2 Acute respiratory distress syndrome

Significant quantities of MIF have been identified in the alveolar air spaces of patients with acute respiratory distress syndrome (ARDS). MIF was observed to augment the release of TNFα and IL-8 by alveolar cells, and this was attenuated by anti-MIF mAb treatment. In addition, MIF was found to override the anti-inflammatory effects of glucocorticoids on cytokine release by these alveolar cells, supporting a

glucocorticoid counter-regulatory role for MIF in human disease (Donnelly et al., 1997).

1.6.5.3 Other human diseases

As detailed in Table 1, MIF expression has been detected in other human pathologic conditions.

Human disease	Reference
Tuberculosis, bronchial carcinoma, sarcoidosis, ideopathic interstitial pneumitis	(Kayser et al., 1993)
Drug-induced urticaria, angiodema	(Livni et al., 1999)
Uveitis	(Kitaichi et al., 1999)
Metastatic prostate cancer	(Meyer-Siegler and Hudson, 1996)
Pituitary adenomas	(Tampanaru-Sarmesiu et al., 1997)
Renal allograft rejection	(Lan et al., 1998)

1.6.6 MIF and intracellular signalling mechanisms

With a membrane receptor for MIF not yet identified, the principal molecular pathways mediating MIF function remains unresolved. In a murine fibroblast cell line, upregulation of cell proliferation by MIF was associated with the activation of the p44/42 ERK MAP kinases (Mitchell et al., 1999). Moreover, the activation of these kinases was dependent on protein kinase A activity. In these cells, MIF regulated $cPLA_2$ via an ERK-dependent pathway.

Much knowledge of NFκB signalling mechanisms in synovial cells arises from studies examining the effects of IL-1. Like IL-1, MIF is a pleiotropic cytokine with known actions in a range of immune and inflammatory processes. Considering the broad spectrum of actions mediated by MIF, these findings by Mitchell et al prompted the hypothesis that MIF regulates RA FLS eicosanoid generation via a signalling pathway involving NFκB. Results of these experiments are presented in Chapter 6.

More recently, MIF was observed to upregulate collagenase, stromelysin and IL-1β mRNA in cultured RA FLS. These events were preceded by upregulation of c-jun and c-fos mRNA. Inhibitors of tyrosine kinase, protein kinase C and AP-1 suppressed mRNA upregulation by MIF. Conversely, the addition of a c-AMP dependent kinase inhibitor, or IL-1 receptor antagonist, failed to inhibit the mRNA upregulation (Onodera et al., 2000). These findings suggest that MIF upregulates collagenase and stromelysin via tyrosine kinase-, protein kinase C-, and AP-1-dependent pathways, and is not dependent on IL-1β. Clearly, increased knowledge of signal transduction mechanisms utilised by MIF is needed to provide further understanding of its role in immune and inflammatory processes.

1.7 Summary and conclusions

Rheumatoid arthritis is a chronic inflammatory disease of unknown aetiology. Current theories suggest that synovial hyperplasia and inflammation in the rheumatoid synovium result from altered systemic immune responses. These events are thought to be mediated by an interaction between synovial lining cells, immune-derived cells and their products. Glucocorticoids have a profound effect on these aspects of inflammation, and this finding has resulted in their widespread use in the treatment of RA over the last 50 years.

Recently, advances in understanding the clinical utility of glucocorticoids have been matched by scientific developments investigating the mechanisms of glucocorticoid actions in inflammation. Annexin I is an anti-inflammatory, glucocorticoid-inducible protein involved in the therapeutic regulation of inflammation by glucocorticoids and in inflammatory autoregulation. In contrast, the pro-inflammatory cytokine, macrophage migration inhibitory factor (MIF), is also released in response to glucocorticoids. This cytokine exhibits the unique capacity to counter-regulate glucocorticoid effects.

The contribution of glucocorticoids and these mediators in the control of RA inflammatory processes requires further understanding. In accordance with this, studies presented in this thesis examine the effect of annexin I, glucocorticoids and MIF in the regulation of inflammation and other functions relevant to RA synovial cells.

CHAPTER TWO: ANNEXIN I SURFACE BINDING SITES AND THEIR REGULATION ON HUMAN FIBROBLAST-LIKE SYNOVIOCYTES.

2.1 Chapter Summary

Annexin I (or lipocortin 1) is a glucocorticoid-inducible protein, whose expression in rheumatoid synovium and inhibitory actions in animal models of arthritis suggests its involvement in human arthritis. The potential for annexin I to mediate its anti-inflammatory actions via specific cell surface binding sites on human fibroblast-like synoviocytes (FLS) has not been directly explored.

Annexin I binding sites on human osteoarthritic (OA) and rheumatoid (RA) FLS were determined by ligand-binding flow cytometry. Elastase activity released in FLS supernatants was measured as release of *p*-nitroaniline. Phospholipase A_2 (PLA_2) activity was measured by ^{3}H-arachidonic acid release.

OA and RA FLS were incubated with annexin I (0-10 μM) and demonstrated concentration-dependent annexin I binding site fluorescence, with more than 99% of OA FLS exhibiting surface binding at 10 μM annexin I. In RA FLS, annexin I binding was notably lower, being 92% at 10 μM annexin I. These observations led to the examination of binding site regulation on human FLS in response to proteolytic enzymes and proinflammatory cytokines. FLS annexin I binding sites were not affected by elastase or a specific elastase inhibitor, and elastase release did not differ between RA and OA cells. In contrast, collagenase increased annexin I binding sites on OA ($p < 0.03$) and near-significantly on RA ($p = 0.06$) FLS. TNFα increased annexin I binding sites on OA ($p < 0.03$) and RA FLS ($p < 0.05$). IL-1β increased annexin I binding on OA FLS ($p < 0.03$) and a non-significant trend was observed in RA FLS ($p = 0.12$). Dexamethasone (DEX) exerted no significant effect on OA or RA FLS annexin I binding sites. Treatment of RA FLS with an annexin I N-terminal peptide significantly inhibited RA FLS PLA_2 activity ($p < 0.01$).

This is the first study to demonstrate annexin I binding sites on human fibroblast-like synoviocytes, with notably reduced annexin I binding sites observed on RA FLS compared to OA FLS. The results indicate regulation of annexin I binding sites on human FLS in response to proinflammatory cytokine and enzyme treatment. These data suggest that annexin I binding site capacity can be altered at sites of inflammation. Reduced annexin I binding sites in RA FLS may impair sensitivity of certain pro-inflammatory events to glucocorticoids.

2.2 Introduction

Annexin I (previously known as lipocortin-1) is a 37 kDa glucocorticoid-inducible protein, which belongs to the annexin family of at least 13 structurally related proteins that bind to anionic phospholipids in a Ca^{2+}-dependent manner (Goulding et al., 1996). The involvement of annexin I in the regulation of joint inflammation has been deduced from its expression in human arthritic synovium, leukocytes, and from several rat models of arthritis (Yang et al., 1998; Yang et al., 1997; Yang et al., 1999). To date, the molecular mode of action of annexin I remains unknown. Considerable evidence suggests that annexin I is exported from target cells, enabling it to bind to cell surface receptors and exert its biological actions. For example, annexin I is translocated to the cell surface in response to glucocorticoids (Solito et al., 1991; Solito et al., 1994). Moreover, anti-annexin I antibodies, which are unable to penetrate the cell, deplete intracellular annexin I and significantly reverse the anti-inflammatory effects of glucocorticoids (Yang et al., 1997; Yang et al., 1999). Finally, the blockade of cell surface annexin I binding sites by inactivated annexin I protein prevents the biological activity of extracellular annexin I peptide (Perretti et al., 1993a). Saturable, specific annexin I binding sites were first identified on human peripheral blood leukocytes (Goulding et al., 1990). At sites of inflammation *in vivo* and in rheumatoid (RA) peripheral blood, leukocyte annexin I binding sites are markedly reduced (Perretti et al., 1993a; Goulding et al., 1992). The demonstration that annexin I is expressed in RA synovium, in addition to its role in joint inflammation, led to the examination of annexin I binding sites on human FLS obtained from RA and osteoarthritic (OA) subjects. The finding of lower annexin I binding on RA FLS led to the investigation of factors regulating annexin I binding sites on FLS.

2.3 Methods

2.3.1 Culture Media

RPMI 1640 (ICN Laboratories, USA) was prepared from 10x concentrated liquid media, diluted and reconstituted with sterile endotoxin-free water (Baxter Healthcare Pty Ltd, NSW, Australia). Media were supplemented with (final concentrations) penicillin 50 IU/ml, streptomycin 50 mg/ml and L-glutamine 2mM (all purchased from ICN). Where noted, media were supplemented with foetal calf serum (FCS, TRACE Biosciences Pty Ltd, Melbourne, Australia). Serum was routinely heat inactivated by immersion in a 55°C water bath for 30 minutes.

2.3.2 Solutions and Buffers

Phosphate buffered saline (PBS) solution

Ca^{2+} and Mg^{2+}-free PBS was prepared from powder (ICN Laboratories, Australia), and contained (final concentrations) NaCl 8000 mg/l, Na_2HPO_4 1150 mg/l, KCl 200 mg/l, and KH_2PO_4 200 mg/l.

Hank's buffered saline solution (HBSS)

HBSS was prepared by dissolving (final concentrations) NaCl (8000 mg/l), KCl (400 mg/l), $CaCl_2.2H_2O$ (186 mg/l), $MgSO_4.7H_2O$ (200 mg/l), Na_2HPO_4 50 mg/l), KH_2PO_4 60 mg/l), $NaHCO_3$ 350 mg/l), $C_6H_{12}O_6$ (1000 mg/l) in 1L with sterile endotoxin-free water. The pH was adjusted to 7.6 with HCl and filtered using a 0.2 μm filter.

EDTA/PBS

1 mM EDTA/PBS was prepared by dissolving EDTA (74.5 mg/l) in 1L of PBS.

0.1% BSA/PBS/1.3 mM $CaCl_2$ (PBC)

PBC solution was prepared by dissolving $CaCl_2$ (Sigma) in a solution of PBS containing 0.1% Bovine serum albumin (BSA).

Flow cytometry (FACS) wash buffer

Washes between incubations in flow cytometry labelling procedures was performed using a solution of PBS containing 1% BSA and 0.01% sodium azide (Sigma).

Tris buffered saline (TBS) solution

TBS was prepared by dissolving (final concentrations) Tris HCl (2220 mg/l, Sigma), Trizma base (1330 mg/l, Sigma), NaCl (5840 mg/l, Sigma) and 5 µl of Brij 35 (Sigma) in 1L with sterile endotoxin-free water. The pH was adjusted to 8.0 with HCl.

2.3.3 Purification of annexin I protein

2.3.3.1 Annexin I production

Using the fission yeast *Schizosaccharomyces pombe* as an expression vector, human annexin I was produced by Dr Giga-Hama (Giga-Hama et al., 1994) and kindly donated. This protein was generated as follows. Human annexin I cDNA (1,300 bp) was isolated from the cDNA library prepared from human fibroblasts and was inserted into the human cytomegalovirus (hCMV) promoter in a pRL2L vector that contains a neomycin resistance gene under the control of the SV40 promoter. The vector was then transfected to *S.pombe* and transformed yeast cells were grown at 32°C in a shaker flask in medium containing antibiotic G418 for 3 days. The increasing copies of annexin I was followed by increasing concentrations of antibiotic G418. Yeast cells were disrupted and centrifuged at 8000 x g for 10 minutes. The clear supernatant was stored at 4°C. Annexin I at levels exceeding 50% of the soluble cellular protein was obtained by this method. The purified protein has been

demonstrated to be equivalent to its native counterpart with respect to antigenicity and biochemical properties such as phospholipase A_2 inhibition, actin binding and N-terminal acetylation.

2.3.3.2 Annexin I protein purification

Crude human annexin I protein extract was purified by mAb affinity gel chromatography by my colleague Dr Yuan-Hang Yang. Mouse anti-human annexin I mAb was covalently coupled to Affi-Gel 10 (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Briefly, 6 ml of slurry (approximately 3 ml neat gel) was transferred to a glass funnel. After draining the supernatant solvent, the gel was washed with three bed volumes of cold 10 mM sodium acetate (pH 4.5, Sigma) and transferred into a 10 ml test tube. 4.5 ml of antibody ligand (21 mg) in 100 mM HEPES buffer (pH 8.0, Sigma) was added and rotated at 4°C overnight. To block any active esters, 1 M glycine (pH 8.0, Sigma) was added and incubated at 4°C for 4 hours. Under these conditions, 85% of total mAb was absorbed to the solid support, as determined by measuring the unbound protein at O.D. 280 nm. The gel was then transferred to a disposable column and processed by washing in 10-column volumes of 100 mM HEPES (pH 8.0), 20-column volumes of 10 mM HCl, 10-column volumes of PBS (pH 8.5) containing 0.5 M NaCl. For immunoaffinity purification of annexin I, crude extract was added to 3 ml of gel, and the mixture was incubated overnight at 4°C using a disposable column. The column was washed with 40 ml of PBS (pH 8.5) and bound annexin I was eluted in 10 ml of 10 mM HCl. The eluate was neutralised immediately with 50 mM Tris-HCl (pH 8.9), and dialyzed against PBS. The recovered protein has a concentration of 150-200 μg/ml by measuring OD at 280 nm. The samples were dissolved in sample buffer for SDS-PAGE and separated on a 12% gel. A single protein band was visualized by Western blotting with an anti-

annexin I mAb at a molecular weight of 37 kDa. Aliquots of annexin I protein were stored at –70°C and thawed immediately prior to use.

2.3.4 Antibody purification

2.3.4.1 Production of murine monoclonal anti-annexin I antibodies

Annexin I monoclonal antibodies were produced by hybridomas of annexin IB (1B), which was kindly donated by Dr J Browning (Biogen Inc, Cambridge, MA). One ml of frozen mAb hybridoma cells of 1B was cultured in serial dilutions in DMEM/20% FCS and placed in 24 well plates at 37°C and 5% CO_2 in a humidified incubator. Confluent hybrid cells were transferred into flasks and propagated until confluent for antibody production. Once confluent, cell lines were passaged and the supernatant was stored at -20°C. Pelleted cells were collected and washed twice with serum free medium at a concentration of 4 x 10^6/ml. Five hundred µl of cell suspension was intraperitoneally injected into BalB/c mice which were previously immunised with 150 µl of incomplete Freund's adjuvant (IFA, Sigma) or pristane (Sigma) one to two weeks prior to hybridoma cell injection. Ascites developed from one to two weeks later, as indicated by bloating of the abdomen. Mice were killed and ascites collected by lavage of the peritoneum. Protein contents of ascitic fluids were measured at OD 280 nm. The Animal Ethics Committee of Monash University approved all studies using mice.

2.3.4.2 Purification of anti-annexin I mAb

Mouse ascites at a protein concentration of 20-30 mg/ml were subjected to a purification procedure using saturated (50%) ammonium sulfate precipitation (SAS) and a Protein A-Sepharose affinity chromatography (Pharmacia). Three ml of SAS cut product was filtered through 0.2 µm filter and pipetted onto an immobilising protein

G column which was equilibrated with binding buffer (0.05 M Na_3BO_3, 0.15 M NaCl, pH 8.0). The bound protein was then eluded with elude buffer (0.1 M glycine HCl, pH 2.8). The fractions with the highest protein content, as assessed by absorption at 280 nm were then dialyzed against PBS. The 1B mAb used in flow cytometry applications described in this thesis was reconstituted with PBS. Aliquots of 1B were stored at -70°C and thawed immediately prior to use.

2.3.5 Synthesis of annexin I N-terminal peptide, ac 2-26

The bioactive N-terminal 2-26 amino acid fragment of annexin I (acetyl-AMVSEFLKQAWFIENEEQEYVQTVK-OH) (Perretti et al., 1995) was prepared by use of solid phase step-wise synthesis and obtained from Prof Milton Hearn (Monash University, Melbourne, Australia). Purity was more than 99% as determined by HPLC. Annexin I fragment was stored at -20°C and thawed immediately prior to use.

2.3.6 Isolation of fibroblast-like synoviocytes

Fibroblast-like synoviocytes were obtained from synovium of osteoarthritic (n=6) and rheumatoid (n=6) patients undergoing joint replacement surgery. All patients satisfied the American College of Rheumatology criteria for the classification of OA (Altman et al., 1991; Altman et al., 1986) and RA (Arnett et al., 1987). All procedures involving human subjects were approved by the appropriate hospital, institutional, and/or university human research ethics committees prior to commencement. Human synovial tissue specimens from OA and RA patients were delivered sterile at 4°C and processed immediately. All procedures were undertaken in a Class II laminar flow biosafety hood under aseptic conditions.

FLS were isolated and cultured as described by Koch et al (Koch et al., 1986). The synovial lining tissue was dissected and minced into 2-3 mm pieces and rotated in 10

ml/2 g of enzyme solution containing 2.4 mg/ml Dispase (grade II, 5 U/mg, Boehringer Mannheim, Sydney, Australia), 1 mg/ml collagenase (type II, Sigma) and 1 mg/ml DNase (type I, Boehringer Mannheim) in Ca^{2+} and Mg^{2+} free Hank's balanced saline solution.

The tissue suspension was gently stirred for 1 hour at 37°C. To stop the reaction, an equal volume of RPMI /20% FCS was added and placed directly on ice. The digests were sequentially filtered through 60 μm and 150 μm meshes and dispensed into centrifuge tubes containing RPMI/20% FCS. Isolated cells were washed three times with RPMI by repeat centrifugation at 300 x g. Cells were plated onto 75 cm^2 tissue culture plates at a density of approximately 10^6 cells/8 ml RPMI/20% FCS, and cultured at 37°C in a 5% CO_2 humidified incubator. Medium was replaced with fresh RPMI/20% FCS every 2-3 days.

2.3.7 Propagation of fibroblast-like synoviocytes

As proliferating synoviocytes approached 90% confluence, a single cell suspension was obtained by incubating adherent cells with 3 ml of 1x Trypsin/EDTA solution (ICN) for 5 mins at 37°C in 5% CO_2. Cells were passaged with a 1:3 split and were frozen in liquid nitrogen at passage 3. Cells at 3rd passage were more than 99% FLS, as defined by dendritic, spindle morphology, and negative staining for the pan-leukocyte antigen CD45 by flow cytometry. Thawed cells were used in experiments between passages 4 and 9.

2.3.8 Culture of fibroblast-like synoviocytes

For all experiments, FLS were seeded at 1 x 10^5 cells per well in 24 well culture plates in RPMI /10% FCS and allowed to adhere overnight, prior to medium being replaced with RPMI/0.1% BSA (Sigma) for 24 hours for experimental purposes. Cells

were > 95% viable by trypan blue exclusion. In each group of experiments, n refers to the number of individual human OA and RA donor FLS used.

2.3.9 Detection of annexin I binding sites

FLS were examined for saturable specific annexin I binding sites, as described by Perretti et al (Perretti et al., 1993). Cells were treated with 0.1 ng/ml human recombinant IL-1β, 10 ng/ml human recombinant TNFα, 10^{-7} M DEX for 24 hours, or elastase 10 μg/ml, ONO-5046 (elastase inhibitor, 10 μM, a generous Gift from Dr Mauro Perretti, The William Harvey Research Institute, London, UK), collagenase 5 U/ml, 250 μg/ml tryspin for 30 mins. Unless specified, all reagents were purchased from Sigma. Duplicate cultures were used for each determination.

Surface bound annexin I was removed by washing FLS with 1 mM EDTA/PBS. FLS were then washed with 0.1% BSA/PBS/1.3 mM $CaCl_2$ (PBC), trypsinized and resuspended in 20 μl RPMI/0.2% BSA. The following procedures were undertaken at 4°C. FLS were plated onto flat bottomed 96 well tissue culture plates at 1 x 10^5 cells/well and incubated with 0-10 μM of human recombinant annexin I for 60 mins. FLS were washed, then incubated with 60 μg/ml annexin I monoclonal antibody 1B (or matched isotype control IgG_{2a}, Silenus, Australia), for 30 mins, in the presence of 5 mg/ml human IgG (Intragam, CSL Pty Ltd, Australia), to block non-specific binding sites. After washing, FLS were incubated with FITC-conjugated anti-mouse IgG (Silenus) for 30 mins and annexin I binding sites on FLS were analysed by flow cytometry.

Each determination was performed in duplicate, with annexin I binding represented as a percentage (%) or mean fluorescence intensity (MFI) following substraction of negative control antibody fluorescence.

2.3.10 Elastase release assay

RA and OA FLS were incubated in 0.1% BSA/RPMI, for 8 hours. Elastase activity released from FLS supernatants was measured as described by Perretti et al (Perretti et al., 1995). A 50 µl aliquot of sample or standard human leukocyte elastase (Sigma) was added to a 96-well culture plate containing TBS buffer (pH 8.0). After mixing at room temperature, the specific elastase substrate, methoxy-Suc-Ala-Ala-Pro-Val-p-nitroanilide (1 mM, Sigma) in N-methyl-2-pyrrolidinone (Sigma) was added, and incubated for 2 hours in TBS buffer at 37°C. The reaction was terminated by the addition of 100 µl of 50% (v/v) aqueous acetic acid (Sigma). Duplicate cultures were used for each determination. The concentration of *p*-nitroaniline released at 405 nm (Dynatech MR7000, Dade International, Sydney, Australia) was calculated from a standard curve (0-50 nmol *p*-nitroaniline). Elastase activity in FLS supernatants was expressed as *p*-nitroaniline formed in nmol/min/10^5 FLS.

2.3.11 Flow cytometric analysis

Labelled FLS were analysed on a Cytomation Mo-Flo flow cytometer (Cytomation, Fort Collins, CO, USA) with a single protocol used in each group of experiments. Each determination was performed in duplicate. The viable cell population of interest was gated from debris on the basis of forward and 90 degree light scatter characteristics and FITC fluorescence measured. At least 5000 events were analysed for each sample and logarithmic amplification of the signal was used. Proportions of cells positive for the phenotype-antigen labelling are expressed as the percentage of cells exhibiting fluorescence above 5% cutoff gates set with isotype control antibody labelling, and mean fluorescence intensity (MFI) expressed following substraction of negative control antibody fluorescences.

2.3.12 Assessment of phospholipase A_2 activity

PLA_2 activity in FLS was determined according to Croxtall et al (Croxtall et al., 1995). FLS were labelled for 18 h with 1 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (Amersham International plc, Sydney, Australia) in 0.5 ml of 0.1% BSA/RPMI. Cells were treated with 0-200 μg/ml annexin I ac2-26 for 8 hours. Duplicate cultures were used for each determination. To quantify released [^{3}H]arachidonic acid following treatment, 0.4 ml of incubation medium was aspirated and transferred to tubes containing 1.5 ml of Ultima-Gold scintillation fluid (Packard Instrument Co, Inc, IL, USA). Radioactivity in the supernatant was determined using a Wallac 1409 liquid scintillation counter (Pharmacia, Finland), with results expressed as [^{3}H]arachidonic acid released (cpm).

2.3.13 Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed using the Mann-Whitney and Wilcoxon tests for unpaired and paired data respectively, with values of $p < 0.05$ regarded as statistically significant.

2.4 Results

2.4.1 Detection of annexin I binding sites on OA and RA FLS

Initial investigations sought to determine the presence of annexin I binding sites on OA FLS. Results demonstrated concentration-dependent annexin I binding, with more than 99% of cells exhibiting cell-surface annexin I fluorescence at 10 μM annexin I (n = 6) (Figure 2.6.1, Table 2.6.1).

Additional studies addressed the presence of annexin I binding sites on RA FLS. RA FLS also exhibited concentration-dependent annexin I binding. Annexin I binding site fluorescence was significantly lower on RA FLS than OA FLS at all concentrations of annexin I ($p < 0.01$) (Table 2.6.1, Figure 2.6.2).

2.4.2 Analysis of elastase release in human FLS

The presence of reduced annexin I binding sites on RA FLS led to the concept that pro-inflammatory mediators might downregulate binding site numbers on FLS. As Perretti et al (Perretti et al., 1995) demonstrated reduced leukocyte annexin I binding after exposure of cells to elastase, subsequent studies addressed the hypothesis that elastase constitutively released by RA FLS might contribute to reduced annexin in binding sites on these cells. Elastase release by RA (84 ± 7 nmol *p*-nitroaniline/10^5 cells/min) and OA (77 ± 4) FLS was not significantly different.

2.4.3 Enzyme regulation of annexin I binding sites on human FLS

To further explore potential enzymatic regulation of annexin I binding sites on FLS, cells were treated with elastase, trypsin and collagenase. Elastase (10 μg/ml) had no significant effect on either OA (1916 ± 340) or RA (1354 ± 186) annexin I binding

site numbers. Moreover, the specific elastase inhibitor ONO-5046 (Iwamura et al., 1993) had no significant effect on OA (1842 ± 441) or RA (1077 ± 79) binding sites. Trypsin (250 μg/ml) had no effect on OA (1158 ± 251) or RA (954 ± 238) annexin I binding sites. In contrast, collagenase (5 U/ml) significantly increased the number of annexin I binding sites on OA FLS ($p < 0.03$), with a similar trend seen in RA FLS ($p = 0.06$) (Figure 2.6.3).

Thus no evidence was obtained to suggest that FLS-derived enzymes could contribute to the lower annexin I binding site numbers on RA cells.

2.4.4 Cytokine regulation of annexin I binding sites on human FLS

Investigations next examined the effect of the pro-inflammatory cytokines IL-1β and TNFα. TNFα (10 ng/ml) increased annexin I binding sites on both OA ($p < 0.03$) and RA FLS ($p < 0.05$) (Figure 2.6.3). IL-1β (0.1 ng/ml) also significantly increased annexin I binding sites on OA FLS ($p < 0.03$) and there was a trend towards increased annexin I binding on RA cells ($p = 0.12$) (Figure 2.6.3).

2.4.5 Dexamethasone regulation of annexin I binding sites on human FLS

Glucocorticoids are key regulators of annexin I expression, although no study has addressed the effects of glucocorticoids on cell surface binding sites. The regulatory effect of glucocorticoids on annexin I binding sites on human FLS was determined using the synthetic glucocorticoid, dexamethasone (DEX). Dexamethasone (10^{-7} M) had no significant effect on OA (1297 ± 316) or RA FLS (1216 ± 175) annexin I binding sites.

2.4.6 Effect of annexin I N-terminal peptide, ac 2-26 on constitutive PLA_2 activity

The demonstration of the presence of annexin I binding sites on the cell surface of FLS raises the hypothesis that annexin I modulates synoviocyte activation. Among the

most important functions of FLS in arthritis is the production of arachidonic acid, mediated by the action of phospholipase A_2 (PLA_2). We therefore examined the effects of annexin I on PLA_2 activity in RA FLS, using a bioactive N-terminal annexin I peptide ac2-26 (Perretti et al., 1995). Constitutive PLA_2 activity was present in RA FLS, and annexin I ac2-26 treatment (100-200 μg/ml) significantly reduced PLA_2 activity ($p < 0.01$) (Figure 2.6.4).

2.5 Discussion

Glucocorticoid treatment was first established 50 years ago, but the potent anti-inflammatory and immunosuppressive mechanisms of action of glucocorticoids are still not fully elucidated. The hypothesis that annexin I is an anti-inflammatory mediator in arthritis originated from the detection of annexin I in human rheumatoid synovium and infiltrating leukocytes (Goulding et al., 1995; Yang et al., 1997). This has been supported by studies in animal models of arthritis. Administration of a bioactive annexin I N-terminal peptide substantially inhibited carrageenan-induced arthritis whilst anti-annexin I antibody exacerbated arthritis severity (Yang et al., 1997). In the rat adjuvant arthritis model, annexin I neutralization also exacerbated disease severity and increased synovial production of PGE_2 and TNFα (Yang et al., 1999). In both these models, annexin I was shown to be necessary for the therapeutic effect of exogenous glucocorticoids, in that monoclonal antibody antagonism of annexin I prevented the anti-inflammatory effect of DEX (Yang et al., 1997; Yang et al., 1999). Annexin I is also necessary for the effect of glucocorticoids on adjuvant arthritis synovial macrophage nitric oxide generation (Yang et al., 1998). Glucocorticoids induce annexin I synthesis and export to the cell surface (Yang et al., 1998; Solito et al., 1994). Several lines of evidence suggest that cellular exportation of annexin I is imperative for its biological function, by allowing access to cell surface

binding sites and thereby exerting its anti-inflammatory effect in a paracrine or autocrine manner. This concept originated from the identification of specific annexin I binding sites on peripheral blood leukocytes (Goulding et al., 1996). Moreover, extracellular anti-annexin I mAb deplete intracellular annexin I (Yang et al., 1999), and blockade of cell surface binding sites by inactivated annexin I prevents the biological activity of annexin I N-terminal peptide (Perretti et al., 1993a). The anti-inflammatory actions of glucocorticoids are specifically reversed by anti-annexin I mAb, despite annexin I being virtually undetectable in sera and extracellular fluids. In spite of this evidence, the mechanism(s) whereby annexin I is exported are unclear, since the protein lacks a signal sequence and is therefore unlikely to access secretory vesicles for release via the conventional method of exocytosis. In conjunction, the molecular identity of the annexin I binding site has not yet been elucidated.

Flow cytometric analysis has proved invaluable in the identification of specific, saturable cell surface annexin I binding sites on human and murine peripheral blood monocytes and neutrophils, and on rat pituitary cells (Goulding et al., 1992; Goulding et al., 1996; Perretti et al., 1993; Christian et al., 1997). This study represents the first description of annexin I binding sites in synovial cells. The finding of reduced annexin I binding sites on RA compared to OA FLS is novel, but consistent with other studies. Annexin I binding sites are reduced on leukocytes at sites of inflammation *in vivo*, and studies in RA peripheral blood have shown reduced leucocyte annexin I binding sites compared to controls (Perretti et al., 1993; Goulding et al., 1992). Recently, Euzger et al have observed almost complete loss of annexin I binding capacity after treatment of monocytes with trypsin, elastase, cathepsin G or collagenase. Moreover, removal of annexin I binding sites from monocytes with elastase leads to loss of the inhibitory action on the respiratory burst displayed by

annexin I or its N-terminal peptide (Euzger et al., 1999). Together, the existing data suggest that annexin I binding site numbers are important in the anti-inflammatory effects of annexin I and glucocorticoids. This raises the hypothesis that that reduced binding sites on RA cells demonstrated in this chapter may contribute to incomplete control of inflammation by endogenous and therapeutic glucocorticoids in RA. Generation of annexin I binding site-deficient mice to test this hypothesis awaits the molecular definition of the binding site itself. Strong supportive evidence for the importance of annexin I binding sites comes from studies in which depletion of cell surface binding sites prevented the anti-inflammatory effect of annexin I (Euzger et al., 1999).

The demonstration of reduced annexin I binding sites on RA FLS led to the hypothesis that these results could be explained by enzymatic degradation of cell surface binding sites, as first proposed by Goulding et al, and recently confirmed by others (Goulding et al., 1996; Euzger et al., 1999). The present study was unable to demonstrate decreased binding site numbers on FLS in response to enzyme treatment. Indeed, collagenase induced a significant increase in annexin I binding sites on OA FLS. Of note, annexin I binding site levels on collagenase-treated RA FLS remained consistently lower than on OA FLS. The discrepancy between these results and those of other authors may relate to constitutive differences between FLS and leukocytes, or to the different milieu from which they were derived, for example, the *in vivo* exposure of FLS to metalloproteinases (Goulding et al., 1996; Euzger et al., 1999). The mechanism(s) of cytokine and enzyme regulation of annexin I binding sites are unknown. Exploration of these mechanisms is difficult in the absence of the molecular identification of the annexin I binding site, but such studies are clearly required.

Cytokines such as IL-1β and TNFα are important in the pathogenesis of RA. The effect of proinflammatory cytokines on the regulation of FLS annexin I binding sites was next explored. Tumor necrosis factorα increased annexin I binding sites on RA and OA FLS, while IL-1β signficantly increased OA FLS annexin I binding sites and showed a trend towards increased annexin I binding sites in RA cells. These results suggest that reduced annexin I binding sites on RA FLS are not a consequence of prior exposure to these cytokines, but instead are consistent with an intrinsic difference between OA and RA FLS. Morand et al have recently shown induction of annexin I synthesis in response to IL-1β in human RA FLS (unpublished observations). The capacity of these cells to increase their production of an anti-inflammatory mediator and its binding site in response to pro-inflammatory stimulation is consistent with the concept of intrinsic auto-regulatory networks within cells participating in the inflammatory response.

Dexamethasone did not affect FLS annexin I binding sites. Glucocorticoids are known to induce cell-surface translocation of annexin I in synovial macrophages (Yang et al., 1998), and studies have recently determined synthesis and translocation of annexin I in human RA FLS in response to DEX (Morand et al, unpublished observations). The essential role of annexin I in the anti-inflammatory effects of glucocorticoids in arthritis models (Yang et al., 1997; Yang et al., 1999) may therefore be mediated independently through changes in synthesis, translocation, and/or binding site numbers.

Evidence of direct anti-inflammatory effects of extracellular annexin I on FLS activation has been demonstrated in these experiments. A significant reduction in FLS PLA_2 activity was demonstrated in response to annexin I N-terminal peptide ac2-26 treatment. Previous studies have demonstrated that ac2-26 acts as a mimic of full-

length annexin I, albeit at a lower molar potency. Moreover, it has been shown that blockade of cell surface annexin I binding sites prevents the action of ac2-26, consistent with a requirement for peptide-binding site interaction for its biological effect (Perretti et al., 1993a). The anti-inflammatory effects of extracellular annexin I on FLS activation will be further discussed in Chapter 3.

At present, the identity of annexin I binding sites on FLS remains unclear. However, important interactions between members of the annexin family have been observed to bind to members of the S100 protein family (Maillard et al., 1996). Interestingly, these S100 proteins have been implicated in many cellular processes attributable to annexins, including proliferation, secretion and leukocyte chemotaxis (Maillard et al., 1996). That the specific complex-formation is mediated through the N-terminal domain of annexin I supports the concept that the unique N-terminus of annexin I carries the functional specificity, as seen with annexin I N-terminal peptides in inflammation (Perretti et al., 1995). To date, however, the physiological consequence of the annexin I-S100C interaction remains unknown and no study has demonstrated that the annexin I binding site is identical to any S100 protein.

Definition of annexin I binding sites on FLS has the potential to open the way for the development of annexin I analogues for the treatment of RA. Such analogues may have an anti-inflammatory profile similar to those of glucocorticoids but a different, and hopefully improved, toxicity profile. Reduced annexin I binding site numbers on RA FLS is consistent with the intrinsically pro-inflammatory phenotype of these cells.

2.6 Table and Figures

Table 2.6.1

Annexin I binding sites on human OA and RA fibroblast-like synoviocytes (FLS). Annexin I binding sites on OA (n = 6) and RA (n = 6) FLS were detected using a concentration range of annexin I (0-10 μM), and quantitated by flow cytometry. Values are represented as a percentage (%) of cells exhibiting fluorescence greater than the 5% negative control mAb cutoff gate, and are the mean ± SEM of 6 experiments for both OA and RA FLS. All concentrations of annexin I exhibited significant fluorescence (* $p < 0.01$) compared to cells incubated without annexin I.

	Annexin I binding (%)	
Annexin I concentration (μM)	OA	RA
0	3.7 ± 0.5	5.3 ± 1.3
1	76.7 ± 4.2*	61.9 ± 13.3*
5	97.0 ± 0.4*	90.3 ± 3.7*
10	99.5 ± 0.1*	92.4 ± 4.3*

Figure 2.6.1

Fluorescence histograms of annexin I binding sites on human FLS

OA FLS were incubated with annexin I and annexin I binding sites detected by flow cytometry. Data is representative of n = 6 experiments using 6 individual human donors. Annexin I binding fluorescence on human FLS using A, 0 μM and B, 10 μM annexin I are depicted. The x-axis represents fluorescence and the y-axis the relative cell number. The fluorescence intensity cutoff gate at 5% is shown: > 99% of cells exhibit annexin I binding at 10 μM.

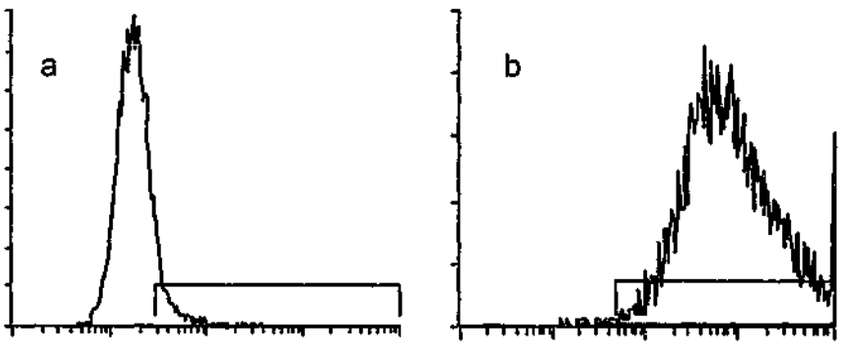

a
b

Figure 2.6.2

Annexin I binding sites on OA and RA FLS.

Annexin I binding site numbers were detected on FLS using ligand binding flow cytometry and expressed as (mean ± SEM) mean fluorescence intensity (MFI) in n=6 OA and n=6 RA experiments. Each increment in annexin I concentration resulted in an increase in annexin I fluorescence (* $p < 0.01$) in both OA and RA FLS. At all concentrations of annexin I (1-10 μM), RA cells exhibited lower annexin I binding site fluorescence than OA cells († $p < 0.01$).

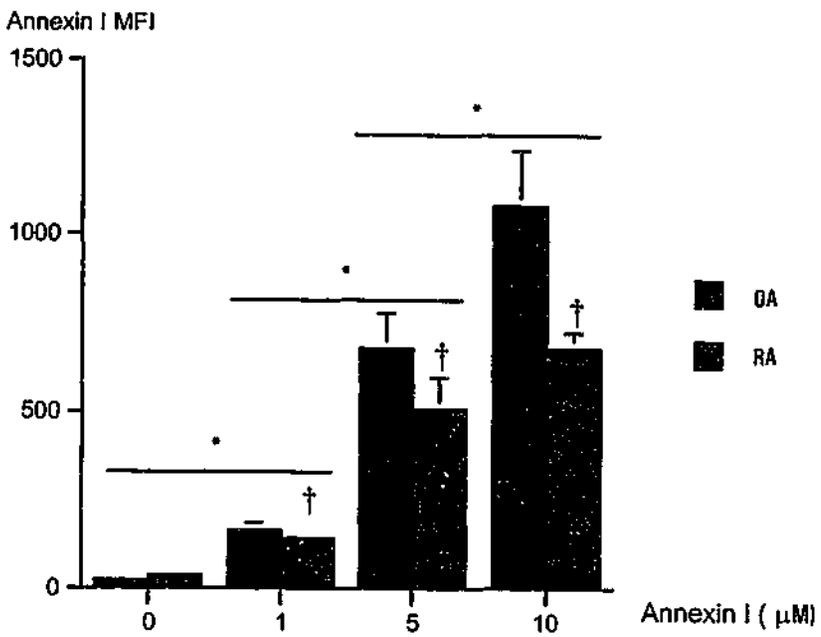
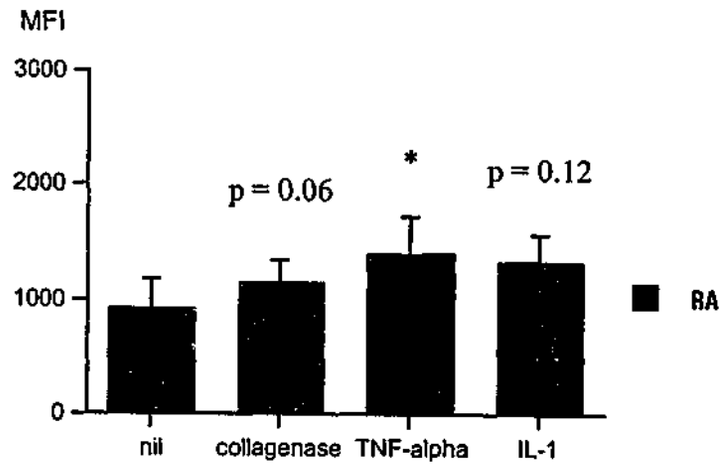

Annexin I MFI
1500
1000
500
0
0
1
5
10
Annexin I (μM)
OA
RA
*
*
*
†
†
†

Figure 2.6.3

Effect of enzyme and cytokine treatment on annexin I binding sites on human FLS. Human RA (upper panel) and OA (lower panel) FLS were treated with medium alone, collagenase (5 U/ml) for 30 minutes, or IL-1β (0.1 ng/ml) or TNFα (10 ng/ml) for 24 hours. Annexin I binding sites were analysed by ligand-binding flow cytometry. Binding site values are expressed as mean fluorescence intensity (MFI) after subtraction of the MFI of the negative control mAb. Values are the mean ± SEM of 6 experiments using 6 individual human donors for each of RA and OA. * $p < 0.05$ versus untreated FLS; † $p < 0.05$ versus untreated RA FLS, ¥ $p < 0.05$ versus untreated OA FLS.


MFI
3000
2000
1000
0
p = 0.06
*
p = 0.12
nil
collagenase
TNF-alpha
IL-1
RA

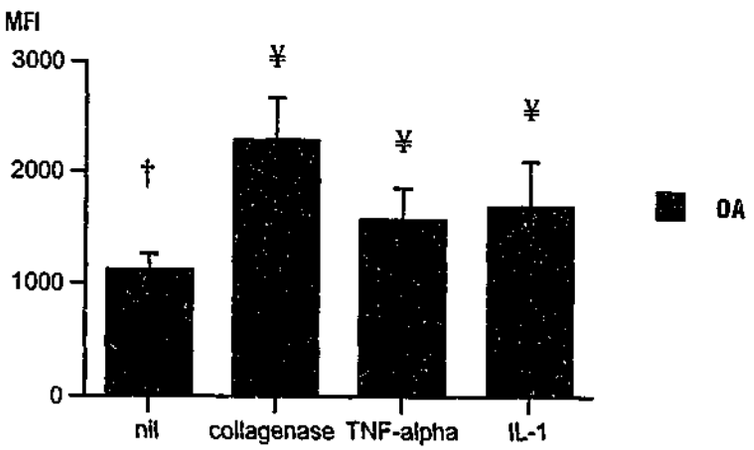
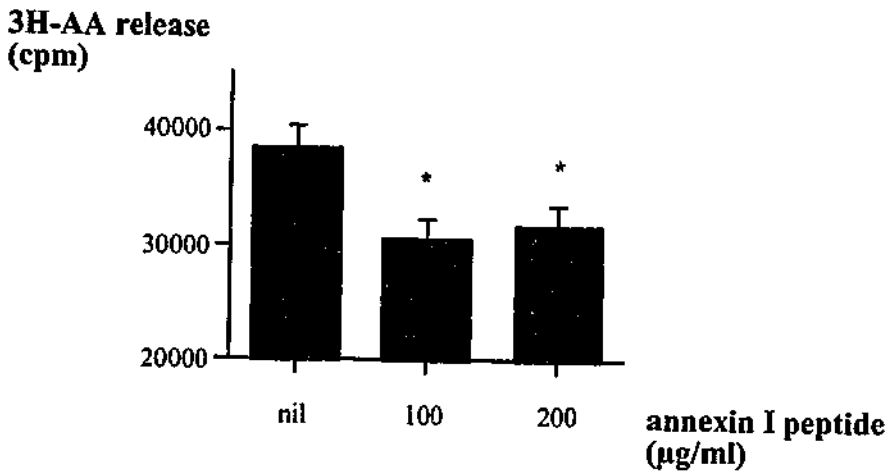

MFI
3000
2000
1000
0
†
¥
¥
¥
nil
collagenase
TNF-alpha
IL-1
OA

Figure 2.6.4

Effect of annexin I on human FLS phospholipase A_2 activity.

Human RA FLS were exposed to annexin I N-terminal peptide ac2-26 for 8 hours and phospholipase A_2 (PLA_2) activity measured by ^{3}H-arachidonic acid release as described in Methods. Values are the mean ± SEM of 6 experiments for RA FLS. * $p < 0.01$ versus untreated FLS.


3H-AA release
(cpm)
40000
30000
20000
*
*
nil
100
200
annexin I peptide
(µg/ml)

CHAPTER THREE: EFFECTS OF ANNEXIN I N-TERMINAL PEPTIDE AND DEXAMETHASONE ON PLA_2 AND COX ACTIVITY IN HUMAN FIBROBLAST-LIKE SYNOVIOCYTES

3.1 Chapter Summary

The glucocorticoid-inducible protein annexin I is expressed in human rheumatoid synovium and is a critical anti-inflammatory effector in animal models of rheumatoid arthritis. Its precise relationship with the anti-inflammatory effects of glucocorticoids has not been resolved, and its mechanisms of action *in vivo* are uncertain.

In this study, the effects of a bioactive annexin I peptide, ac 2-26, the synthetic glucocorticoid dexamethasone (DEX), and the proinflammatory cytokine interleukin-1β (IL-1β) on OA FLS activation were investigated.

PLA_2 activity was measured using [^{3}H]arachidonic acid release. PGE_2 release and COX activity were determined by PGE_2 ELISA. Intracellular COX2 expression in FLS was assessed by permeabilisation flow cytometry.

Annexin I peptide ac2-26 exerted a significant concentration-dependent inhibition of constitutive FLS PLA_2 activity, which was reversed by IL-1β. In contrast, DEX inhibited IL-1β-induced PLA_2 activity but not constitutive activity. DEX but not annexin I peptide inhibited IL-1β-induced PGE_2 release. COX activity and intracellular COX2 expression were significantly increased by IL-1β. Annexin I peptide demonstrated no inhibition of constitutive or IL-1β-induced COX activity. DEX exerted a concentration-dependent inhibition of IL-1β-induced but not constitutive COX activity.

The effect of annexin I but not DEX on constitutive PLA_2 activity suggests a glucocorticoid-independent role for annexin I in autoregulation of arachidonic acid production. These studies demonstrate uncoupling of inhibition of PLA_2 and COX by annexin I and glucocorticoids. The independence of annexin I inhibition of constitutive PLA_2 activity and DEX inhibition of COX activity is consistent with the

hypothesis that COX is rate-limiting for PGE_2 synthesis in FLS. The lack of effect of annexin I on cytokine-induced PGE_2 production suggests PGE_2-independent mechanisms for the anti-inflammatory effects of annexin I *in vivo*.

3.2 Introduction

Prostaglandins, such as PGE_2, mediate the pain and edema associated with arthritis, leading to the widespread use of non-steroidal anti-inflammatory drugs in the treatment of arthritis. Within the arthritic synovial lesion, fibroblast-like synoviocytes (FLS) have been implicated as a primary source of PGE_2 (Arend and Dayer, 1990). In eicosanoid generation, phospholipase A_2 (PLA_2) and cyclooxygenase (COX, or prostaglandin H synthase) have been described as important regulatory enzymes. The hydrolytic release of arachidonic acid from membrane phospholipids is catalyzed by PLA_2, and arachidonic acid is then catalysed by COX enzymes for the subsequent production of PGE_2. PLA_2 exists as several isoforms which include a secretory group II isoform ($sPLA_2$) and an arachidonic acid-selective cytosolic form ($cPLA_2$) (Murakami et al., 1997). Two isoforms of cyclooxygenase have also been identified: a constitutive (COX1) and a mitogen/growth factor inducible (COX2) form (Crofford et al., 1994). At present, the enzymatic events responsible for the enhanced production of PGE_2 in RA are not fully understood and knowledge pertaining to which enzyme is rate-limiting in eicosanoid generation in synovium is not fully elucidated.

In arthritic synovial tissue, expression of $cPLA_2$ and COX2 are observed. It is known that expression of the $cPLA_2$ and COX2 are increased by interleukin-1β (IL-1β) (Croxtall et al., 1996; Angel et al., 1994) and that this induction is inhibited by the anti-inflammatory action of dexamethasone (DEX) (Crofford et al., 1994; Angel et al., 1994; Wilborn et al., 1995).

As described in Chapter 1, annexin 1 was originally defined as a glucocorticoid-inducible *in vitro* inhibitor of PLA_2 activity (Morand et al., 1995b). The involvement of annexin I in the regulation of joint inflammation has now been demonstrated in several rat models of arthritis, in which a range of biological actions extending beyond effects on PLA_2 activity has been suggested (Yang et al., 1998; Yang et al., 1997; Yang et al., 1998). The possibility that annexin I has anti-inflammatory activity in human arthritis has not been previously explored. Studies presented in Chapter 2 demonstrate the presence of cell surface annexin I binding sites on FLS, suggesting the hypothesis that annexin I can exert regulatory effects on these cells. With evidence that annexin I is expressed in arthritic synovium, I sought to further define the effects of annexin I on PLA_2, COX and prostaglandin production in human FLS. Studies also utilised the known PLA_2 inhibitory properties of annexin I to investigate the rate limiting step in synoviocyte prostaglandin production.

3.3 Methods

3.3.1 Solutions and buffers

2% Paraformaldehyde (PFA)/PBS

Two ml of PFA (Sigma) was added to 98 ml of PBS.

0.2% saponin/PBS

0.2% saponin/PBS solution was prepared by dissolving 20 g of saponin (Sigma) in 500 ml of PBS at 60°C.

3.3.2 Synthesis of annexin I N-terminal peptide, ac 2-26

The bioactive N-terminal 2-26 amino acid fragment of annexin I (acetyl-AMVSEFLKQAWFIENEEQEYVQTVK-OH) (Perretti et al., 1995) was prepared by

use of solid phase step-wise synthesis and obtained from Prof Milton Hearn (Monash University, Clayton, Australia). Purity was more than 99% as determined by HPLC. Annexin I fragment was stored at -20°C and thawed immediately prior to use.

3.3.3 Isolation and culture of fibroblast-like synoviocytes

OA FLS were used in the following experiments. Fibroblast-like synoviocytes were obtained, propagated and cultured as described (sections 2.3.6-2.3.8) from synovium of osteoarthritic (OA) patients (n = 8) undergoing joint replacement surgery. All patients satisfied the American College of Rheumatology criteria for the classification of OA (Altman et al., 1991; Altman et al., 1986). Thawed cells were used in experiments between passages 4 and 9. For all experiments, FLS were seeded at 1 x 10^5 cells per well in 24 well culture plates in RPMI/10% FCS and allowed to adhere overnight, prior to medium being replaced with RPMI/0.1% BSA for experimental purposes. Cells were >95% viable by trypan blue exclusion. In each group of experiments, n refers to the number of individual human OA donor FLS used.

3.3.4 Assessment of phospholipase A_2 activity

PLA_2 activity in FLS was determined according to Croxtall et al (Croxtall et al., 1995). FLS were labelled for 18 hours with 1 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (Amersham International plc, Sydney, Australia) in 0.1% BSA/RPMI. Cells were treated with 0-100 μg/ml annexin I peptide; 0-0.1 ng/ml human recombinant IL-1β and 10^{-9} - 10^{-7} M DEX for 8 hours. Triplicate cultures were used for each determination. Radioactivity in the supernatant was determined using a Wallac 1409 liquid scintillation counter (Pharmacia, Finland), with results expressed as [^{3}H]arachidonic acid released (cpm), or as a percentage of the mean result of unstimulated cells.

3.3.5 Determination of prostaglandin E_2 levels

The concentration of PGE_2 in the treated FLS supernatants was measured by PGE_2 ELISA kit, according to the manufacturer's instructions (Neogen Corporation, Lexington, KT). Cells were treated with 0-100 μg/ml annexin I peptide; 0, 0.1 ng/ml human recombinant IL-1β and 10^{-9} - 10^{-7} M DEX for 8 hours. Duplicate cultures were used for each determination. Supernatants were aspirated immediately and stored at -20°C prior to assay. PGE_2 standards and samples (50 μl) were loaded onto a PGE_2 antibody pre-coated 96 well plate in duplicate and 50 μl of PGE_2-horseradish peroxidase conjugate was then added. After incubation for 1 hour at room temperature, the bound enzyme conjugate was detected by the addition of TMB and hydrogen peroxide mixture. The reaction was stopped by acidification (1 M HCl) and results were obtained by absorbance plate reader at 450 nm (Dynatech MR7000). The PGE_2 concentrations were then determined using a standard curve (0-10 ng/ml PGE_2) produced on each plate. PGE_2 release from treated FLS supernatants were expressed as ng/ml of PGE_2 or as a percentage of the mean result of the unstimulated cells. The detection limit for this assay was <0.1 ng/ml.

3.3.6 Assessment of cyclooxygenase activity

COX activity was measured according to Wilborn et al (Wilborn et al., 1995). PGE_2 release reflects the actions of both PLA_2 and COX. To bypass PLA_2 activity, and estimate maximal COX activity, FLS were incubated for 30 minutes with 10 μM exogenous [5,6,8,9,11,12,14,14-H]arachidonic acid (Sigma). Previous studies establish this concentration of arachidonic acid to be saturating (Wilborn et al., 1995). Duplicate cultures were used for each determination. Supernatants were aspirated immediately and stored at -20°C prior to assay. PGE_2 content in treated FLS

supernatants was determined using PGE_2 ELISA (see section 3.3.5). COX activity was expressed as ng/ml of PGE_2 or as a percentage of the mean result of the unstimulated cells.

3.3.7 Determination of intracellular cyclooxygenase2

Intracellular expression of COX2 in FLS was determined using permeabilisation flow cytometry, as described by Morand et al (Morand et al., 1995a). Cells were treated with 0-100 μg/ml annexin I peptide; 0, 0.1 ng/ml human recombinant IL-1β and 10^{-9} - 10^{-7} M DEX for 8 hours. Duplicate cultures were used for each determination. FLS were then washed with HBSS, trypsinised and fixed by suspension in 2% paraformaldehyde (PFA, Sigma)/PBS for 5 mins. All subsequent procedures were undertaken at 4°C. Cells were washed with FACS buffer and then permeablised by incubation in 0.2% saponin (Sigma)/PBS. FLS were then sequentially incubated with 20 μg/ml COX2 mAb (or isotype-matched mAb control, IgG_1) and FITC-conjugated anti-mouse IgG, each of which were diluted in 0.2% saponin/PBS. Permeabilisation of the cells was reversed by suspension of the cells in PBS. Labelled FLS were analysed using flow cytometry. At least 5000 cells were used for each determination. Intracellular COX2 protein was expressed as mean fluorescence intensity after subtraction of mean fluorescence intensity obtained with negative control mAb.

3.3.8 Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed using the Student's t-test, with values of $p < 0.05$ regarded as statistically significant.

3.4 Results

3.4.1 Effect of annexin I N-terminal peptide ac 2-26 on constitutive and IL-1β -stimulated PLA_2 activity

To define the effect of annexin I on synoviocyte PLA_2 activity, FLS were treated with annexin I N-terminal peptide, ac 2-26 (0-100 μg/ml). Annexin I peptide induced a significant concentration-dependent inhibition of constitutive PLA_2 activity (Figure 3.6.1), confirming the results presented in Chapter 2. Inhibition of PLA_2 activity by annexin I was prevented when cells were co-treated with IL-1β (Figure 3.6.1).

3.4.2 Effect of DEX on constitutive and IL-1β -stimulated PLA_2 activity

I next assessed the effects of DEX on synoviocyte PLA_2 activity. DEX treatment did not significantly inhibit constitutive PLA_2 activity over the concentration range 10^{-9}-10^{-7} M. In contrast, DEX exerted a significant concentration-dependent reduction of PLA_2 activity in IL-1β-stimulated FLS (Figure 3.6.2).

3.4.3 Effect of annexin I N-terminal peptide ac 2-26 on constitutive and IL-1β -stimulated PGE_2 release

Since annexin I peptide inhibited constitutive PLA_2 activity, investigations next determined whether treatment with annexin I peptide affected PGE_2 release. Constitutive PGE_2 synthesis was detected in FLS. Annexin I peptide did not significantly reduce constitutive PGE_2 synthesis. A significant increase in PGE_2 release was observed following IL-1β-stimulation ($p=0.001$), and annexin I peptide did not inhibit IL-1β-stimulated PGE_2 release (Figure 3.6.3).

3.4.4 Effect of DEX on constitutive and IL-1β -stimulated PGE_2 release

DEX (10^{-9} - 10^{-7} M) had no effect on constitutive PGE_2 release. In contrast, DEX significantly inhibited IL-1β-induced PGE_2 release in a concentration-dependent manner (Figure 3.6.4).

3.4.5 Effect of annexin I N-terminal peptide ac 2-26 on constitutive and IL-1β - stimulated COX activity

To further address the differential inhibitory actions of annexin I on PLA_2 activity and PGE_2 synthesis, studies were designed to evaluate the effects of annexin I peptide and DEX on constitutive and IL-1β-induced COX activity. Since the synthesis of PGE_2 from endogenous arachidonic acid results from the activities of both PLA_2 and COX, to estimate maximal COX activity, PLA_2 activity was bypassed by addition of exogenous arachidonic acid (Wilborn et al., 1995). Low levels of constitutive COX activity were detected in FLS. IL-1β significantly increased COX activity ($p=0.0004$). Annexin I peptide exerted no significant effect on constitutive or IL-1β-stimulated COX activity (Figure 3.6.5).

3.4.6 Effect of DEX on constitutive and IL-1β -stimulated COX activity

DEX exerted no significant effect on constitutive COX activity in DEX treated FLS. In contrast, DEX resulted in a concentration-dependent reduction of IL-1β -induced COX activity (Figure 3.6.6).

3.4.7 Effect of annexin I N-terminal peptide ac 2-26 on constitutive and IL-1β - stimulated COX2 protein expression.

The question of whether modulation of COX activity reflected its intracellular expression of COX2 was investigated using permeabilisation flow cytometry. Low levels of constitutive expression of intracellular COX2 protein were detected in FLS,

and COX2 expression was increased by IL-1β. Annexin I peptide had no significant effect on constitutive or IL-1β-stimulated COX2 protein in FLS (Figure 3.6.7).

3.4.8 Effect of DEX on constitutive and IL-1β -stimulated COX2 protein expression

DEX did not significantly inhibit constitutive intracellular expression of COX2 (Figure 3.6.7). In contrast, DEX exerted a concentration-dependent inhibition of intracellular COX2 expression in IL-1β-stimulated FLS. These results demonstrate consistency between modulation of intracellular expression of COX2 and its activity (Figure 3.6.8).

3.5 Discussion

As discussed in Chapters 1 and 2, the hypothesis that annexin I is an anti-inflammatory mediator in arthritis originated from the detection of annexin I in human rheumatoid synovium (Goulding et al., 1995). Animal models of arthritis have subsequently demonstrated that annexin has important inhibitory effects *in vivo*. For example, administration of annexin I peptide substantially inhibited carrageenan-induced arthritis, whilst anti-annexin I antibody exacerbated arthritis severity and reversed the effect of exogenous DEX in this model (Yang et al., 1997). Similarly, exacerbation of disease and increased synovial production TNFα and PGE_2 were observed in adjuvant arthritic rats administered anti-annexin I antibody (Yang et al., 1998). Moreover, annexin I neutralisation reversed the effects of DEX on disease severity in adjuvant arthritis (Yang et al., 1998) and on rat synovial macrophage nitric oxide production (Yang et al., 1998).

In Chapter 2, I established the presence of biologically active annexin I binding sites on human FLS, with notably reduced binding sites on RA FLS compared to OA FLS.

Regulation of annexin I binding sites on FLS was observed in response to proinflammatory cytokine and enzyme exposure, which suggests the capacity of annexin I binding site capacity to be altered at sites of inflammation. In the current study, I sought to assess the effects of annexin I on these cells. An annexin I N-terminal peptide of 25 amino acids, annexin I ac 2-26, has been shown to mimic anti-inflammatory actions of annexin I (Cirino et al., 1993; Perretti et al., 1993a) including inhibition of arthritis models (Yang et al., 1998). Perretti et al have established that the biological activity of annexin I ac2-26 is comparable with that of recombinant human annexin I, albeit at a lower molar potency, and that it requires binding to cell surface binding sites to exert its biological effects (Perretti et al., 1993a).

As reviewed in Chapter 1, previous investigations have evaluated the effects of glucocorticoids and cytokines on the regulation of FLS PLA_2 and COX at the levels of transcription (Angel et al., 1994; Newton et al., 1997) or translation (Hulkower et al., 1994). Studies have also compared the level of activity of these enzymes in various cell types, following IL-1β (Szczepanski et al., 1994) and DEX treatments (Wilborn et al., 1995), but studies of the rate-limiting step at the enzyme activity level in human FLS have not been undertaken. The concept that IL-1β induced PLA_2 and COX activity measured reflect effects on $cPLA_2$ and COX2 mRNA expression will also be shown in Chapters 4 and 5, where I was able to reproduce published effects of IL-1β on $cPLA_2$ and COX2 expression in FLS.

The results presented in this chapter indicate a significant concentration-dependent inhibition of constitutive but not IL-Iβ-induced PLA_2 activity by annexin I ac 2-26. Annexin I ac 2-26 inhibition of arachidonic acid release has previously been noted in A549 cells (Croxtall et al., 1998) and in human neutrophils (Perretti et al., 1995). In A549 cells, inhibition of arachidonic acid release by annexin I peptide is believed to

be mediated through inhibition of the activation of $cPLA_2$, whereby $cPLA_2$ is not phosphorylated in the presence of annexin I (Pistritto et al., 1998). Details of how this is accomplished are still uncertain, but binding-site dependent effects of ac2-26 are unlikely to relate to the previously proposed "substrate binding" hypothesis for the effects of annexin I on PLA_2. The selective inhibitory action of DEX on IL-1β-stimulated but not constitutive $cPLA_2$ transcription (Angel et al., 1994; Newton et al., 1997) and expression (Hulkower et al., 1994) has been described in other investigations, but is confirmed at the enzyme activity level for the first time in the current study. The uncoupling of the effects of annexin I and glucocorticoids on PLA_2 supports the contention that the constitutive anti-inflammatory effects of annexin I are only in part related to mediating the effects of glucocorticoids.

As previously demonstrated, constitutive PGE_2 synthesis was detected in cultured FLS. Cytokine upregulation of PGE_2 synthesis was observed, again consistent with previous findings (Angel et al., 1994; Hulkower et al., 1994; Szczepanski et al., 1994). Annexin I peptide did not demonstrate any significant inhibitory effect on constitutive or IL-1β -stimulated PGE_2 production. In rat astrocytes, annexin I peptide reduced by did not abolish LPS-induced PGE_2 release (Pistritto et al., 1998). Consistent with the observed effects on PLA_2 activity, my studies demonstrate that DEX significantly reduced IL-1β-stimulated but not constitutive PGE_2 release. Annexin I mAb has been observed to reverse the effects of DEX inhibition of PGE_2 release (Newman et al., 1994), and the current study suggests that glucocorticoid suppression of COX occurs via an annexin I-independent mechanism. Glucocorticoid-induced suppression of COX2 appears to be independent of annexin I and is almost certainly explained by the direct interaction of the steroid-receptor complex with nuclear factor-kappa B (Barnes and Karin, 1997). DEX inhibition of

cytokine- or mitogen-induced COX regulation has been previously reported (Wilborn et al., 1995; Szczepanski et al., 1994; Pistritto et al., 1998; Angel et al., 1994; Morand et al., 1995b; Masferrer et al., 1990). In the current study, regulation of intracellular COX2 expression was found to be consistent with regulation of its activity, suggesting that the level of COX activity is dependent upon intracellular levels of COX2 (Kujubu and Herschman, 1992). The differential effects of annexin I peptide and DEX on PLA_2 activity, COX, and PGE_2 production support the hypothesis that COX is the rate-limiting enzyme in IL-1β-induced PGE_2 synthesis in FLS. Conversely, the lack of effect of glucocorticoids on constitutive PLA_2 activity suggests that annexin I may have a glucocorticoid-independent role in constitutive regulation of PLA_2 activity.

Biological activity of annexin I peptide has been demonstrated *in vivo* in rat carrageenan arthritis (Yang et al., 1997), a model mediated by eicosanoids in addition to nitric oxide and reactive oxygen species. That annexin I peptide has previously demonstrated to have inhibitory effects on nitric oxide and reactive oxygen species production in other systems highlights the importance of viewing annexin I not solely as an inhibitor of PLA_2 activity.

In summary, two novel findings are reported in this study. Firstly, the uncoupling of the effects of annexin I and glucocorticoids on constitutive and cytokine-induced arachidonic acid generation suggests that annexin I has glucocorticoid-independent regulatory activities in inflammation. Results herein are consistent with the conclusion that COX2 is the rate-limiting step in synoviocyte PGE_2 synthesis, suggesting that PLA_2-directed strategies may not successfully inhibit eicosanoid production in arthritis. Secondly, the lack of effect of annexin I on PGE_2 synthesis, despite clear effects on inflammation *in vivo* suggests the anti-inflammatory effects of

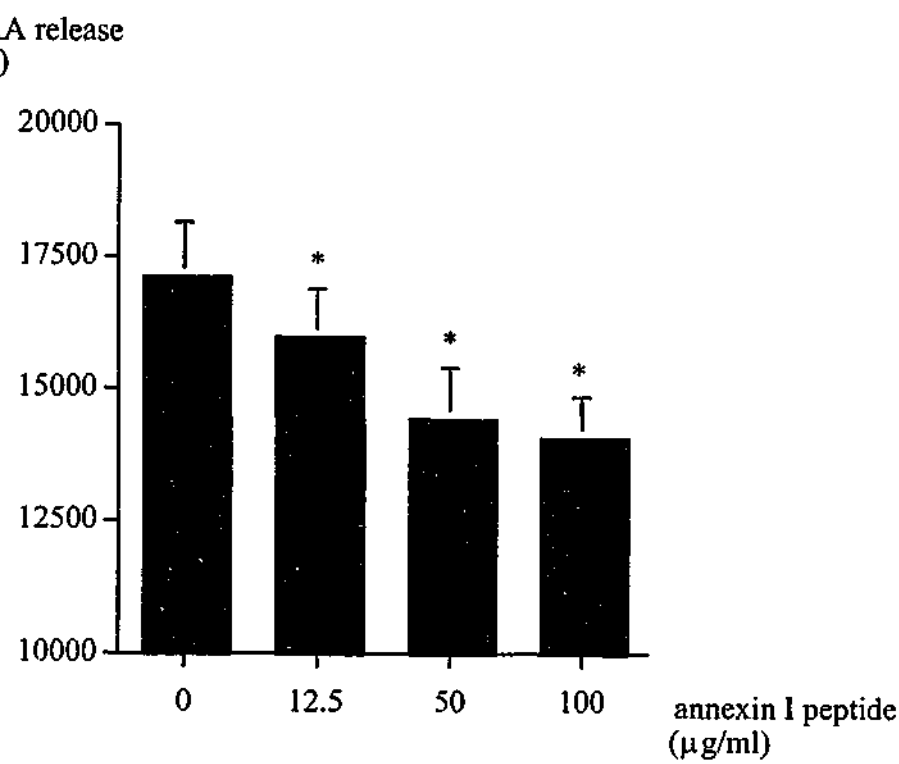
annexin I are eicosanoid-independent. The observed effect of annexin I neutralization of synovial PGE_2 in rat adjuvant arthritis may reflect a secondary effect via the increase in synovial TNFα, rather than a direct interaction with PLA_2 and COX. Annexin I may also influence arachidonic acid-mediated intracellular signal transduction. Arachidonic acid influences activation of the JNK/SAPK subgroups of the MAP kinase family of signal transduction enzymes, via an eicosanoid-independent pathway (Rizzo and Carlo-Stella, 1996). This important pro-inflammatory signal pathway is influenced by reactive oxygen species (Cui and Douglas, 1997), and is also known to be inhibited by annexin I (Maridonneau-Parini et al., 1989). The possibility that annexin I modulates inflammation via eicosanoid-independent mechanisms remains to be further investigated. Studies directed at understanding the role of annexin I in such pathways may have therapeutic benefit in the treatment of arthritis.

3.6 Figures

Figure 3.6.1

Effect of annexin I peptide on human FLS PLA_2 activity. FLS were treated with (a) annexin I ac 2-26 (0-100 μg/ml), and/or (b) IL-1β (0.1 ng/ml) plus annexin I peptide for 8 hours. PLA_2 activity is expressed as ^{3}H-AA released. Values are the mean ± SEM of 8 experiments. * $p < 0.05$ versus untreated FLS, † $p < 0.01$ versus untreated FLS.

a


^{3}H-AA release
(cpm)
20000
17500
15000
12500
10000
*
*
*
0
12.5
50
100
annexin I peptide
(μg/ml)

b

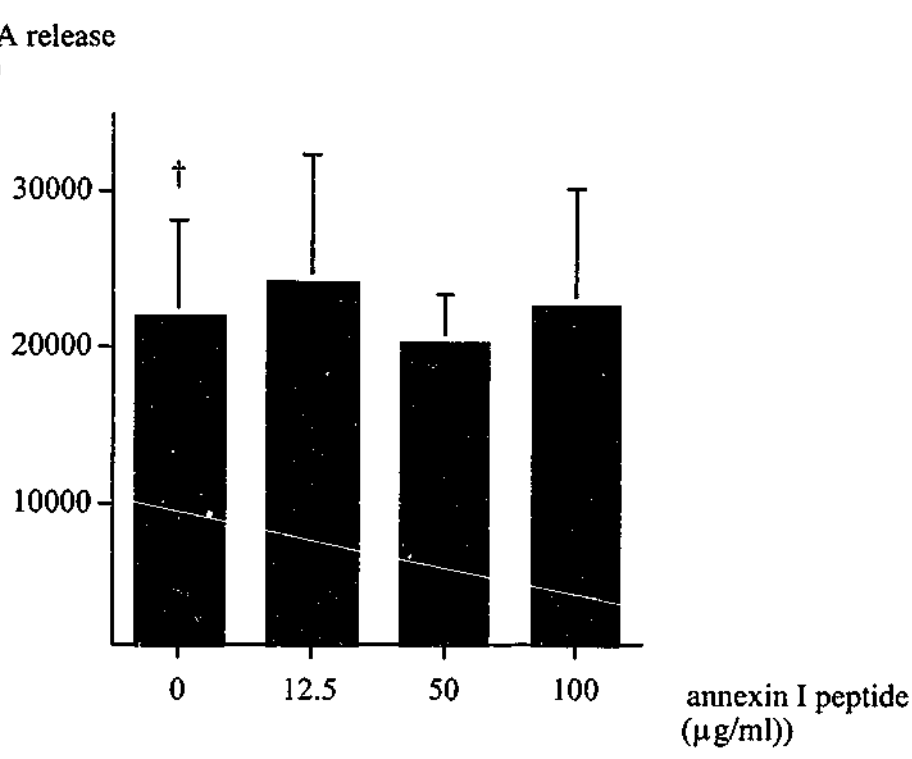

^{3}H-AA release
(cpm)
30000
20000
10000
†
0
12.5
50
100
annexin I peptide
(μg/ml))

Figure 3.6.2

Effect of DEX on human FLS PLA_2 activity. FLS were treated with DEX (10^{-9}-10^{-7} M), and/or IL-1β (0.1 ng/ml) for 8 hours. PLA_2 activity (^{3}H-AA released) is expressed as a percentage of untreated FLS. Values are the mean ± SEM of 8 experiments. * $p < 0.01$ versus untreated FLS, † $p < 0.05$ versus IL-1β-treated FLS.

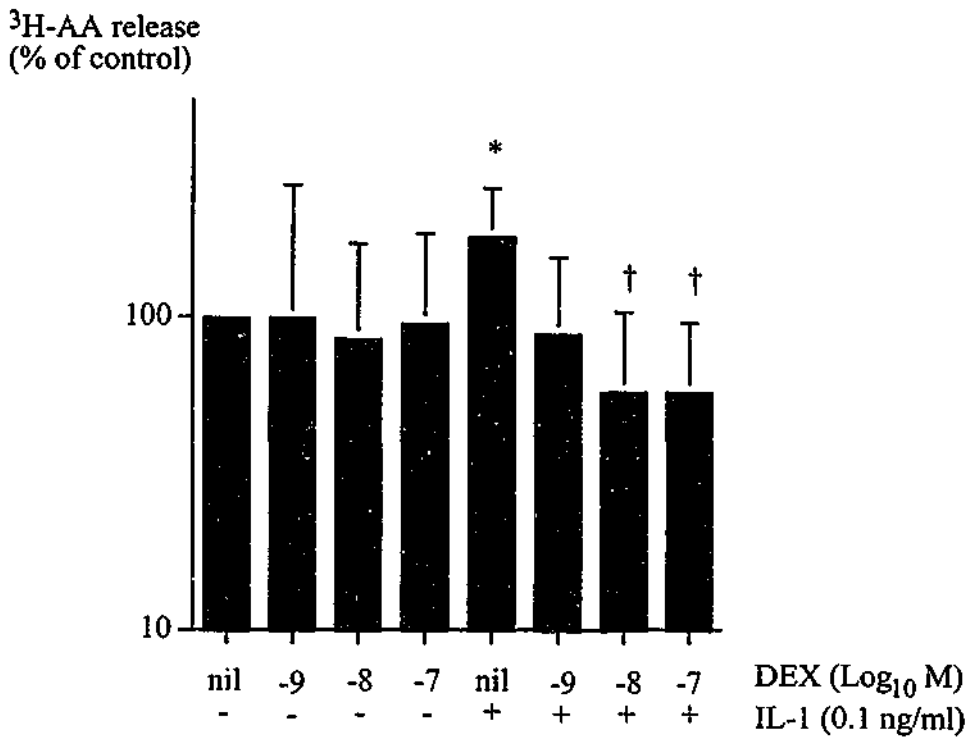

^{3}H-AA release
(% of control)
100
10
*
†
†
nil
-9
-8
-7
nil
-9
-8
-7
DEX (Log$_{10}$ M)
-
-
-
-
+
+
+
+
IL-1 (0.1 ng/ml)

Figure 3.6.3

PGE_2 release in human FLS. Human FLS were treated with IL-1β (0.1 ng/ml), and/or annexin I ac 2-26 (100 μg/ml) for 8 hours. PGE_2 release was quantitated by assaying supernatants using ELISA and expressed as a percentage relative to untreated FLS. Values are the mean ± SEM of 4 experiments. * $p < 0.01$ versus untreated FLS.

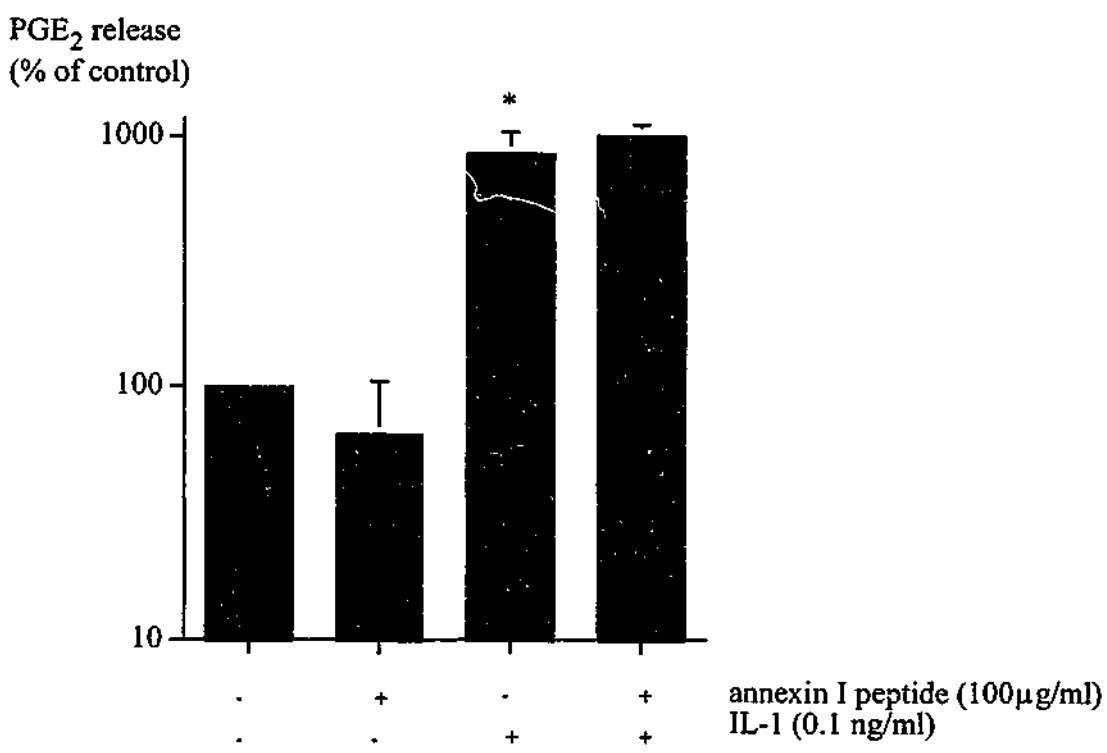

PGE2 release
(% of control)
1000
100
10
*
annexin I peptide (100µg/ml)
IL-1 (0.1 ng/ml)

Figure 3.6.4

PGE_2 release in human FLS. Human FLS were treated with IL-1β (0.1 ng/ml), and/or DEX (10^{-9}-10^{-7} M) for 8 hours. PGE_2 release was quantitated by assaying supernatants using ELISA and expressed as a percentage relative to untreated FLS. Values are the mean ± SEM of 4 experiments. ** $p < 0.01$ versus untreated FLS, * $p < 0.05$ versus IL-1β-treated FLS.

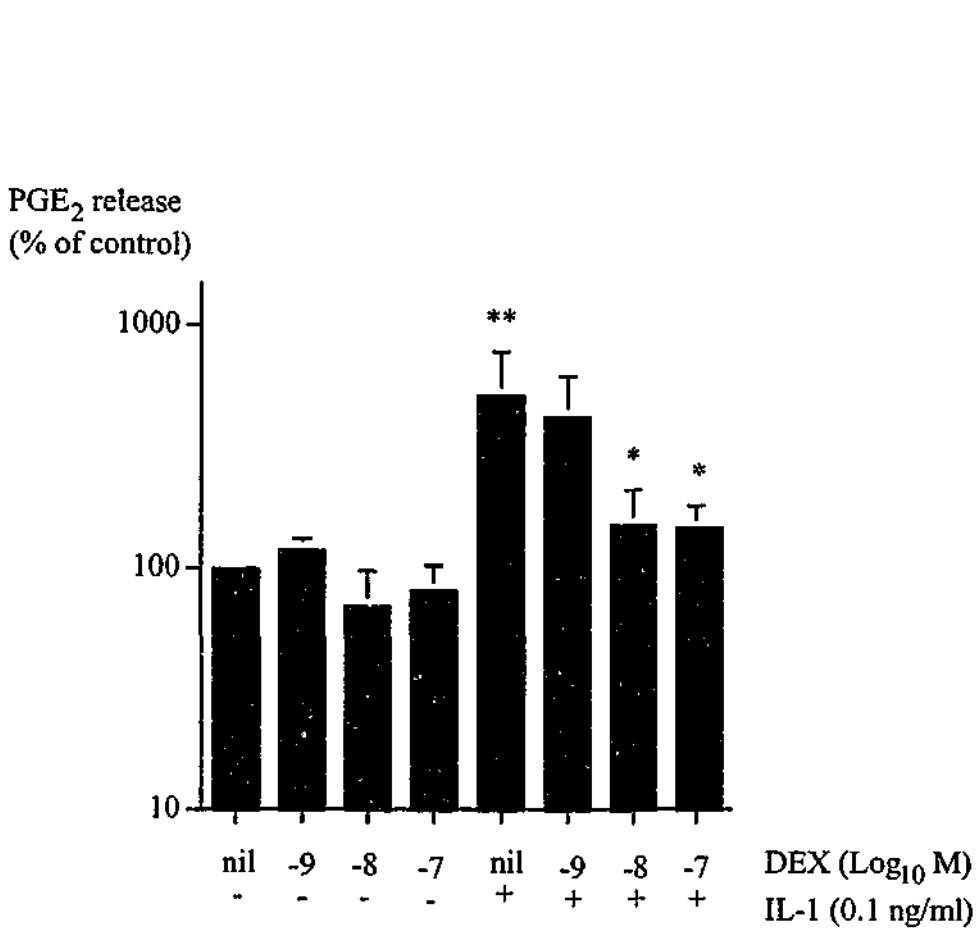

PGE2 release
(% of control)
1000
100
10
**
*
*
nil
-9
-8
-7
nil
-9
-8
-7
DEX (Log10 M)
-
-
-
-
+
+
+
+
IL-1 (0.1 ng/ml)

Figure 3.6.5

COX activity in human FLS. Human FLS were treated with IL-1β (0.1 ng/ml), and/or annexin I ac 2-26 (100 μg/ml) for 8 hours and COX activity measured as described. COX activity is expressed as a percentage relative to untreated FLS. Values are the mean ± SEM of 4 experiments. ** $p < 0.01$ versus untreated FLS.

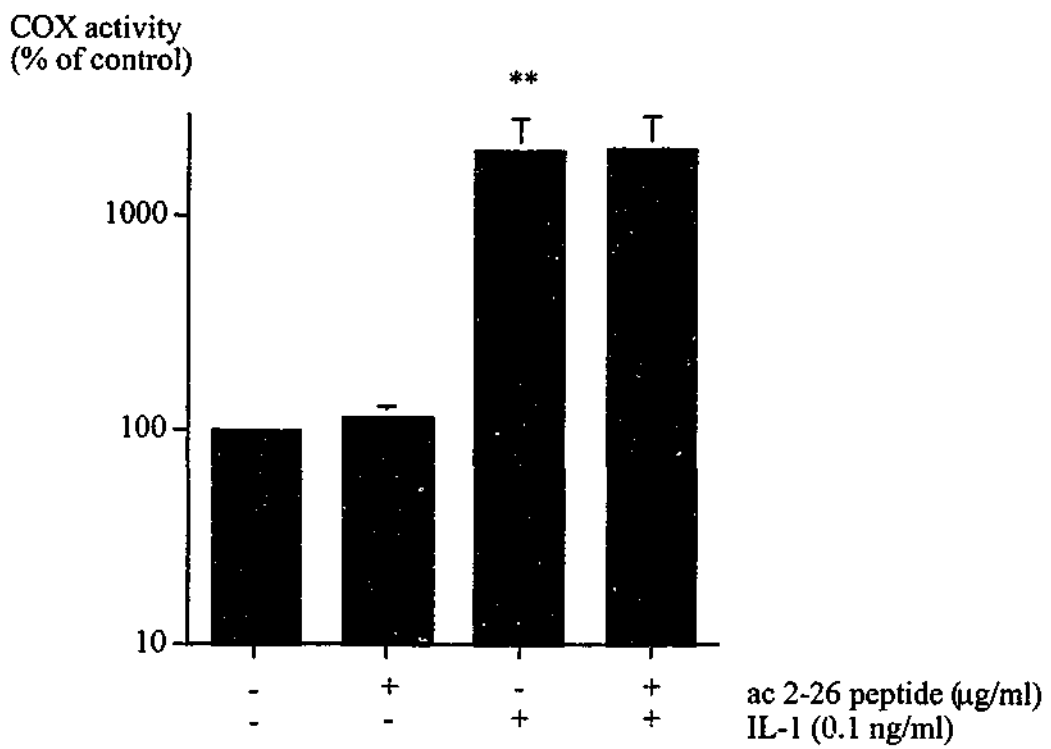

COX activity
(% of control)
**
1000
100
10
-
+
-
+
ac 2-26 peptide (μg/ml)
-
-
+
+
IL-1 (0.1 ng/ml)

Figure 3.6.6

COX activity in human FLS. Human FLS were treated with IL-1β (0.1 ng/ml), and/or DEX (10^{-9}-10^{-7} M) for 8 hours and COX activity measured as described. COX activity is expressed as a percentage relative to untreated FLS. Values are the mean ± SEM of 4 experiments. ** $p < 0.01$ versus untreated FLS, * $p < 0.05$ versus IL-1β-treated FLS.

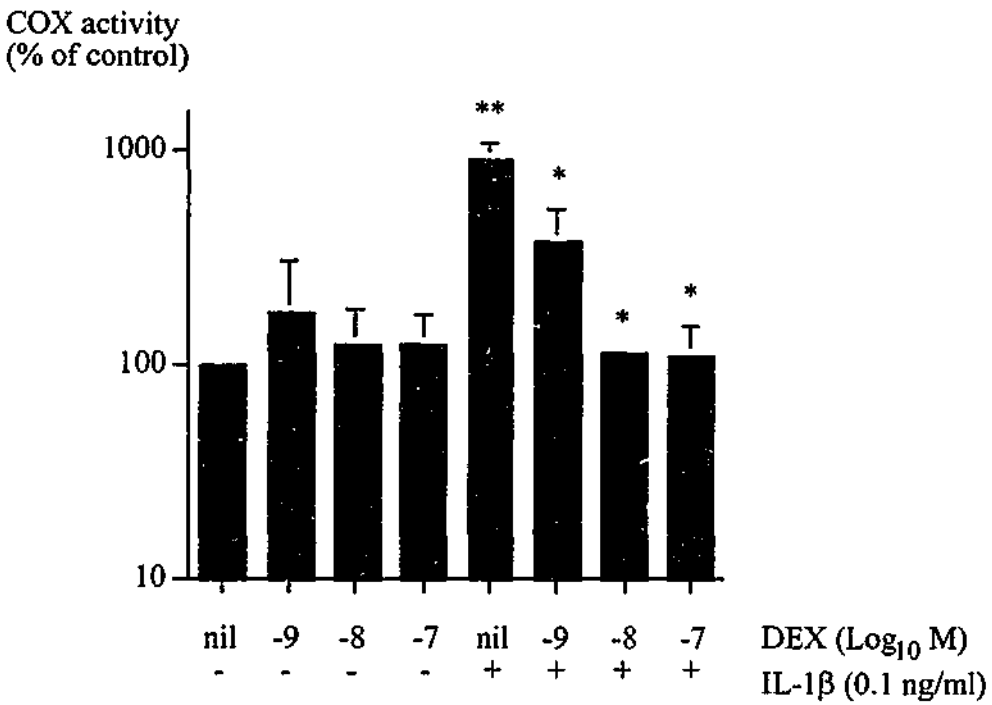

COX activity
(% of control)
1000
100
10
**
*
*
*
nil
-9
-8
-7
nil
-9
-8
-7
DEX (Log10 M)
-
-
-
-
+
+
+
+
IL-1β (0.1 ng/ml)

Figure 3.6.7

Intracellular COX2 expression in human FLS. Human FLS were treated with IL-1β (0.1 ng/ml), and/or annexin I ac 2-26 (100 μg/ml) for 8 hours. Intracellular COX2 was detected by permeabilization flow cytometry and expressed as the mean fluorescence intensity (MFI) after subtraction of MFI obtained with the negative control mAb. Values are the mean ± SEM of 4 experiments. ** $p < 0.01$ versus untreated FLS.

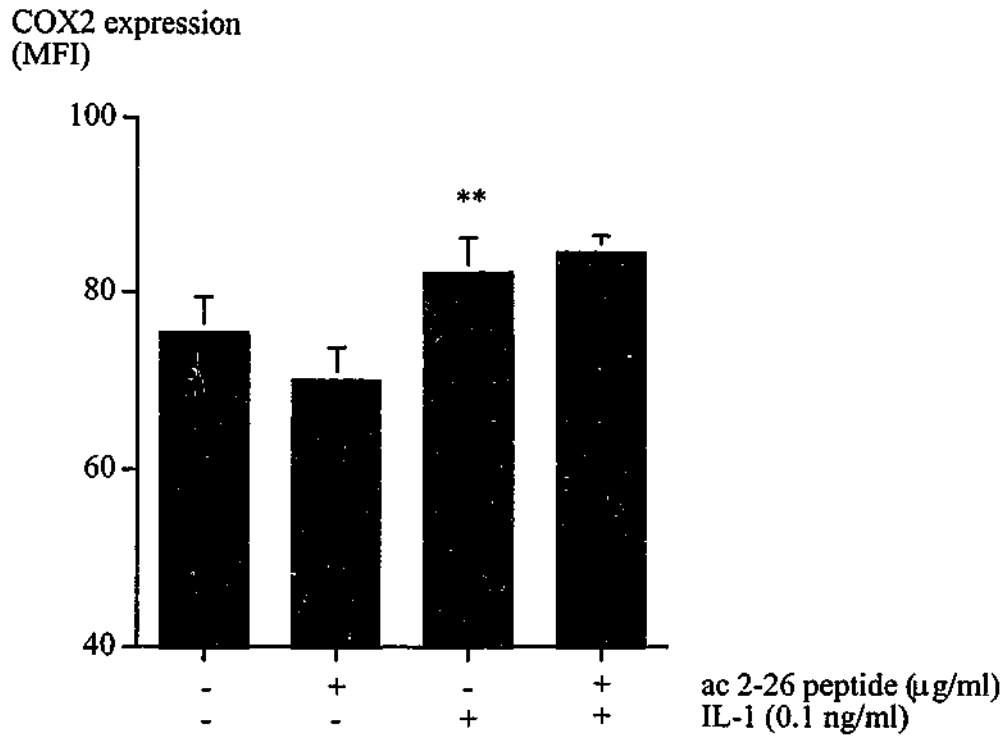
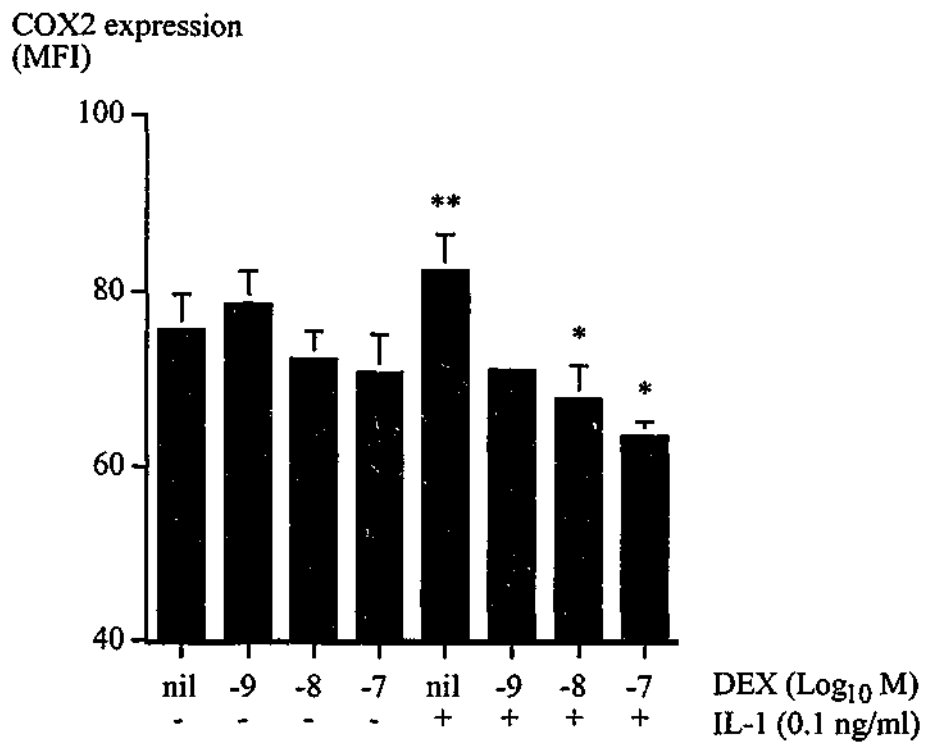

COX2 expression
(MFI)
100
80
60
40
**
- + - +
- - + +
ac 2-26 peptide (µg/ml)
IL-1 (0.1 ng/ml)

Figure 3.6.8

Intracellular COX2 expression in human FLS. Human FLS were treated with IL-1β (0.1 ng/ml), and/or DEX (10^{-9}-10^{-7} M) for 8 hours. Intracellular COX2 was detected by permeabilization flow cytometry and expressed as the mean fluorescence intensity (MFI) after subtraction of MFI obtained with the negative control mAb. Values are the mean ± SEM of 4 experiments. ** $p < 0.01$ versus untreated FLS, * $p < 0.05$ versus IL-1β-treated FLS.


COX2 expression
(MFI)
100
80
60
40
**
*
*
nil -9 -8 -7 nil -9 -8 -7
DEX (Log10 M)
- - - - + + + +
IL-1 (0.1 ng/ml)

CHAPTER FOUR: EFFECTS OF MIF ON RHEUMATOID FIBROBLAST-LIKE SYNOVIOCYTE PHOSPHOLIPASE A_2 ACTIVITY

4.1 Chapter Summary

Macrophage migration inhibitory factor (MIF) is expressed in human rheumatoid (RA) synovium and fibroblast-like synoviocytes (FLS). The involvement of MIF in T-cell activation and monocyte TNFα production implicates a role in the pathogenesis of RA. MIF is unique in its ability to override the anti-inflammatory actions of glucocorticoids. In synovial inflammation, PLA_2 is a critical enzyme involved in the hydrolytic release of arachidonic acid, leading to PGE_2 generation. The effects of MIF on FLS activation phenomena such as PLA_2 induction have not been previously explored. In the studies presented in this chapter, I investigated the effects of MIF on FLS phospholipase A_2 (PLA_2) mRNA and activity in RA FLS. The ability of MIF to override the inhibitory actions of glucocorticoids in IL-1β-stimulated PLA_2 activity in FLS was also examined.

PLA_2 activity was measured by [^{3}H]arachidonic acid released from treated FLS supernatants. $cPLA_2$ mRNA was determined using semi-quantitative RT-PCR. Constitutive PLA_2 activity was detected in cultured RA FLS. MIF upregulated PLA_2 activity and $cPLA_2$ mRNA expression. IL−1β also induced PLA_2 activity and $cPLA_2$ mRNA expression and this effect of IL−1β was inhibited by MIF mAb. Dexamethasone (10^{-8}M) inhibited IL−1β-stimulated PLA_2 activity, and MIF antagonised this inhibition. This study represents the first demonstration of MIF reversal of DEX inhibitory actions in human cells derived from the site of inflammatory disease.

The findings that MIF directly induces PLA_2 activity and mRNA expression, and that MIF mAb is inhibitory of IL−1β activation of FLS, implies upstream and downstream actions of MIF in RA inflammatory processes. Moreover, MIF exerts a

glucocorticoid-counter regulatory action in RA FLS. These data suggest an important therapeutic potential for MIF antagonism in RA.

4.2 Introduction

As reviewed in Chapter 1, glucocorticoids mediate a wide range of inhibitory effects on immune and inflammatory activation. In rheumatoid arthritis (RA), a prominent effect is inhibition of cytokine synthesis. Glucocorticoids also upregulate synthesis of anti-inflammatory molecules such as interleukin-10, and as reviewed in the preceding chapters, annexin I.

The proinflammatory cytokine, macrophage migration inhibitory factor (MIF), has a wide range of effects consistent with a role in the pathogenesis of RA. As detailed in Chapter 1, animal and human studies support the hypothesis that MIF is an important cytokine in RA. My interest in this molecule arises from the unusual characteristic that, although pro-inflammatory, the synthesis and release of MIF are induced by glucocorticoids. Thus, glucocorticoids can induce both anti-inflammatory and pro-inflammatory regulatory proteins.

The effects of MIF on synoviocyte activation have not been previously explored. As reviewed in Chapter 1, MIF has emerged as an important pro-inflammatory cytokine in inflammatory and immune responses. MIF was originally defined by its ability to inhibit the random migration of macrophages *in vitro*. The recent cloning of mouse and human MIF has enabled additional pro-inflammatory actions of MIF to be elucidated which transcend the original nomenclature of this cytokine (Geiler et al., 1994; Mitchell et al., 1995). Important proinflammatory actions of MIF have been demonstrated in the development of endotoxic shock, delayed-type hypersensitivity and T-cell activation (Calandra and Bucala, 1996; Bernhagen et al., 1996; Bacher et al., 1996). A unique aspect of MIF among proinflammatory cytokines is its induction by low concentrations of glucocorticoids. In concert with its ability to counter-

regulate the immunosuppressive effects of glucocorticoids, this suggests a unique MIF-glucocorticoid interaction in the control of the immune response (Calandra et al., 1995; Leech et al., 1999; Bucala, 1996; Calandra and Bucala, 1997).

Rheumatoid arthritis (RA) is characterized by synovial inflammation mediated by macrophages, T cells and fibroblast-like synoviocytes (FLS). Several lines of evidence support a potential role for MIF in RA. Its constitutive expression in macrophages and T-cells, its ability to induce macrophage TNFα release and nitric oxide production, and its essential role in T-cell activation, indicate a role for MIF in immune cell activation (Bacher et al., 1996; Attur et al., 1997; Liew, 1994). This concept is supported by studies in animal models of arthritis, wherein monoclonal antibody immunoneutralization of MIF inhibits disease severity, frequency and onset of disease (Leech et al., 1998; Mikulowska et al., 1997). Moreover, the constitutive expression of MIF in human rheumatoid synovium, serum, synovial fluid and cultured FLS suggests the involvement of MIF in the initiation and/or perpetuation of inflammatory processes in RA (Leech et al., 1999). For example, RA FLS-derived MIF induces monocyte TNFα release (Leech et al., 1999). The potential effects of MIF on FLS activation, however, have not been previously examined.

Phospholipase A_2 (PLA_2) has been detected in a wide variety of inflammatory conditions including RA, in which serum levels of this enzyme have been found to correlate with disease activity. In the synovial joint, sources of PLA_2 include FLS and infiltrating leukocytes. PLA_2 is a critical enzyme involved in the hydrolytic release of arachidonic acid from phospholipid membranes, leading to PGE_2 generation. The major isotypes of PLA_2 consist of a secretory ($sPLA_2$) and a mitogen/cytokine inducible cytosolic form ($cPLA_2$) (Murakami et al., 1997).

In vitro studies suggest that $cPLA_2$ is regulated by elevated cytokine levels present in RA synovium. Cytokine-activated RA FLS are believed to contribute to important RA inflammatory phenomena through activation of $cPLA_2$ (Croxtall et al., 1998). For example, treatment of cultured FLS with IL-1β increases $cPLA_2$ activity and expression (see Chapter 3) (Hulkower et al., 1992; Hulkower et al., 1994; Gilman et al., 1988; Roshak et al., 1996; Angel et al., 1993; Hulkower et al., 1993), which correlated with increased PGE_2 production. As described in Chapter 3 and other studies, cytokine-induced $cPLA_2$ activation is inhibited by DEX treatment.

The described proinflammatory actions of MIF, in addition to the eicosanoid generating capacity of RA FLS, led to the study of the effects of MIF on PLA_2 activity and mRNA expression in RA FLS. Investigations also examined MIF reversal of the anti-inflammatory actions of DEX in IL-1β-induced FLS.

4.3 Methods

4.3.1 Isolation and culture of fibroblast-like synoviocytes

As described in Chapter 2, fibroblast-like synoviocytes (FLS) were obtained from synovium of rheumatoid (RA) patients (n = 6) undergoing joint replacement surgery. All patients satisfied the American College of Rheumatology criteria for the classification of RA (Arnett et al., 1987). FLS were isolated using enzyme digestion procedures and cultured in RPMI/10% FCS (see sections 2.3.6-2.3.8). Thawed cells were used in experiments between passages 4 and 9. For all experiments, FLS were seeded at 1 x 10^5 cells per well in 24 well culture plates in RPMI/10% FCS and allowed to adhere overnight, prior to medium being replaced with RPMI/0.1% BSA for experimental purposes. Cells were >95% viable by trypan blue exclusion. In each group of experiments, n refers to the number of individual human RA donor FLS used.

4.3.2 Assessment of Phospholipase A_2 activity

PLA_2 activity in FLS was determined as described in section 3.3.4. In brief, FLS were labelled for 18 hours with 1 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid in 0.1% BSA/RPMI. Cells were treated with human recombinant MIF (5-500 ng/ml), a neutralizing anti-human MIF mAb (50 μg/ml; or isotype-matched negative control, IgG_1) (Leech et al., 1999), human recombinant IL–1β (0.1 ng/ml; Sigma), and/or DEX (10^{-8} M; Sigma) for 8 hours. Duplicate cultures were used for each determination. Radioactivity in the supernatant was determined using a Wallac 1409 liquid scintillation counter (Pharmacia, Finland), with results expressed as [^{3}H]arachidonic acid released (cpm).

4.3.3 $cPLA_2$ mRNA RT-PCR

Cells were treated with human recombinant MIF (500 ng/ml), anti-human MIF mAb (50 μg/ml; or isotype-matched negative control, IgG_1) (Leech et al., 1999), and/or human recombinant IL–1β (0.1 ng/ml; Sigma) for 8 hours. Duplicate cultures were used for each determination. Following treatment, total RNA was extracted from treated FLS using TRIzol (Gibco BRL, Melbourne, Australia). RNA was reverse transcribed in a 20 μl volume containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM MgCl, 10 mM DTT, 500 μM dNTPs, 500 nM oligo dT primers, 0.5 μg total RNA and 200 units of Superscript II reverse transcriptase (Gibco BRL). PCR was performed in a 25 μl volume of 20 mM Tris HCl (pH 8.4), 50 mM KCl, 3 mM MgCl, 400 μM dNTPs, 500 nM each of forward and reverse cDNA primers, 2 μl cDNA and 2.5 units of Taq polymerase (Gibco BRL). The cycling parameters were denaturation at 94°C for 1 min, annealing at 54°C for 1 min and extension at 72°C for 1 min, for 30 cycles, using a DNA thermal cycler (Hybaid, Omnigene). The primers used were: $cPLA_2$: 5' GAG CTG ATG TTT GCA GAT TGG GTT G 3' (forward), 5' GTC ACT CAA AGG AGA CAG TGG ATA AGA 3' (reverse); and GAPDH: 5' CGT CTT CAC CAC CAT GGA GA 3' (forward), 5' CGG CCA TCA CGC CAC AGT TT 3' (reverse), yielding PCR products of 510 bp and 300 bp, respectively. Following PCR, the amplified products were electrophoresed on a 2% agarose gel containing ethidium bromide with a size marker (123 bp ladder, Sigma) and with gel loading normalised for GAPDH products. Visual analysis and image analysing software (Kodak EDAS 120 System, Gibco BRL) were used for the comparison of band intensities. These experiments were performed with the assistance of Ms Pamela Hall, Monash University.

4.3.4 Statistical Analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed using the Student's t-test, with values of $p < 0.05$ regarded as statistically significant.

4.4 Results

4.4.1 Effect of rMIF on PLA_2 activity

In this study, the effect of MIF on FLS PLA_2 activity was first examined. Constitutive PLA_2 activity, as measured by [^{3}H]arachidonic acid release, was detected in RA FLS. MIF (5-500 ng/ml) induced a significant concentration-dependent increase in PLA_2 activity (Fig. 4.6.1).

4.4.2 Effect of rMIF on $cPLA_2$ mRNA expression

Results from PLA_2 activity studies suggested that the effects of MIF were mediated by actions on $cPLA_2$. The effect of MIF on $cPLA_2$ mRNA expression in RA FLS has not been previously explored. Consistent with the effects on PLA_2 activity, RT-PCR demonstrated MIF induction of $cPLA_2$ mRNA in RA FLS (Fig. 4.6.2).

4.4.3 MIF reversal of the inhibitory actions of DEX on IL-1β stimulated PLA_2 activity

A unique aspect of MIF is its antagonism of glucocorticoid anti-inflammatory effects. Investigations sought to confirm that this phenomenon was present in RA FLS. IL–1β-stimulated PLA_2 activity ($p < 0.01$) was significantly inhibited by DEX (10^{-8} M) ($p < 0.01$). MIF (50 ng/ml) antagonized this inhibition by DEX, resulting in a significant restoration of PLA_2 activity ($p < 0.05$) (Table 4.6.1).

4.4.4 Effect of MIF immunoneutralization on constitutive and IL-1β stimulated PLA_2 activity

The effect of FLS-derived MIF on PLA_2 activity was examined by MIF immunoneutralization. IL–1β (0.1 ng/ml) significantly upregulated PLA_2 activity ($p < 0.01$), and monoclonal antibody MIF immunoneutralization (50 μg/ml) significantly

inhibited this induction by IL–1β ($p < 0.05$; Fig. 4.6.3). Of note, MIF immunoneutralization did not inhibit constitutive PLA_2 activity.

4.4.5 Effect of MIF immunoneutralization on constitutive and IL-1β stimulated $cPLA_2$ mRNA expression

Consistent with PLA_2 activity, IL–1β up-regulated the expression of $cPLA_2$ mRNA. MIF immunoneutralization inhibited IL-1-induced $cPLA_2$ mRNA expression in RA FLS (Fig. 4.6.4).

4.5 Discussion

The current study extends the diverse range of proinflammatory actions ascribed to MIF in immune/inflammatory settings. As reviewed in Chapter 1, MIF has previously been shown to induce macrophage secretion of TNFα and promotes IFN-γ-induced production of nitric oxide by macrophages (Attur et al., 1997; Liew, 1994; Bernhagen et al., 1994). In addition to its effects on macrophages, MIF is a critical factor in T-cell activation *in vitro* (Bacher et al., 1996). A key role for MIF in basic immune and inflammatory responses is supported by its observed importance in animal models of inflammatory disease. For example, immunoneutralization of MIF confers protection against lethal endotoxemia, and the role of MIF in endotoxic shock has been confirmed in studies using MIF -/- mice (Calandra and Bucala, 1996; Bozza et al., 1999). MIF is also essential for delayed-type hypersensitivity responses *in vivo* (Calandra et al., 1995). The potential role of MIF in the pathogenesis of autoimmune diseases has recently been reported. For example, in rat immune glomerulonephritis, MIF immunoneutralization profoundly inhibited disease severity (Lan et al., 1997). In concert with other autoimmune diseases whose pathology is dominated by multiple cell types, these studies raise the possibility of a pro-inflammatory role for MIF in RA.

RA is a chronic, systemic disease whose manifestations are dominated by synovial hyperplasia and inflammation in the synovial joint. The aetiology of RA is unknown, but current theories suggest the involvement of T cells, macrophages and resident synovial cells in the initiation and/or perpetuation of inflammatory processes in RA. Coworkers have demonstrated the overexpression of MIF in RA macrophage-like synoviocytes, cultured FLS, synovial fluid and RA patient serum (Leech et al., 1999). Monoclonal immunoneutralization of MIF profoundly inhibited adjuvant arthritis,

reducing synovial macrophage and T-cell accumulation (Leech et al., 1998). In murine collagen-induced arthritis, MIF immunoneutralization lowered the onset and frequency of disease (Mikulowska et al., 1997). MIF derived from RA FLS induced monocyte TNFα release *in vitro*, which suggests that locally produced MIF is capable of up-regulating the RA inflammatory process and has an upstream regulatory action in TNFα release (Leech et al., 1999).

In RA, prostaglandin E_2 is the major eicosanoid generated in the synovial tissue, and is implicated as an important mediator of inflammation in RA (Davies et al., 1984). As described in section 1.2.4.2 and Chapter 3, PLA_2 is a pivotal enzyme in eicosanoid generation that is expressed in RA synovium and FLS. Considerable evidence documents both the expression and induction of PLA_2 by proinflammatory cytokines in human FLS (Hulkower et al., 1992; Hulkower et al., 1994; Gilman et al., 1988; Roshak et al., 1996; Angel et al., 1993; Hulkower et al., 1993) (refer to Chapter 3). To date, however, the mechanisms involved in upregulation of PLA_2-induced PGE_2 production in RA remain incompletely elucidated. In the present study, I demonstrate for the first time the role of MIF in PLA_2 induction in RA FLS. The coordinate induction of $cPLA_2$ mRNA and activity by MIF demonstrates that MIF has a stimulatory role on a key aspect of FLS activation. The concentrations of MIF used in these experiments are within the range detected in human RA synovial fluid (Leech et al., 1999). Studies by my coworker Ms Pamela Hall, not included in this thesis, demonstrate that the effect of MIF is limited to $cPLA_2$. No induction of $sPLA_2$ expression was observed in MIF-treated RA FLS.

Interleukin-1β induction of $cPLA_2$ mRNA and activity in human FLS, and other cell types, has been reported previously (Hulkower et al., 1994; Angel et al., 1994; Newton et al., 1997; Szczepanski et al., 1994). Anti-MIF mAb administration

inhibited IL–1β-induced $cPLA_2$ mRNA expression and activity. Cultured RA FLS constitutively release MIF, but interestingly IL–1β does not upregulate the release of MIF by RA FLS (Leech et al., 1999). This implies that, in addition to its direct effects on $cPLA_2$, MIF is an essential cofactor in the upregulation of $cPLA_2$ by IL–1β. The ability of anti-MIF mAb to act in a system where MIF expression is stable is consistent with published *in vitro* studies on T cell activation (Bacher et al., 1996). In these studies, MIF had no mitogenic effect in isolation but MIF immunoneutralization prevented the activation of T cells by mitogens or anti-CD3 antibody. The mechanism through which MIF exerts this cofactor role is currently unknown, but may involve the activation of signal transduction pathways required for the effects of other stimuli. Although the signal transduction events activated by MIF are incompletely documented, these data confirm and extend recent findings observed in a cultured fibroblast line, in which MIF was found to induce $cPLA_2$ activity via upregulation of p44/p42 ERK MAP kinases (Mitchell et al., 1999). The current data is the first demonstration of PLA_2 upregulation in human cells derived from the site of inflammatory disease. Moreover, this study is the first to establish that the effects of MIF on PLA_2 involve increases in $cPLA_2$ gene transcription. Signal transduction events operative in synovial cells are the subject of studies presented in Chapter 6. Glucocorticoids are potent anti-inflammatory and immunosuppressive drugs, whose therapeutic efficacy has led to their widespread clinical utilisation. Despite the sensitivity of RA to the action of glucocorticoids, incomplete efficacy of glucocorticoids is observed clinically (Van Riel et al., 1999). Biphasic regulation of MIF release by glucocorticoids, wherein low concentrations of glucocorticoids stimulate MIF synthesis and release, has been described in monocytes and macrophages, and more recently in human RA FLS (Calandra et al., 1995; Leech et

al., 1999). The paradoxical finding that glucocorticoids induce secretion of a pro-inflammatory cytokine led to the concept that MIF might counter-regulate the anti-inflammatory and immunosuppressive effects of glucocorticoids (Calandra et al., 1995). In this context, MIF has been shown override glucocorticoid-inhibition of cytokine production in LPS-stimulated macrophages, T-cell proliferation, and production of IL-2 and IFN-γ (Bacher et al., 1996; Calandra et al., 1995). In murine endotoxemia, MIF was able to overcome the protective effects of glucocorticoids (Calandra and Bucala, 1996). More recently, a study using a fibroblast cell-line has demonstrated the ability of MIF to override the inhibitory actions of DEX in TNFα-induced PLA_2 activity (Mitchell et al., 1999). Results herein are the first to demonstrate MIF reversal of the inhibitory actions of glucocorticoids in human cells derived from an inflammatory disease. The failure of glucocorticoids to completely control RA pathology may potentially be explained by counter-regulatory phenomena such as the induction of MIF. The physiological relevance of these results is supported by *in vivo* studies of arthritis, whereby administration of MIF was observed to counteract the inhibitory actions of glucocorticoids in a murine arthritis model (Santos L., and Morand E.F., Clin. Exp. Immunol., in press). Specific antagonism of MIF may enhance the therapeutic efficacy of glucocorticoids in diseases such as RA. In summary, these results demonstrate that MIF directly induces PLA_2 mRNA expression and activity in RA FLS, and establish the essential role of MIF in IL-1β-upregulation of PLA_2 in RA FLS. Results also confirm that the phenomenon of MIF antagonism of glucocorticoid anti-inflammatory effects is present in RA FLS. These results further support an important role for MIF in a broad range of pro-inflammatory actions in RA pathology. The actions of MIF in FLS activation studies, taken with its constitutive expression in RA synoviocytes, its role in macrophage and T-cell

activation processes, and its ability to induce TNFα secretion, implicates both upstream and downstream regulatory actions for MIF in the inflammatory cascade. These observations are consistent with its demonstrated roles across innate and cognate immune responses *in vivo*. The breadth of involvement of MIF in the immune response suggests it is a therapeutic target in RA with potentially greater benefit than that gained from targeting monokines alone. Moreover, the ability of MIF to antagonise the anti-inflammatory action of glucocorticoids in cytokine-activated FLS provides evidence that this counter-regulatory system is operative in RA.

4.6 Table and Figures

Table 4.6.1

MIF reversal of the inhibitory actions of DEX on IL-1β stimulated PLA_2 activity. FLS were treated with IL-1β (0.1 ng/ml), and/or DEX (10^{-8}M) and/or MIF (50 ng/ml). PLA_2 activity is expressed as ^{3}H-AA release (cpm). Values are the mean ± SEM of 4 experiments. * p versus untreated, † p versus IL-1 β treated FLS, ‡ p < versus DEX plus IL-1β treated FLS.

Treatment	**^{3}H-AA release (cpm)**	**p value**
control	12246 ± 2348	
IL-1β	22270 ± 6094	* $p < 0.01$
IL-1 β + DEX	16428 ± 2003	† $p < 0.01$
IL-1 β + DEX + MIF	18578 ± 2386	‡ $p < 0.05$

Figure 4.6.1

Effect of rMIF on PLA_2 activity in FLS.

FLS were treated with human recombinant MIF (5-500 ng/ml). PLA_2 activity is expressed as ^{3}H-AA released. Values are the mean ± SEM of 4 experiments. * $p < 0.01$ versus untreated FLS.

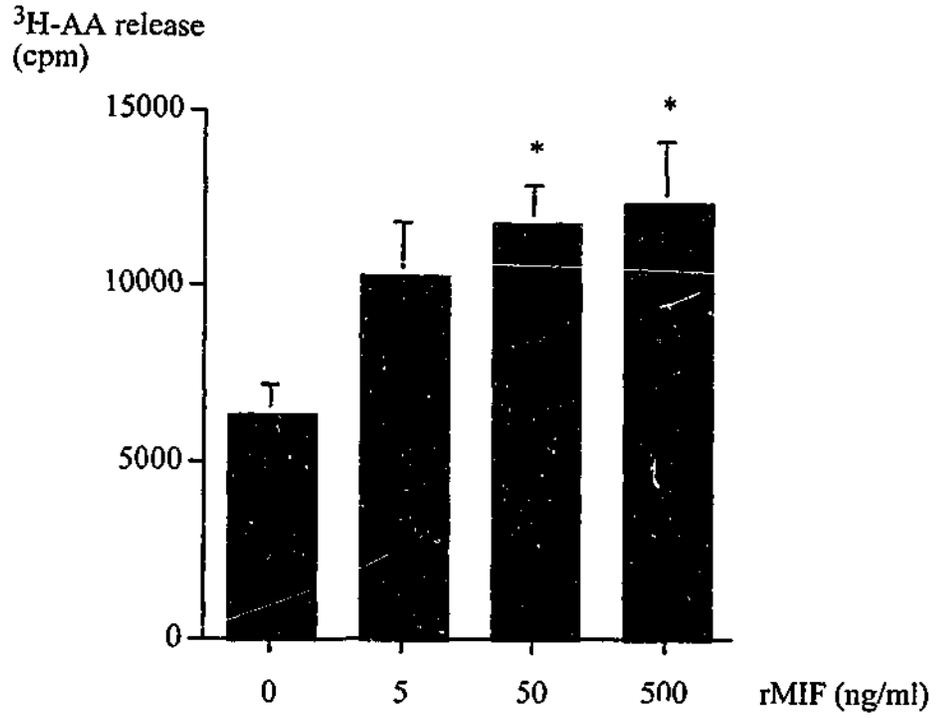

^{3}H-AA release
(cpm)
15000
10000
5000
0
*
*
0
5
50
500
rMIF (ng/ml)

Figure 4.6.2

Effect of rMIF on $cPLA_2$ mRNA expression.

FLS were treated with human recombinant MIF. A PCR product of 300 bp was obtained for GAPDH and a PCR product of 510 for $cPLA_2$. Lane 1: untreated FLS; Lane 2: MIF (500 ng/ml); Representative data of n = 3 experiments.

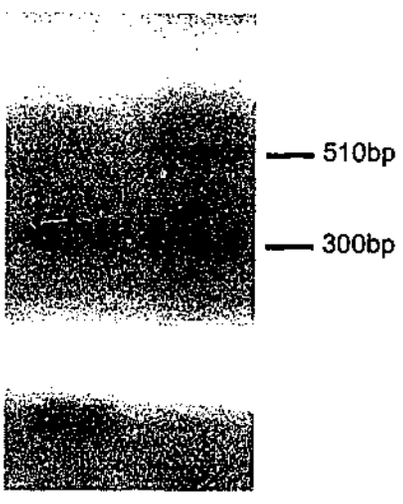

510bp
300bp

Figure 4.6.3

Effect of MIF immunoneutralization on constitutive and IL-1β stimulated PLA_2 activity.

FLS were treated with MIF mAb (50 μg/ml; or isotype-matched control Ab (NC Ab), IgG_1) and/or IL–1β (0.1 ng/ml). PLA_2 activity is expressed as ^{3}H-AA released. Values are the mean ± SEM of 5 experiments. * $p < 0.01$ versus untreated, † $p < 0.05$ versus negative control antibody.

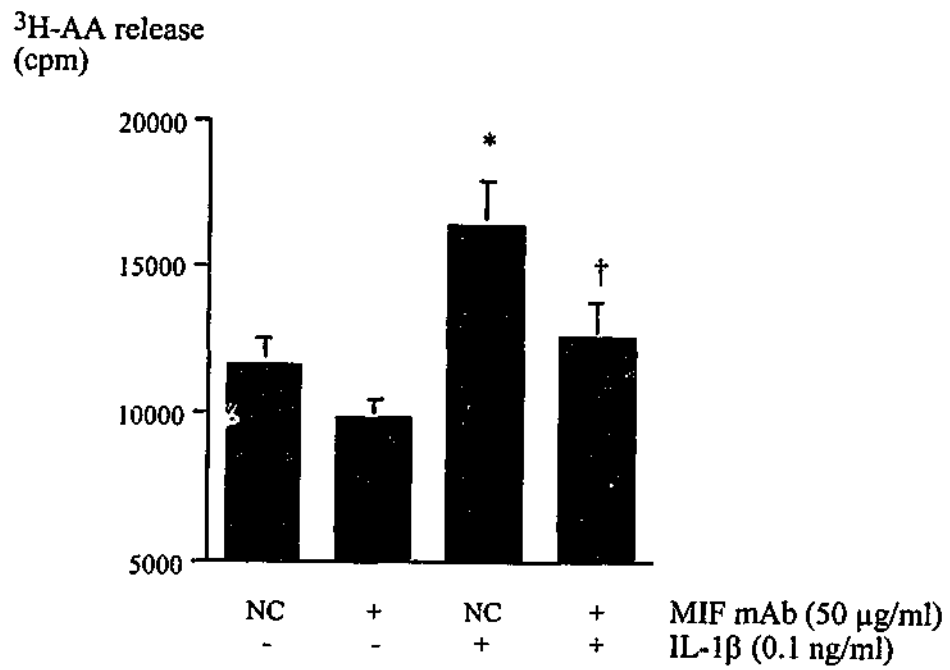
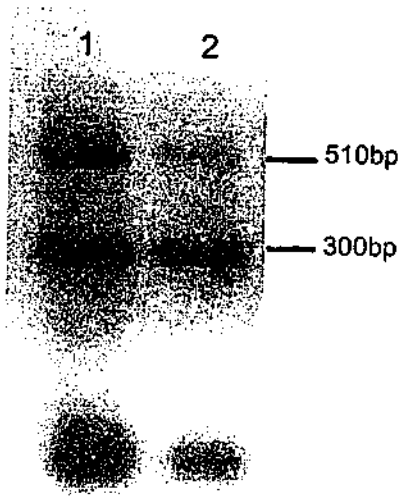

^{3}H-AA release
(cpm)
20000
15000
10000
5000
*
†
NC
+
NC
+
MIF mAb (50 μg/ml)
-
-
+
+
IL-1β (0.1 ng/ml)

Figure 4.6.4

Effect of MIF mAb on IL-1β-stimulated $cPLA_2$ mRNA transcription.

FLS were treated with human recombinant IL–1β and negative control IgG_1, or MIF mAb. A PCR product of 300 bp was obtained for GAPDH and a PCR product of 510 for $cPLA_2$. Lane 1: IL–1β (0.1 ng/ml) and negative control IgG_1 (50 μg/ml); Lane 2: IL–1β and MIF mAb (50 μg/ml). Representative data of n = 3 experiments.


1
2
510bp
300bp

CHAPTER FIVE: EFFECTS OF MIF ON RHEUMATOID FIBROBLAST-LIKE SYNOVIOCYTE COX2 AND PGE_2 PRODUCTION

5.1 Chapter Summary

As discussed in Chapters 1 and 4, macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine with known actions in macrophage and T cell activation. In synovial inflammation, PGE_2 production is catalyzed by the enzymatic actions of phospholipase A_2 and cyclooxygenase (COX). Although COX2 is increased in RA synovium both *in vivo*, and *in vitro* following treatment with proinflammatory cytokines, the enzymatic events responsible for PGE_2 production in RA remains controversial. In this chapter, the effects of MIF on FLS COX2 induction and PGE_2 release in RA FLS were investigated.

COX activity was measured by PGE_2 ELISA after exogenous arachidonic acid was added to treated FLS. COX2 mRNA was determined using semi-quantitative RT-PCR. PGE_2 release from FLS supernatants was measured by PGE_2 ELISA.

RA FLS exhibited low levels of constitutive COX activity. MIF upregulated FLS COX activity, implying an effect on COX2, and RT-PCR confirmed MIF induction of COX2 mRNA. IL-1β induced COX activity and COX2 mRNA expression. MIF immunoneutralization inhibited IL-1β-stimulated COX activity and COX2 mRNA. Constitutive PGE_2 release in FLS, as measured by PGE_2 ELISA, was upregulated by MIF. Interleukin-1β upregulated PGE_2 release and MIF immunoneutralization exhibited a trend towards inhibition of IL-1β stimulated PGE_2 release.

MIF exerts a proinflammatory effect on key aspects of RA FLS activation. The demonstration that MIF directly induces COX activity, COX2 mRNA expression and PGE_2 release, and that MIF mAb is inhibitory of IL–1β activation of FLS, implies downstream and upstream actions of MIF in the inflammatory cascade. These results are consistent with a key role for MIF in RA eicosanoid production and support a therapeutic potential for MIF antagonism in RA.

5.2 Introduction

As discussed in section 1.6 and Chapter 4, macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine with an emerging importance in immune and inflammatory responses. An essential role for MIF is observed in the development of endotoxic shock, delayed-type hypersensitivity and T-cell activation (Calandra and Bucala, 1996; Bernhagen et al., 1996; Bacher et al., 1996). Its induction by low concentrations of glucocorticoids and ability to override the anti-inflammatory actions of glucocorticoids implicates an endogenous glucocorticoid counter-regulatory action for MIF in inflammatory processes (Calandra et al., 1995; Leech et al., 1999; Bucala, 1996; Calandra and Bucala, 1997).

In chronic inflammatory diseases such as RA, whose pathology is targeted to the synovial joints, much attention has focused on the contribution of infiltrating T cells, macrophages and resident FLS, and their products in the disease process. The evidence supporting a role for MIF in RA has been presented in Chapters 1 and 4. The potential effects of MIF in FLS eicosanoid generation have not been explored.

Prostaglandin E_2 (PGE_2) is an important mediator of pain and oedema associated with RA synovitis. Prostaglandin E_2 synthesis is initiated by the release of arachidonic acid from membrane phospholipids by phospholipase A_2 (PLA_2). Arachidonate is then converted to PGH_2 by cyclooxygenase (COX). The recent discovery of a second isoform of COX, COX2, has prompted new questions concerning the regulation of PGE_2 synthesis. As noted in Chapter 1, data from *in vitro* studies suggest that expression of the COX2 gene is regulated by proinflammatory stimuli, whilst COX1 is constitutively expressed.

In RA, elevated levels of COX have been observed in synovial tissue, with both COX1 and COX2 detected in RA FLS (Sano et al., 1992). Cytokines present in RA

synovium are believed to contribute to increased COX2. For example, treatment of cultured FLS with proinflammatory cytokines dramatically increases PGE_2 production. To date, the enzymatic events responsible for elevated PGE_2 generation in inflammation remain unresolved. For example, in a COX2 gene knock out model, inflammation still persists despite the absence of this enzyme.

In Chapter 4, I established that MIF directly induces PLA_2 activity and $cPLA_2$ mRNA expression in RA FLS. To further explore the action of MIF in the RA FLS eicosanoid generation pathway, I investigated the effects of MIF on COX activity, COX2 mRNA expression and PGE_2 release in RA FLS.

5.3 Methods

5.3.1 Isolation and culture of FLS

Fibroblast-like synoviocytes (FLS) were obtained from synovium of rheumatoid (RA) patients (n = 6) undergoing joint replacement surgery. All patients satisfied the American College of Rheumatology criteria for the classification of RA (Arnett et al., 1987). FLS were isolated using enzyme digestion procedures and cultured in RPMI/10% FCS (see sections 2.3.6-2.3.8). Thawed cells were used in experiments between passages 4 and 9. For all experiments, FLS were seeded at 1 x 10^5 cells per well in 24 well culture plates in RPMI/10% FCS and allowed to adhere overnight, prior to medium being replaced with RPMI/0.1% BSA for experimental purposes. Cells were >95% viable by trypan blue exclusion. In each group of experiments, n refers to the number of individual human RA donor FLS used.

5.3.2 Determination of PGE_2 release

The concentration of PGE_2 in the treated FLS supernatants was measured using a PGE_2 ELISA kit, according to the manufacturer's instructions (see section 3.3.5).

Cells were treated with human recombinant MIF, anti-human MIF mAb (50 μg/ml; or isotype-matched negative control, IgG_1) (Leech et al., 1999), and/or human recombinant IL-1β (0.1 ng/ml), for 8 hours. PGE_2 released from the treated FLS supernatants was measured by PGE_2 ELISA (see section 3.3.5). In brief, a 50 μl aliquot of PGE_2 standards and samples were loaded onto a PGE_2 antibody precoated 96 well culture plate, in the presence of PGE_2-horseradish peroxidase conjugate. Following incubation, the enzyme conjugate was detected by a 3,3', 5,5' tetramethylbenzidine/hydrogen peroxide mixture. The reaction was terminated and PGE_2 concentrations were measured at Abs_{450nm}. Results were expressed as ng/ml of PGE_2.

5.3.3 Assessment of COX activity

COX activity was measured in RA FLS as described by Wilborn et al (Wilborn et al., 1995) and as detailed in 3.3.6. In brief, cells were treated with human recombinant MIF, anti-human MIF mAb (50 μg/ml; or isotype-matched negative control, IgG_1) (Leech et al., 1999), and/or human recombinant IL-1β (0.1 ng/ml) for 8 hours. Following incubation, FLS were washed and then incubated for 30 minutes with 10 μM exogenous arachidonic acid. Supernatants from treated FLS were aspirated immediately, stored at -20°C, and assayed for PGE_2 release by ELISA (see section 3.3.5). Duplicate cultures were used for each determination and COX activity was expressed as ng/ml PGE_2. This assay measures PGE_2 production in the presence of excess arachidonic acid, thus providing a measure of total COX activity.

5.3.4 COX2 mRNA RT-PCR

Cells were treated with human recombinant MIF, anti-human MIF mAb (50 μg/ml; or isotype-matched negative control, IgG_1), and/or human recombinant IL-1β (0.1

ng/ml; Sigma) for 8 hours. Duplicate cultures were used for each determination. Following treatment, total RNA was extracted from treated FLS using TRIzol (Gibco BRL, Melbourne, Australia). RNA was reverse transcribed in a 20 µl volume containing 50 mM Tris HCl (pH 8.3), 75 mM KCl, 3 mM MgCl, 10 mM DTT, 500 µM dNTPs, 500 nM oligo dT primers, 0.5 µg total RNA and 200 units of Superscript II reverse transcriptase (Gibco BRL). PCR was performed in a 25 µl volume of 20 mM Tris HCl (pH 8.4), 50 mM KCl, 3 mM MgCl, 400 µM dNTPs, 500 nM each of forward and reverse cDNA primers, 2 µl cDNA and 2.5 units of Taq polymerase (Gibco BRL). The cycling parameters were denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute and extension at 72°C for 1 minute, for 30 cycles, using a DNA thermal cycler (Hybaid, Omnigene). The primers used were: COX2: 5' TTC AGC TCC ACA GCC AGA CGC 3' (forward), 5' ATC AGG CAC AGG AGG AAG GGC 3' (reverse); and GAPDH: 5' CGT CTT CAC CAC CAT GGA GA 3' (forward), 5' CGG CCA TCA CGC CAC AGT TT 3' (reverse), yielding PCR products of 499 bp and 300 bp, respectively. Following PCR, the amplified products were electrophoresed on a 2% agarose gel containing ethidium bromide with a size marker (123 bp ladder, Sigma) and with gel loading normalised for GAPDH products. Visual analysis and image analysing software (Kodak EDAS 120 System, Gibco BRL) were used for the comparison of band intensities. These experiments were performed with the assistance of Ms Pamela Hall, Monash University.

5.3.5 Statistical analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed using the Student's t-test, with values of $p < 0.05$ regarded as statistically significant.

5.4 Results

5.4.1 Effect of rMIF on PGE_2 production

The findings that MIF regulated the induction of PLA_2 activity and mRNA expression in FLS (see sections 4.4.1 and 4.4.2) strongly suggested that MIF may also induce PGE_2 release. Constitutive RA FLS PGE_2 release, as detected by ELISA, was upregulated by MIF (1 ng/ml) ($p < 0.05$; Figure 5.6.1).

5.4.2 Effect of rMIF on COX activity

The mechanism of MIF induction of PGE_2 was next explored. The observed induction by MIF on PLA_2 activity and $cPLA_2$ mRNA expression (see sections 4.4.1 and 4.4.2) suggested a similar action by MIF on COX2. To assess total COX activity in FLS, PLA_2 activity was bypassed by the addition of exogenous arachidonic acid (Wilborn et al., 1995). Constitutive COX activity was detected in FLS, and was significantly upregulated by rMIF (5 ng/ml) ($p < 0.05$; Figure 5.6.2).

5.4.3 Effect of MIF on COX2 mRNA expression

The ability of MIF to induce COX activity suggested that the effects of MIF were mediated via actions on COX2. Consistent with the effects on COX activity, MIF upregulated COX2 mRNA expression as measured by RT-PCR (Figure 5.6.3).

5.4.4 Effect of MIF immunoneutralization on constitutive and IL-1β stimulated COX activity

The effect of FLS-derived MIF on COX activity was determined by MIF immunoneutralization. IL–1β significantly increased COX activity ($p < 0.01$), and this effect was significantly reduced by MIF monclonal antibody neutralization ($p < 0.05$; Figure 5.6.4). Of note, MIF immunoneutralization had no effect on constitutive COX activity.

5.4.5 Effect of MIF immunoneutralization on constitutive and IL-1β-stimulated COX2 mRNA expression

Having observed that MIF antagonism inhibits IL–1β induced COX activity, I next sought to examine the effect of FLS-derived MIF on COX2 mRNA expression. Interleukin–1β increased COX2 mRNA expression, and this effect was inhibited by MIF immunoneutralization (Figure 5.6.5).

5.5 Discussion

The recognition of MIF as an important regulator of immune and inflammatory responses has been presented in Chapters 1 and 4. In brief, MIF is released by activated T cells, macrophages, and resident cells and upregulates the pro-inflammatory activity of these cells. Key pro-inflammatory actions exerted by MIF are confirmed in animal models of disease. For example, an essential role for MIF is observed in the development of endotoxic shock, delayed-type hypersensitivity reactions (Calandra and Bucala, 1996; Bernhagen et al., 1996). The importance of MIF in inflammatory diseases is evident from studies where MIF Ab neutralisation profoundly inhibited severity of adjuvant arthritis in rats. MIF antagonism also delayed the onset and frequency of collagen-induced arthritis in the rat. In rat immune glomerulonephritis, administration of MIF mAb markedly inhibited disease severity (Lan et al., 1997).

RA is characterised by synovial inflammation and a cellular infiltrate comprised of T cells, macrophages and resident synovial cells. It is believed that expression of MIF in these cell types (Leech et al., 1999), in addition to elevated levels of MIF in RA FLS, is consistent with its role in inflammatory models of arthritis. More recently, MIF derived from RA FLS has been reported to induce monocyte TNFα release *in vitro*

(Leech et al., 1999). Investigations, however, have not yet determined the potential effects of MIF in RA FLS eicosanoid generation.

Prostaglandin E_2 is the major eicosanoid generated in RA synovial tissue, and is implicated as an important mediator of inflammation in RA (Davies et al., 1984). As reviewed in Chapters 1 and 3, COX2 is considered the major isoform of COX responsible for PGE_2 production in RA. Based on *in vitro* studies, it is assumed that agents such as proinflammatory cytokines are responsible for the upregulation of COX2, thereby leading to increased PGE_2 production (Hulkower et al., 1994; Angel et al., 1994; Newton et al., 1997). The role of COX2 in the pathophysiology of joint inflammation is further supported by the findings of animal studies. For example, the development of rat adjuvant arthritis is strongly associated with increased expression of COX2 and overproduction of PGE_2, and both the high levels of PGE_2 and joint oedema can be reversed by treatment with a selective COX2 inhibitor (Anderson et al., 1996). In the present study, I investigated for the first time the role of MIF in eicosanoid generation in human RA FLS. My results demonstrate that MIF induces FLS PGE_2 production, COX activity and COX2 mRNA expression. Studies by my coworker Ms Pamela Hall, not included in this thesis, demonstrate no effect of MIF on the expression of COX1 in RA FLS.

The direct induction of COX2 mRNA and activity by MIF demonstrates that MIF has a stimulatory role in FLS activation. In accordance with other *in vitro* studies is the demonstration of IL–1β induction of COX2 mRNA and activity in human FLS (Angel et al., 1994; Newton et al., 1997; Mino et al., 1998). The finding that anti-MIF mAb neutralisation inhibited IL–1β-induced COX2 mRNA expression, and COX activity is of great interest. This is because cultured RA FLS constitutively release MIF, but IL–1β does not upregulate the release of MIF in these cells (Leech et al.,

1999). Consistent with the results presented in Chapter 4, this finding implicates an essential 'cofactor' role of MIF in IL-1β-induced FLS activation. The current study is first to confirm a cofactor role of MIF in the upregulation of COX2 expression and activity. This inhibitory action of MIF mAb in an *in vitro* system where MIF expression is stable is consistent with previous investigations, as discussed in Chapter 4. From these studies, it is evident that an increased understanding of signal transduction mechanism(s) through which MIF may exert this cofactor role is required. Moreover, the ability for anti-MIF mAb to inhibit IL–1β induced FLS $cPLA_2$ and COX2 suggests that MIF-directed therapy may have rapid and clinically useful effects in patients with RA.

In summary, the results presented in Chapter 4 and this chapter demonstrate that MIF directly induces both $cPLA_2$ and COX2 in RA FLS. Moreover, I have shown that MIF is essential for the upregulation of $cPLA_2$ and COX2 in RA FLS by IL–1β. This chapter is first to confirm that the proinflammatory effects of MIF involve increases in COX2 gene transcription. The precise mechanisms responsible for MIF-induced PLA_2 and COX2 expression in RA require further definition. The intracellular events involved in the activation of FLS by MIF have not been previously investigated. Both $cPLA_2$ and COX2 genes are believed to be upregulated by cytokines such as IL-1β via effects on the transcription factor nuclear factor kappa B (NFκB). The hypothesis that MIF-induced FLS activation is via NFκB will be explored in Chapter 6.

FLS activation studies presented in this chapter support and extend the involvement of MIF in proinflammatory processes attributed to RA pathogenesis. MIF is constitutively expressed in cultured RA FLS, with a demonstrated role in T cell and macrophage activation. Moreover, coworkers have recently reported the ability of

FLS-derived MIF to induce monocyte TNFα secretion. These studies implicate both upstream and downstream regulatory processes for MIF in RA inflammation.

A further key aspect of synovial inflammation in RA is the control of FLS proliferation. The effect of MIF on FLS proliferation will be examined in Chapter 6.

5.6 Figures

Figure 5.6.1

Effect of rMIF on PGE_2 production.

FLS were treated with human recombinant MIF (1 ng/ml) for 8 hours. PGE_2 release was quantitated by assaying FLS supernatants by PGE_2 ELISA. PGE_2 release is expressed as ng/ml PGE_2. Values are the mean ± SEM of 5 experiments.

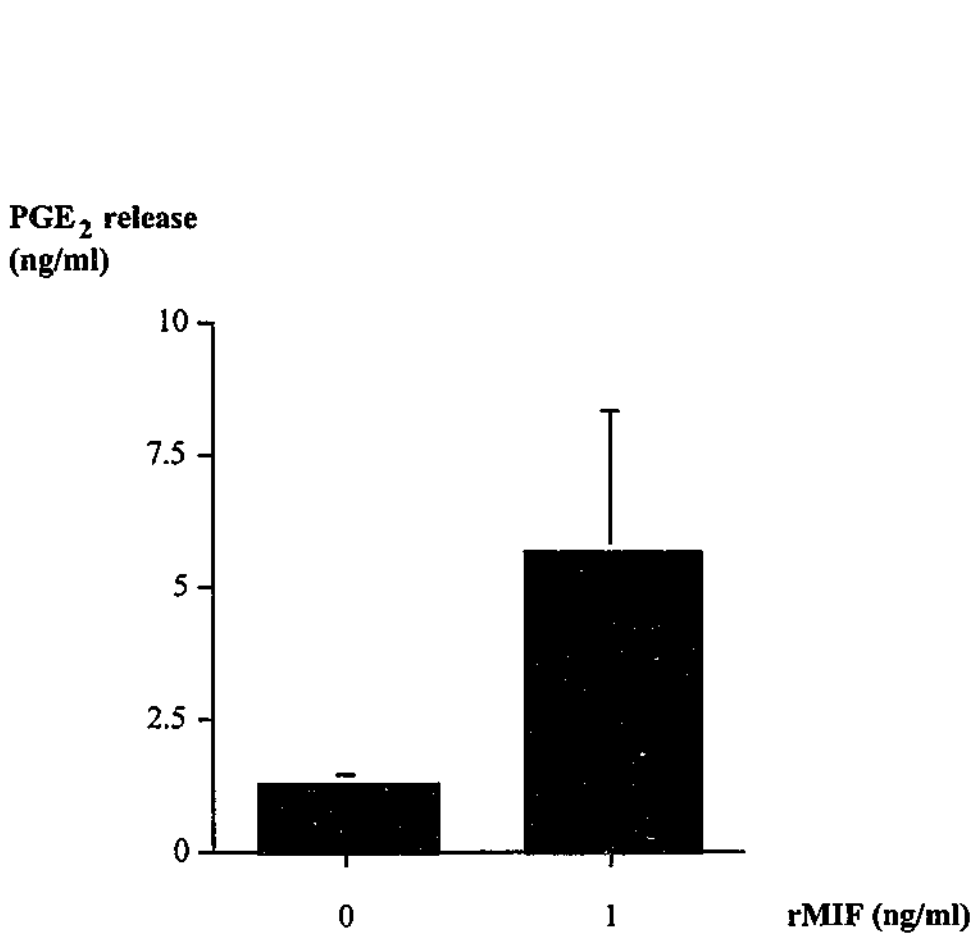

PGE2 release
(ng/ml)
10
7.5
5
2.5
0
0
1
rMIF (ng/ml)

Figure 5.6.2

Effect of MIF on COX activity.

FLS were treated with human recombinant MIF (5 ng/ml) for 8 hours prior to incubation with 10 μM arachidonic acid for 30 minutes. COX activity was quantitated by assaying supernatants by PGE_2 ELISA. COX activity is expressed as ng/ml PGE_2. Values are the mean ± SEM of 5 experiments. * $p < 0.05$ versus untreated FLS.

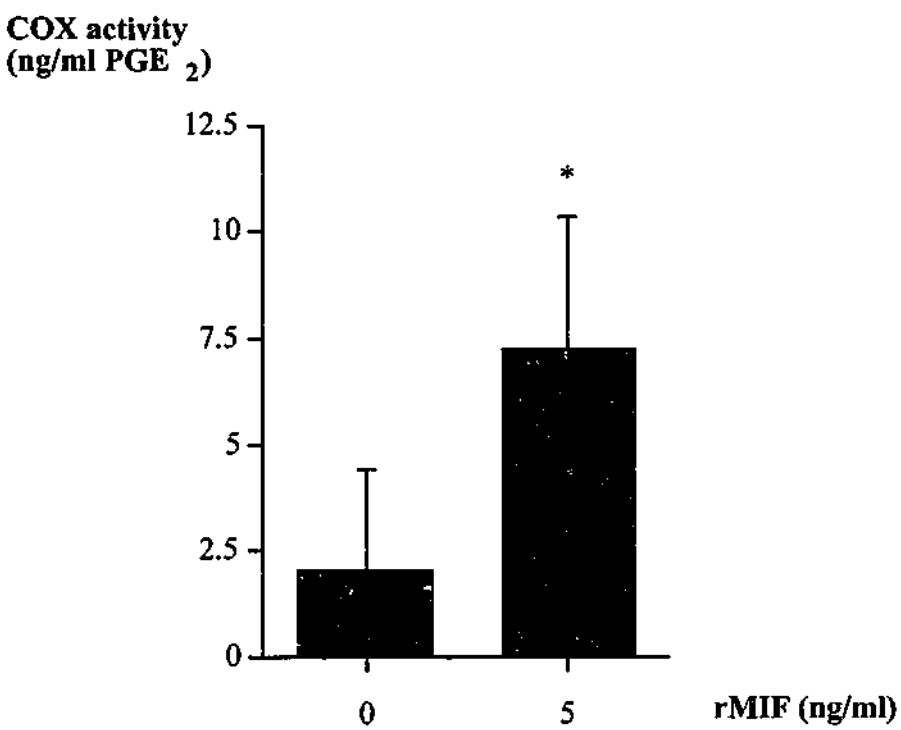

COX activity
(ng/ml PGE 2)
12.5
10
7.5
5
2.5
0
*
0
5
rMIF (ng/ml)

Figure 5.6.3

Effect of MIF on COX2 mRNA expression.

FLS were treated with human recombinant MIF for 8 hours. A PCR product of 300 bp was obtained for GAPDH and a PCR product of 499 bp for COX2. Lane 1: untreated FLS; Lane 2: MIF (500 ng/ml). Representative data of n = 3 experiments.

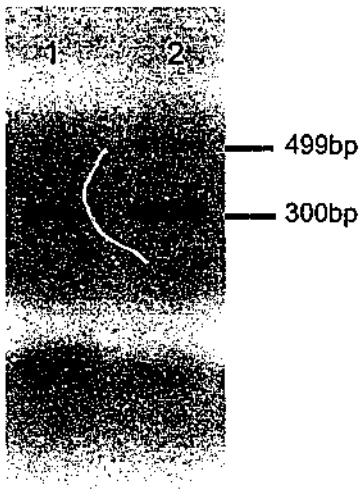

1
2
499bp
300bp

Figure 5.6.4

Effect of MIF immunoneutralization on constitutive and IL-1β stimulated COX activity.

FLS were treated with MIF mAb (50 μg/ml; or isotype-matched control mAb (NC Ab), IgG_1) and/or IL-1β for 8 hours prior to incubation with 10 μM arachidonic acid for 30 minutes. COX activity was quantitated by assaying supernatants by PGE_2 ELISA. COX activity is expressed as ng/ml PGE_2. Values are the mean SEM of 5 experiments. * $p < 0.01$ versus untreated FLS, † $p < 0.05$ versus negative control mAb.

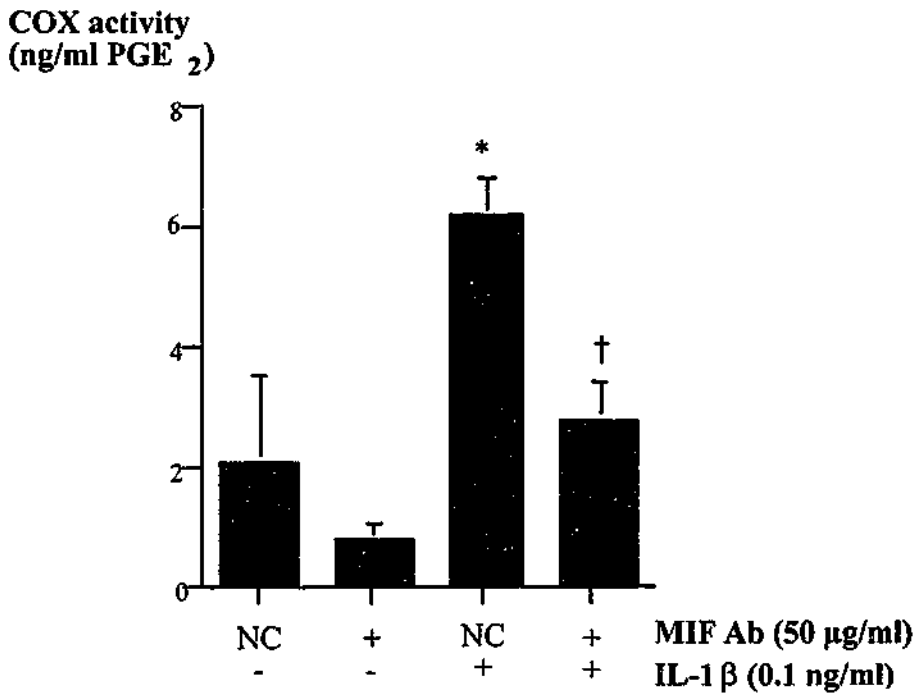
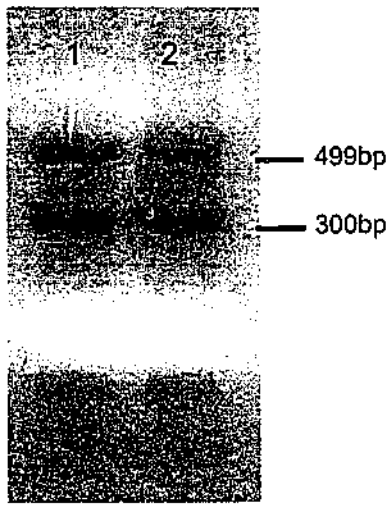

COX activity
(ng/ml PGE 2)
8
6
4
2
0
*
†
NC
+
NC
+
MIF Ab (50 µg/ml)
-
-
+
+
IL-1 β (0.1 ng/ml)

Figure 5.6.5

Effect of MIF immunoneutralization on constitutive and IL-1β stimulated COX2 mRNA expression.

FLS were treated with IL−1β and/or MIF mAb for 8 hours. A PCR product of 300 bp was obtained for GAPDH and a PCR product of 499 bp for COX2. Lane 1: IL−1β (0.1 ng/ml) and negative control IgG_1 (50 μg/ml); Lane 2: IL−1β and MIF mAb (50 μg/ml). Representative data of n = 3 experiments.


1
2
499bp
300bp

CHAPTER SIX: EFFECTS OF MIF ON RHEUMATOID FIBROBLAST-LIKE SYNOVIOCYTE PROLIFERATION AND NFκB

6.1 Chapter Summary

As discussed in previous chapters, macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine expressed in human rheumatoid (RA) synovium and fibroblast-like synoviocytes (FLS). MIF has been implicated as a delayed-early response cytokine, and to be involved in endothelial and T-cell proliferation. In RA, FLS are believed to contribute to hyperplasia and local invasion of activated cells seen in the synovial joint. In the current chapter, the hypothesis that MIF has a role in RA FLS proliferation was examined. Studies presented in Chapters 4 and 5 demonstrate the upregulation by MIF of FLS $cPLA_2$ and COX2 expression. Nuclear factor kappa B (NFκB) is a transcription factor reported to be involved in cytokine-induced FLS activation. I therefore also explored the potential for MIF to activate NFκB in RA FLS.

FLS proliferation was assessed using [^{3}H]thymidine incorporation. Intracellular localization of NFκB subunits p65 and p50 in RA FLS was determined by immunostaining. PLA_2 activity in FLS was determined by [^{3}H]arachidonic acid release. The role of NFκB in FLS proliferation and PLA_2 activity studies was determined using an inhibitor of NFκB.

Constitutive FLS proliferation was detected and was significantly increased by MIF. Interleukin–1β-induced proliferation was inhibited by MIF immunoneutralization. Cytoplasmic but not nuclear staining of p65 and p50 was observed in untreated RA FLS. Nuclear translocation of p65 and p50 was induced by IL-1β, but not by MIF. Anti-MIF mAb had no effect on IL–1β-induced nuclear translocation of p65 and p50. MIF effects on FLS proliferation and FLS $cPLA_2$ activity were not inhibited by specific antagonism of NFκB.

These results establish that MIF is an inducer of RA FLS activation, and that MIF mAb is inhibitory of IL–1β-induced FLS proliferation. In addition, MIF effects on FLS proliferation utilise NFκB-independent signal transduction pathways. These data suggest an important therapeutic potential for MIF antagonism in RA.

6.2 Introduction

As discussed in Chapters 4 and 5, MIF is increasingly recognised as an important regulator in immune and inflammatory responses. The contribution of MIF to cell activation is well described. MIF is released by activated macrophages, T-cells and FLS and upregulates the proinflammatory activity of these cells.

RA is characterised by synovial hyperplasia and inflammation, with FLS implicated in the local invasion of activated cells observed at the cartilage-pannus interface of the synovial joint (Geiler et al., 1994). In this thesis, I have shown the effect of MIF on key aspects of FLS activation, namely $cPLA_2$ and COX2 expression and activity and PGE_2 release. MIF has been implicated as an early response cytokine, with known actions in endothelial and T-cell proliferation (Chesney et al., 1999; Bacher et al., 1996). The critical role of MIF described in human disease and animal models of tumorigenesis, is consistent with its ability to suppress p53-mediated growth arrest in a murine fibroblast cell line (Hudson et al., 1999). Current evidence therefore predicts a role for MIF in RA cell proliferation.

Despite considerable evidence implicating MIF in immune-inflammatory cell activation processes, the signal transduction mechanism(s) through which MIF mediates its action remain unknown. The transcription factor nuclear factor kappa B (NFκB) is a sequence-specific DNA binding protein that is involved in the induction of the expression of a variety of genes that are believed to be central to synovial inflammation (Barnes and Karin, 1997; Lentsch and Ward, 1999). As a direct consequence, the expression and activation of NFκB in RA synovium has been extensively documented. Immunohistochemistry studies have detected nuclear staining of NFκB subunits RelA (p65) and p50 in RA synovium, predominantly

where FLS reside (Handel et al., 1995; Marok et al., 1996; Sioud et al., 1998; Han et al., 1998).

In non-stimulated cells, NFκB exists in an inactive, cytosolic form, bound to its inhibitor, IκB. Upon stimulation, by agents such as IL-1β or TNFα, IκB dissociates from the NFκB-IκB complex, allowing the nuclear localisation sequence to become unmasked. This unmasking enables activation of NFκB, which then translocates to the nucleus, where it controls gene expression by binding to specific promoters.

The emerging evidence for a role of MIF in the control of cell proliferation and my demonstration of FLS activation by MIF led me to investigate the effects of MIF on NFκB in FLS.

6.3 Methods

6.3.1 Isolation and culture of fibroblast-like synoviocytes

Fibroblast-like synoviocytes (FLS) were obtained from synovium of rheumatoid (RA) patients (n = 6) undergoing joint replacement surgery. All patients satisfied the American College of Rheumatology criteria for the classification of RA (Arnett et al., 1987). FLS were isolated using enzyme digestion procedures and cultured in RPMI/10% FCS (see sections 2.3.6-2.3.8). Thawed cells were used in experiments between passages 4 and 9. For all experiments, FLS were seeded at 1 x 10^5 cells per well in 24 well culture plates in RPMI/10% FCS and allowed to adhere overnight, prior to medium being replaced with RPMI/0.1% BSA for experimental purposes. Cells were >95% viable by trypan blue exclusion. In each group of experiments, n refers to the number of individual human RA donor FLS used.

6.3.2 Measurement of fibroblast-like synoviocyte proliferation

To determine the effect of MIF on FLS proliferation, DNA synthesis was measured by [^{3}H]thymidine incorporation into FLS. FLS were seeded overnight at $0.5x10^5$ cells per well in 24 well tissue culture plates in RPMI/10% FCS. Cell growth was synchronised by culturing FLS in RPMI/0.1 % BSA for 24 hours. FLS were treated with human recombinant MIF (5-500 ng/ml), anti-human MIF mAb (50 μg/ml; or isotype-matched negative control, IgG_1), and/or human recombinant IL-1β (0.1 ng/ml) for 48 hours prior to cells being pulsed for 18 hours with 1 μCi/ml [^{3}H]thymidine. Duplicate cultures were used for each determination. FLS were detached using Trypsin-EDTA and harvested using a PHD Cell Harvester (Cambridge Technology Inc., USA). The radioactivity incorporated into DNA was determined by liquid scintillation counting, with results expressed as [^{3}H] thymidine incorporation.

6.3.3 Analysis of nuclear factor kappa B (NFκB)

FLS ($1x10^5$) were treated with human recombinant MIF (5-500 ng/ml), human recombinant IL-1β (0.1 ng/ml) and/or anti-human MIF mAb (50 μg/ml; or isotype-matched negative control, IgG_1), for 30 mins. Cells were then centrifuged onto Superfrost Plus microscope glass slides (Selby Laboratories, Melbourne, Australia) using a cytospin centrifuge (Shandon, Pittsburgh). Single immunohistochemical staining was performed using a streptavidin-biotin method as described (Pettit et al., 1997), with the following modifications. The sections were incubated for 1 hour at room temperature with the primary antibody: goat anti-human NFκB p50 polyclonal Ab, 1:150 dilution (1.3 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-human NFκB p65 polyclonal Ab, 1:800 dilution (0.25 μg/ml; Santa Cruz), or negative control goat anti-human NFκB p50 (1.3 μg/ml) and p65 (0.25 μg/ml) polyclonal Abs, raised against p65 and p50 blocking peptides (Santa Cruz). The primary antibody was

detected using a biotinylated swine anti-goat IgG (DAKO, Sydney, Australia). This was followed by incubation with streptavidin-horseradish peroxidase (DAKO) and the chromagen substrate DAB (3, 3'-diaminobenzidine tetrachloride, DAKO). The sections were counterstained with Meyer's hematoloxylin (Sigma), mounted and examined under light microscopy. Nuclear translocation of p50 or p65 was identified by nuclear staining, as described (Pettit et al., 1997).

In MIF-induced FLS activation studies (refer to sections 6.3.2 and 6.3.4.), nuclear translocation of NFκB was blocked using SN50 (50 μg/ml; Biomol Research Laboratories, Plymouth Meeting, PA), which inhibits translocation of the NFκB active complex into the nucleus (Lin et al., 1995), or control peptide, according to the manufacturer's instructions.

6.3.4 Assessment of Phospholipase A_2 activity

PLA_2 activity in FLS was determined as described in section 3.3.3. In brief, FLS were labelled for 18 hours with 1 μCi/ml [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid in 0.1% BSA/RPMI. Cells were treated with human recombinant MIF (5-500 ng/ml), a neutralizing anti-human MIF mAb (50 μg/ml; or isotype-matched negative control, IgG_1) (Leech et al., 1999), and/or human recombinant IL–1β (0.1 ng/ml) for 8 h. Duplicate cultures were used for each determination. Radioactivity in the supernatant was determined using a Wallac 1409 liquid scintillation counter (Pharmacia, Finland), with results expressed as [^{3}H]arachidonic acid released (cpm).

6.3.5 Statistical Analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed using the Student's t-test, with values of $p < 0.05$ regarded as statistically significant.

6.4 Results

6.4.1 Effect on MIF on FLS proliferation

The effect of MIF on FLS proliferation was first examined. Constitutive FLS proliferation was detected, and was significantly increased by rMIF (5-500 ng/ml) in a concentration-dependent manner ($p < 0.02$; Figure 6.6.1).

6.4.2 Effect on MIF immunoneutralization on constitutive and IL-1β-stimulated FLS proliferation

The effect of FLS-derived MIF on proliferation was determined by monclonal antibody MIF immunoneutralization. IL−1β significantly induced FLS proliferation ($p < 0.02$), and this effect was markedly inhibited by MIF immunoneutralization ($p < 0.04$; Figure 6.6.2). MIF immunoneutralization had no significant effect on constitutive FLS proliferation.

6.4.3 Detection of NFκB subunits p65 and p50 in untreated FLS

With the demonstration that MIF directly induces $cPLA_2$, COX2 and proliferation in RA FLS, and the requirement for MIF in IL−1β-induced FLS activation, subsequent studies investigated the potential involvement of NFκB in the mediation of MIF-induced FLS activation. Cytoplasmic but not nuclear staining of p65 and p50 subunits of NFκB was observed in untreated RA FLS (Figure 6.6.3).

6.4.4 Effect of MIF and IL-1β on NFκB nuclear translocation

Studies next examined the ability of MIF and IL-1β to stimulate nuclear translocation of NFκB in RA FLS. IL-1β induced nuclear translocation of p65 and p50 subunits in RA FLS. In contrast MIF did not induce p65 and p50 nuclear translocation, at concentrations shown to induce FLS activation in RA FLS (Figure 6.6.3).

6.4.5 Effect of MIF immunoneutralization on NFκB nuclear translocation

The observation that MIF immunoneutralisation inhibited IL-1-induced proliferation in RA FLS suggested that MIF antagonism may inhibit IL-1-induced NFκB translocation in these cells. Anti-MIF mAb, however, had no effect on IL–1β-induced translocation of p65 and p50 subunits of NFκB (Figure 6.6.4).

6.4.6 Effect of NFκB inhibition on MIF-induced FLS activation

To confirm findings that MIF-induced FLS activation did not involve NFκB, I next examined the effect of NFκB antagonism in MIF-induced FLS activation studies. NFκB antagonism was achieved with the use of the specific NFκB antagonist SN50, which contains the nuclear localisation sequence of the transcription factor NFκB p50 linked to the hydrophobic region of the signal peptide of Kaposi fibroblast growth factor. The peptide N-terminal K-FGF hydrophobic-region confers cell-permeable peptide import, while the nuclear localisation sequence (360-369) inhibits translocation of the NFκB active complex into the nucleus (Lin et al., 1995). SN50 was used at 1 - 100 μg/ml, based on studies in the human monocytic THP-1 cell line, which shows 85% inhibition of NFκB activation at 100 μg/ml (Lin et al., 1995).
In the present study, FLS proliferation and PLA_2 activity were used as measures of FLS activation. MIF (50 ng/ml) induced an increase in FLS proliferation. SN50 (20 μg/ml) did not significantly effect MIF-induced FLS proliferation (Figure 6.6.5). Similarly, PLA_2 activity in RA FLS was upregulated by MIF (50 ng/ml) and SN50 (20 μg/ml) exerted no significant effect on MIF-induced PLA_2 activity in RA FLS (Figure 6.6.6).

6.5 Discussion

Considering the extensive evidence implicating MIF in immune and inflammatory processes, it is of great interest that studies attribute a role for MIF in invasive and

proliferative processes, such as tumour angiogenesis. For example, anti-MIF antibodies decreased the outgrowth and vascularisation of a murine B cell lymphoma (Chesney et al., 1999). In a rat model of galactose cataract induction, lens epithelial MIF mRNA expression was profoundly increased compared to controls, and was associated with intensified lens epithelial cell proliferation (Wen et al., 1996). MIF protein and mRNA were highly expressed in a murine colon carcinoma cell line, and transfection of these cells with an antisense MIF plasmid was associated with decreases in cell proliferation (Takahashi et al., 1998).

These findings are supported by studies in human tumor cells. Enhanced expression of MIF mRNA has been detected in human metastatic prostate cancer (Meyer-Siegler and Hudson, 1996). In this study, MIF was overexpressed in metastatic prostate cancer compared with focal prostate carcinoma and was barely detectable in normal prostatic tissue. Constitutive mRNA expression of MIF is demonstrated in a human leukemia cell line, HL-60, and its expression is upregulated by exposure of these cells to LPS (Nishihira et al., 1996). Immunohistochemical studies have detected MIF in a variety of pituitary adenomas, especially thyrotrophs and corticotroph adenomas. In addition, MIF was detected in gonadotroph adenomas (Tampanaru-Sarmesiu et al., 1997). Given that synovial pannus demonstrates many invasive and proliferative properties (Zvaifler and Firestein, 1994), these observations suggest that induction of cell proliferation may be another process where MIF contributes to disease expression.

As discussed in Chapters 4 and 5, the contribution of MIF to RA activation mechanisms is suggested by several studies. MIF is expressed in synovial T-cells, macrophages and FLS (Leech et al., 1999). Published studies demonstrate a role for MIF in macrophage and T-cell activation (Calandra et al., 1994; Bacher et al., 1996).

In an animal model of arthritis, immunoneutralization of MIF reduced disease severity and synovial hypercellularity (Leech et al., 1998). Moreover, the ability of MIF to suppress p53-mediated growth arrest in murine fibroblasts (Hudson et al., 1999), is of great interest given the putative role of p53 in the control of FLS proliferation in RA (Aupperle et al., 1998; Firestein et al., 1997) in addition to its expression in RA FLS and RA synovium (Firestein et al., 1996).

Evidence of the contribution of local FLS division to synovial hyperplasia is suggested by the expression of PCNA/cyclin and c-*myc* by these cells. RA FLS are capable of autonomous invasion of human cartilage, as seen in an engrafted co-culture system of the SCID mouse (Geiler et al., 1994). To date, the factors responsible for the proliferation of unstimulated RA FLS are uncertain, especially since these cells do not express TNFα and little IL–1β. In contrast, unstimulated RA FLS release abundant MIF (Leech et al., 1999).

The current study represents the first examination of the effects of MIF on proliferation using human cells derived from the site of inflammatory disease. Consistent with findings described in Chapters 4 and 5, results demonstrate upregulation of FLS proliferation by MIF and an essential role for MIF in the effects of IL–1β on proliferation. Clearly, therapeutic targeting of MIF has the potential not only to limit inflammation but also synovial hyperplasia itself.

Phenomena such as $cPLA_2$ and COX2 induction, PGE_2 release and proliferation have been linked to cytokine activation of NFκB (Nakano et al., 1998; Crofford et al., 1997). The effects of MIF on these phenomena led to the hypothesis that MIF mediates its effects via activation of NFκB.

NFκB expression and activation in RA synovium has been studied extensively. Immunohistochemistry studies have detected nuclear staining of p65 and p50 NFκB

subunits in rheumatoid synovium, predominantly in the intimal lining layer, where FLS reside (Handel et al., 1995; Marok et al., 1996; Sioud et al., 1998; Han et al., 1998). Moreover, the ability of synovial inflammation to activate NFκB is demonstrated in animal models of arthritis. NFκB expression is increased in the synovial lining layer of rats with adjuvant arthritis early in the disease course (Tsao et al., 1997). In collagen-induced arthritis in mice, synovial NFκB activation occurs well before clinical signs of synovitis (Han et al., 1998). Although NFκB is activated in RA synovium, the specific pathways involved in synovial inflammatory processes are not resolved. No prior study has examined the regulation of NFκB by MIF in human FLS activation.

Results herein confirm cytoplasmic but not nuclear expression of p65 and p50 NFκB subunits in unreated FLS, and that IL–1β induces nuclear translocation of p65 and p50 in RA FLS (Handel et al., 1995; Marok et al., 1996; Roshak et al., 1997; Crofford et al., 1997). I found no evidence that MIF induces the nuclear translocation of NFκB in RA FLS, and moreover no evidence that inhibition of IL–1β effects by anti-MIF mAb are mediated via NFκB. Confirming this observation, specific antagonism of NFκB failed to prevent the induction of FLS proliferation and PLA_2 activity by MIF. Previous investigations have established the involvement of IL-1β in the activation of NFκB in RA FLS, where inhibition of NFκB activation resulted in reduced PLA_2 and COX2 induction and PGE_2 production (Roshak et al., 1997; Crofford et al., 1997). The current study suggests that NFκB is not involved in MIF-induced PLA_2 activity or proliferation, or that inhibition of IL–1β effects by anti-MIF mAb are mediated via NFκB. The data therefore supports NFκB-independent mechanisms of action of MIF on FLS activation. Recent observations in a cultured

murine fibroblast line showed MIF was able to induce $cPLA_2$ and proliferation via upregulation of p42/44 MAP kinases (ERK) (Mitchell et al., 1999). Preliminary studies using RA FLS suggest blockade of p44/p42 MAP kinase prevents MIF-induced FLS proliferation (Sampey A, Lacey D, Morand EF, unpublished observation).

In summary, results demonstrate that MIF directly induces FLS proliferation and that MIF is essential for the upregulation of FLS proliferation by IL–1β. Moreover, I have shown that MIF effects on FLS activation are independent of NFκB nuclear translocation mechanisms. Together, these findings implicate a unique therapeutic potential for MIF in RA. It is evident that an increased understanding of signal transduction pathways facilitated by MIF, and how proinflammatory actions of MIF contribute to the emergence of RA inflammation, is still required. Activation of genes via NFκB is exquisitely sensitive to inhibition by glucocorticoids. The lack of involvement of NFκB in MIF-mediated inflammatory events is consistent with the ability of MIF to exert proinflammatory effects despite the presence of glucocorticoids. Alternatively, the recent finding that MIF is capable of functionally inactivating a tumor suppressor, p53, may provide a link between inflammatory responses and tumorigenesis (Hudson et al., 1999). It is hoped that elucidation of signal transduction mechanism(s) utilised by MIF will facilitate the rationale use of pharmacologic glucocorticoids to address the dysregulated physiologic function of glucocorticoids observed clinically in RA patients.

6.6 Figures

Figure 6.6.1

Effect of MIF on FLS proliferation.

FLS were treated with MIF (5-500 ng/ml) for 48 hours and labelled with 1 μCi/ml [^{3}H]thymidine for 18 hours. FLS proliferation was determined from duplicate cultures and expressed as [^{3}H]thymidine incorporation (cpm). Values are the mean ± SEM of 4 experiments. * p < 0.06 versus untreated, † p < 0.02 versus untreated FLS.

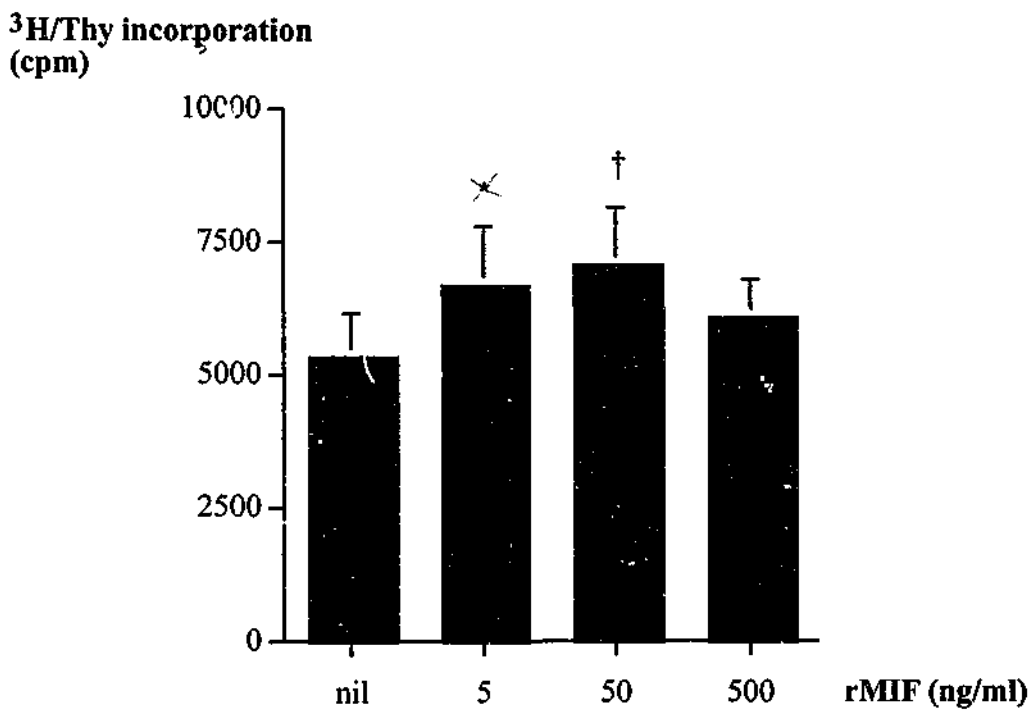

^{3}H/Thy incorporation (cpm)
10000
7500
5000
2500
0
*
†
nil
5
50
500
rMIF (ng/ml)

Figure 6.6.2

Effect of MIF mAb on constitutive and IL-1β-stimulated FLS proliferation.

FLS were treated with MIF mAb(50 μg/ml; or isotype-matched control mAb, IgG_1 (NC)) and/or IL-1β (0.1 ng/ml) for 48 hours and labelled with 1 μCi/ml [^{3}H]thymidine for 18 hours. FLS proliferation was determined from duplicate cultures and expressed as [^{3}H]thymidine incorporation (cpm). Values are the mean ± SEM of 6 experiments. † $p < 0.05$ versus untreated, * $p < 0.02$ versus negative control.

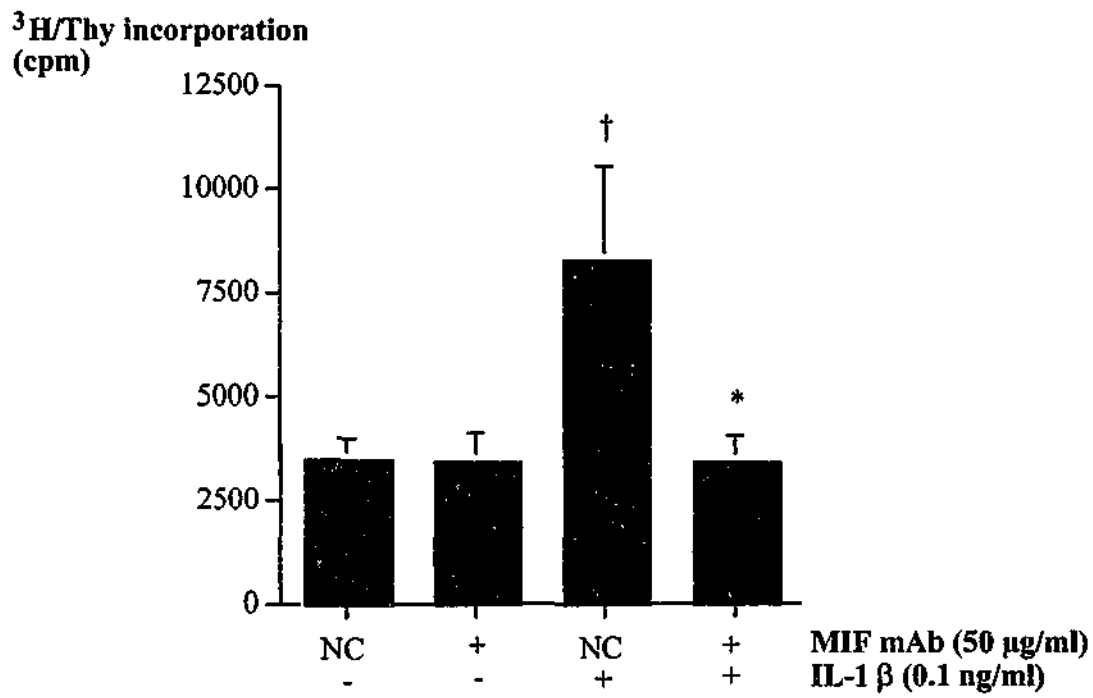

^{3}H/Thy incorporation (cpm)
12500
10000
7500
5000
2500
0
†
*
NC
+
NC
+
-
-
+
+
MIF mAb (50 µg/ml)
IL-1 β (0.1 ng/ml)

Figure 6.6.3

Nuclear translocation of p50 and p65 subunits of NFκB in IL-1β- or MIF-treated FLS. FLS were treated with (a-d) medium; or (e-f) IL-1β (0.1 ng/ml); or (g-h) MIF (500 ng/ml), for 30 minutes and immunostained for the negative control p50 (a); or negative control p65 (b); or p50 (c, e, g); or p65 (d, f, h) subunits of NFκB.

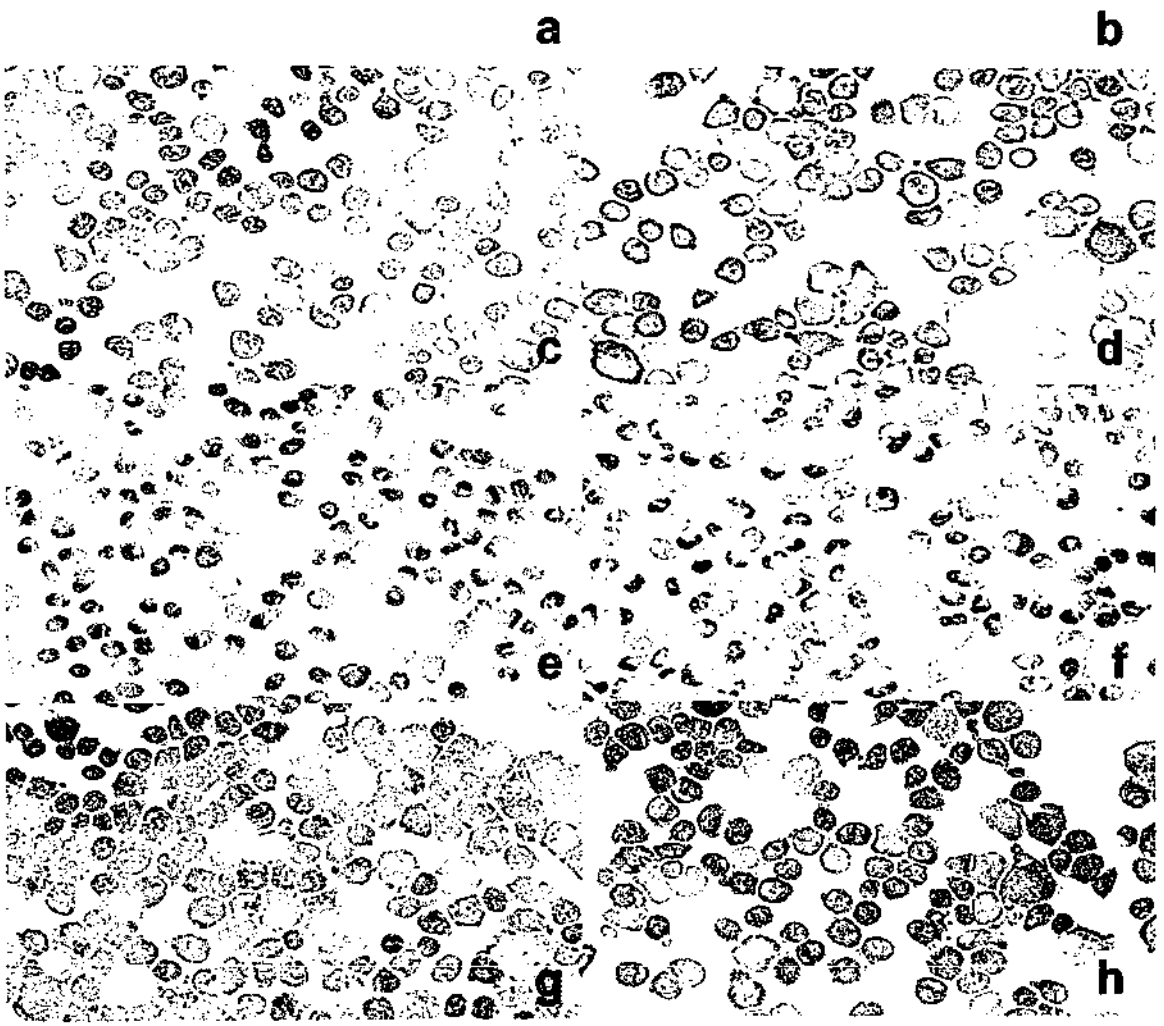

a
b
c
d
e
f
g
h

Figure 6.6.4

Nuclear translocation of p50 and p65 subunits of NFκB in IL-1β-stimulated FLS co-treated with MIF mAb or negative control mAb.

FLS were treated with (a-d) IL-1β (0.1 ng/ml) and (c-d) negative control mAb, IgG_1 (50 μg/ml); or (e-f) IL-1β (0.1 ng/ml) and MIF mAb (50 μg/ml), for 30 minutes and immunostained for the negative control p50 (a); or negative control p65 (b); or p50 (c, e); or p65 (d, f) subunits of NFκB.

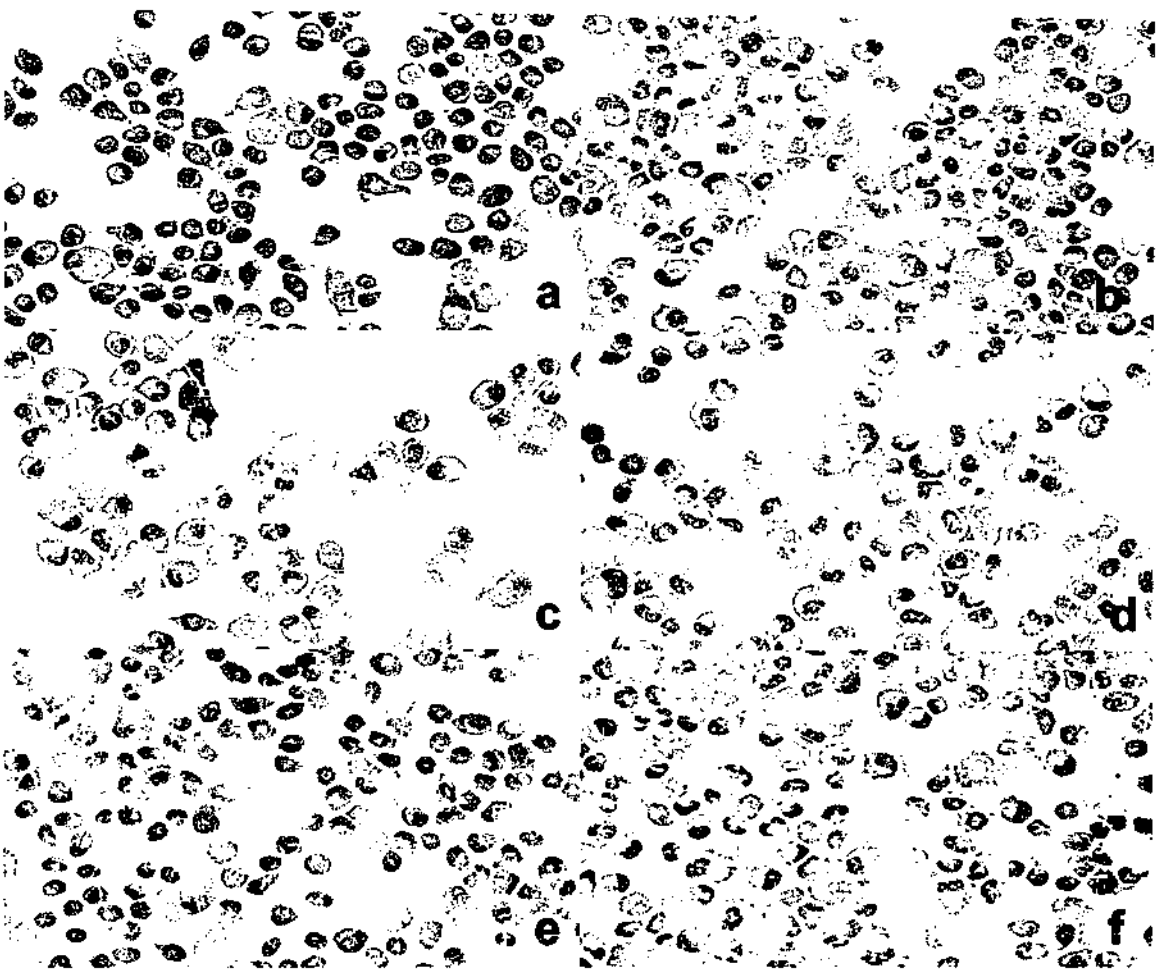

a
b
c
d
e
f

Figure 6.6.5

Effect of NFκB inhibition in MIF-induced FLS proliferation.

FLS were treated with MIF (50 ng/ml) and SN50 peptide (20 μg/ml; or negative control peptide) for 48 hours and labelled with 1 μCi/ml [^{3}H]thymidine for 18 hours. FLS proliferation was determined from duplicate cultures and expressed as [^{3}H]thymidine incorporation (cpm). Values are the mean ± SEM of 4 experiments.

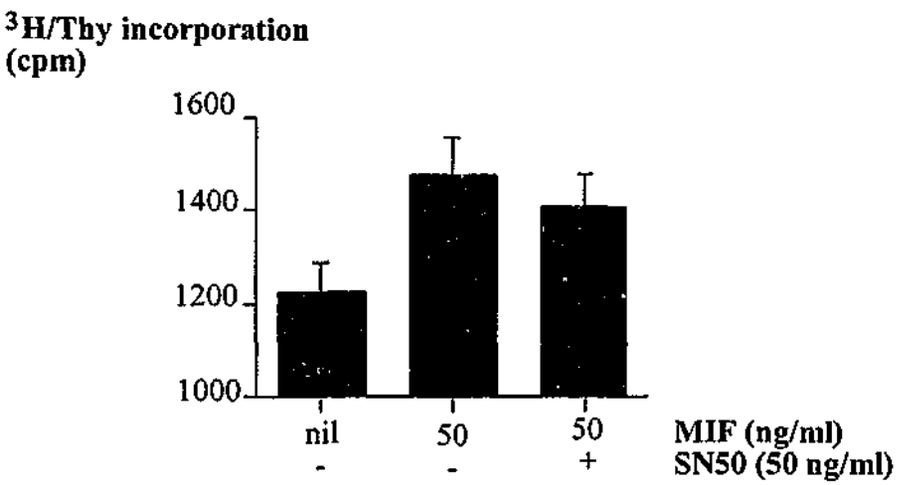
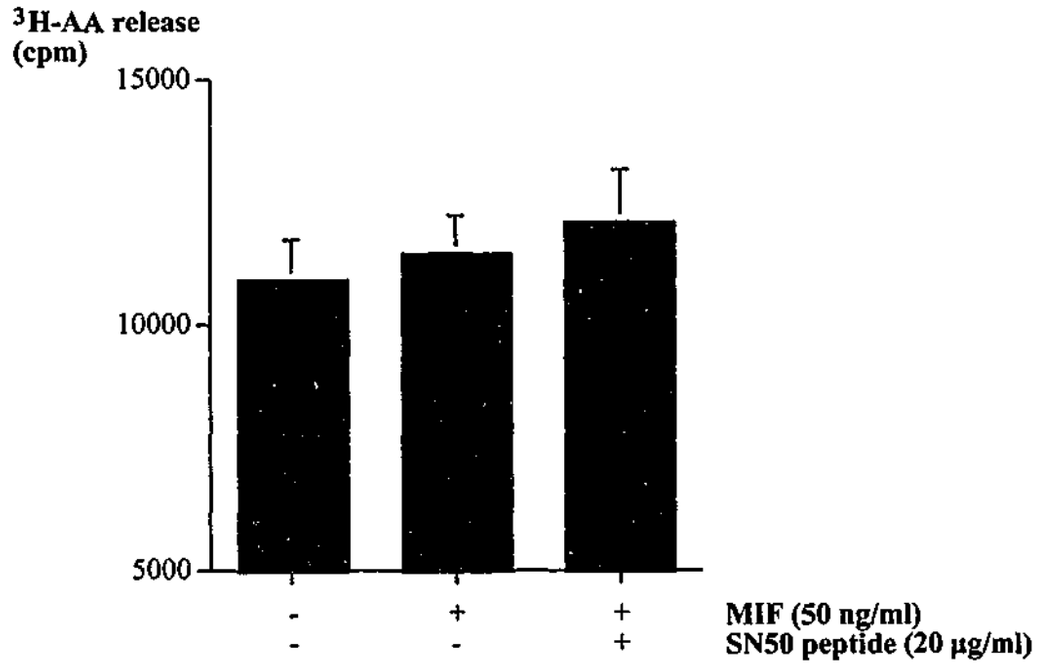

^{3}H/Thy incorporation
(cpm)
1600
1400
1200
1000
nil
50
50
MIF (ng/ml)
-
-
+
SN50 (50 ng/ml)

Figure 6.6.6

Effect of NFκB inhibition in MIF-induced FLS PLA_2 activity.

FLS were treated with MIF (50 ng/ml) and SN50 peptide (20 μg/ml; or negative control peptide) for 8 hours. PLA_2 activity is expressed as ^{3}H-AA release (cpm). Values are the mean ± SEM of 4 experiments.


^{3}H-AA release
(cpm)
15000
10000
5000
-
+
+
MIF (50 ng/ml)
-
-
+
SN50 peptide (20 µg/ml)

CHAPTER SEVEN: SUMMARY AND CONCLUSIONS

Rheumatoid arthritis is a chronic, systemic disease characterised by persistent inflammation, synovial hyperplasia, and altered immune responses, that results in progressive destruction of the affected joints. Although the pathogenesis of the disease remains obscure, it is proposed that overactivity of pro-inflammatory systems and/or the underactivity of anti-inflammatory mechanisms result in the dysfunction of immune-inflammatory processes in RA. In this context, the hypothalamic-pituitary-adrenal (HPA) axis represents a critical modulatory system involved in the RA immune-inflammatory response. Glucocorticoids are released as part of the systemic stress response that is mediated by the HPA axis. Glucocorticoids exert powerful anti-inflammatory and immunosuppressive effects when administered at pharmacological doses in RA. In spite of the widespread use of glucocorticoids, however, there is only partial knowledge of the molecular mechanisms whereby glucocorticoids exert their potent anti-inflammatory and immunosuppressive effects.

As noted previously, annexin I is induced by glucocorticoids in a number of *in vitro* and *in vivo* model systems. In RA, annexin I is an important endogenous mediator involved in the regulation of joint inflammation.

It is expressed in human RA synovium and peripheral blood leukocytes, and is a critical anti-inflammatory mediator in animal models of arthritis. However, the molecular mode of action of annexin I remains obscure, given that annexin I lacks a hydrophobic signal sequence which should otherwise be involved in targeting the protein to the endoplasmic reticulum. It is known that glucocorticoids induce annexin I translocation to the cell surface. Moreover, annexin I antibodies, which are unable to penetrate the cell, deplete intracellular annexin I and significantly reverse the anti-inflammatory effects of glucocorticoids.

Numerous studies have suggested that annexin I is exported from target cells, enabling annexin I to bind to cell surface receptors and exert its biological actions in a paracrine or autocrine manner. In support of this concept, specific annexin I binding sites were first identified on peripheral blood leukocytes.

Studies presented in Chapter 2 represent the first description of annexin I binding sites on human synovial cells. The finding of reduced annexin I binding sites on RA compared to OA FLS is consistent with reduced annexin I binding sites at sites of inflammation *in vivo*, and on leukocytes derived from RA peripheral blood. These findings support the hypothesis that reduced annexin I binding sites on RA FLS may contribute to incomplete control of inflammation by endogenous and therapeutic glucocorticoids in RA.

Whilst increased production of proinflammatory cytokines and metalloproteinases at sites of inflammation are believed to contribute to the perpetuation of inflammatory arthritis, the regulatory effect of these mediators on annexin 1 binding sites on human FLS has not previously been examined. Regulation of annexin I binding sites on human FLS was observed in response to pro-inflammatory cytokine and enzyme treatment, suggesting that annexin I binding sites are regulated at sites of inflammation. The regulation of annexin I binding sites on human FLS is clearly a complex phenomenon, and findings in one cell type do not necessarily predict those in another. Reduced annexin I binding sites on RA FLS is consistent with the intrinsically pro-inflammatory phenotype of these cells. However, differences between these results and those of other groups may be associated with constitutive differences between FLS and leukocytes, or to the different milieu in which they were derived.

Whilst the mechanisms attributed to cytokine and enzyme regulation of annexin I binding sites remains unresolved, the lack of molecular identification of the annexin I binding site makes elucidation of these mechanisms difficult. The finding of inhibition of FLS PLA_2 activity in response to annexin I N-terminal peptide, provides evidence of direct anti-inflammatory effects of annexin I binding sites. Definition of annexin I binding sites on FLS suggests the potential for the development of annexin I analogs for the treatment of arthritis. Clearly, such analogs may have an anti-inflammatory profile similar to those of glucocorticoids but a different and improved toxicity profile.

The regulatory activity of annexin I in human FLS eicosanoid generating mechanisms has not previously been examined. In RA synovitis, prostaglandins are desribed as important mediators of inflammation, and RA FLS are a key source of prostaglandin E_2 (PGE_2). Prostaglandin synthesis in RA results from the activities of two pivotal enzymes, phospholipase A_2 (PLA_2) and cyclooxygenase-2 (COX2).

The study presented in Chapter 3 addresses the effects of annexin-I and dexamethasone on PLA_2 and COX activities and PGE_2 release in cultured human FLS. In this study, uncoupling of the effects of annexin-I and glucocorticoid effects on constitutive and IL-1β-stimulated arachidonic acid production suggests glucocorticoid-independent regulatory activities for annexin I in inflammatory processes. These findings are also consistent with the hypothesis that COX2 is the rate-limiting enzyme in FLS PGE_2 generation. Moreover, the lack of effect of annexin I on FLS PGE_2 synthesis, despite anti-inflammatory effects observed *in vivo*, suggests that annexin I modulates inflammation via eicosanoid-independent mechanisms. For example, arachidonic acid-mediated intracellular signal transduction mechanisms are important in the powerful *in vivo* anti-inflammatory effects of annexin I. This

possibility requires further investigation, and studies examining the role of annexin I in intracellular signal transduction mechanisms may significantly impact on the current understanding of glucocorticoid inflammatory regulation.

Glucocorticoids are inhibitory modulators of inflammatory and immune responses. Until recently, no systemic mediator had been identified that could counter-regulate the powerful inhibitory effects of glucocorticoids on the immune system. Macrophage migration inhibitory factor (MIF) has now been identified as a critical regulatory mediator whose secretion is induced by glucocorticoids and which has the capacity to directly counter-regulate the inhibitory effects of glucocorticoids. Coworkers have recently reported the expression of MIF in human RA synovium and FLS. MIF is a potent pro-inflammatory cytokine, which has the capacity to induce monocyte TNFα release, placing it high in the putative cytokine "hierarchy" of RA. MIF has also recently shown to have a critical role in animal models of RA. The effects of MIF in the functional activation of these cells have not been previously explored. The data presented in Chapters 4 and 5 establish MIF as an inducer of synoviocyte activation in rheumatoid arthritis. I show that MIF induces FLS PGE_2 production, in addition to activation of FLS PLA_2 and COX2 at the levels of their expression and activity. Moreover, I show an essential role for MIF in the activation of these phenomena in FLS by IL-1β. In this study, the ability of MIF to antagonise glucocorticoid inhibitory actions in IL-1β-induced FLS is consistent with findings both *in vivo* and *in vitro* and suggests that this counter-regulatory system may be operant in RA.

It is also of great interest that studies implicate MIF in the perpetuation of cell activation processes. MIF is released by activated T cell and macrophages and has been shown to upregulate the pro-inflammatory activities of these cells. The early description of MIF as an early response cytokine is consistent with its reported actions

in endothelial and T cell proliferation and as presented in this thesis, in FLS proliferation and FLS activation processes (Chapters 4, 5 and 6). Moreover, the reported ability of MIF to suppress p53-mediated growth arrest *in vitro* is in keeping with its suggested role in tumorigenesis. In accordance with these findings, it is noteworthy that RA is characterised by synovial hyperplasia and inflammation, with FLS believed to participate in the local invasion of activated cells seen at the cartilage-pannus junction of the synovial joint.

Despite evidence attributing MIF in immune-inflammatory events, the membrane receptor of MIF remains unidentified, a fact which has made studies investigating principal cellular pathways governing MIF function difficult. It is known however that upregulation of cell proliferation in a fibroblast cell line by MIF was associated with p44/42 ERK MAP kinase activation, which was also dependent on protein kinase A activity. Moreover, studies using RA FLS have shown that MIF upregulates collagenase and stromelysin via tyrosine kinase-, protein kinase C-, and AP-1-dependent pathways, and is not dependent on IL-1β. Presumably, increased knowledge of such signal transduction mechanisms utilised by MIF will facilitate greater understanding of the role of MIF in immune and inflammatory processes.

Nuclear factor kappa B (NFκB) is a key transcription factor believed to be involved in the induction of a variety of genes attributed to synovial inflammatory processes. In addition, NFκB is expressed in RA synovial tissue and FLS, with known actions in cytokine-induced FLS activation. However, the effects of MIF on FLS proliferation, and the potential for MIF to activate NFκB in RA FLS, have not been previously examined. Studies presented in Chapter 6 show an additional and critically important effect of MIF on synoviocyte proliferation, and provide evidence that MIF-induced PLA_2 and COX2 activities, in addition to FLS proliferation, are not mediated via

NFκB. Moreover, I demonstrate that the activating effect of IL-1 on these three aspects of synoviocyte activation is dependent upon MIF via NFκB-independent mechanisms. The activation of FLS via NFκB-independent mechanisms also suggests a basis for the ability of MIF to antagonise the effects of glucocorticoids.

Together, these data establish MIF as a key therapeutic target in human RA with potential anti-inflammatory and disease-modifying effects.

Studies presented in this thesis extend the current knowledge of two endogenous, glucocorticoid-inducible proteins, annexin I and MIF in glucocorticoid-regulated inflammatory processes of relevance to human synovial cells.

The use of glucocorticoids for over 50 years in the treatment of RA has not until recently been accompanied by systematic examination of their mechanisms of action. Future studies on annexin I and MIF have the potential to greatly increase our understanding of the regulation of inflammation. Demonstration of the activity of annexin I and MIF in synovial cells is this thesis also support the development of therapeutic strategies based on manipulation of these molecules.

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