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PHD Thesis
NFκB independent pathway activation of rheumatoid arthritis FLS by macrophage migration inhibitory factor (MIF)
December 2003
Derek Lacey

Addendum

Chapter 1

1.2.3.6 Osteoclasts and RA

Osteoclasts are key regulates of bone loss in many forms of arthritis, including RA. They are believed to play a pivotal role in bone erosion at sites of periarticular inflammation. The inflammatory environment created in the RA pannus contains numerous inflammatory mediators which have been shown to recruit and activate osteoclasts, which perpetuates bone loss and joint damage in RA.

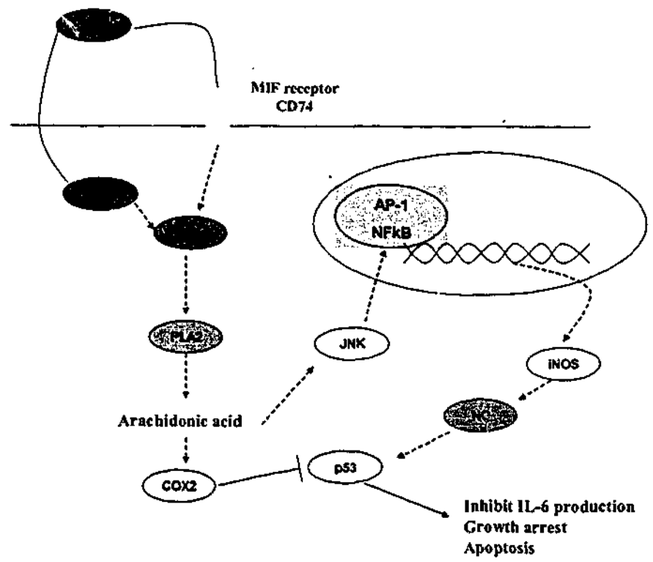
The receptor activator of NFκB ligand (RANKL), osteoprotegerin (OPG) and receptor activator of NFκB (RANK) have all been found to be highly expressed in RA synovium (Horwood et al. 1999). In addition RANKL, OPG and RANK have been shown to activate osteoclast and increase bone loss and joint damage (Horwood et al. 1999; Romas et al. 2000; Haynes et al. 2001). Although osteoclasts play an important role in RA joint destruction, a full discussion of the role and activation mechanisms of osteoclasts is beyond the scope of this thesis. It is at least hypothetically possible that MIF is an influential cytokine in osteoclast biology, but no studies of osteoclast function were undertaken in this thesis.

1.3.1 p53 and RA

Dominant negative p53 mutations do not suppress cytokine production or increase pro-apoptotic genes. This form of mutation results in over production of non-functional p53 which blocks wild type p53 from performing its function of suppressing cytokines and upregulation of pro-apoptotic genes.

Figure 1.3.1

This is a descriptive figure of the role of MIF in relation to the p53 pathway.



Chapter 2

The MIF protein and anti-MIF mAb employed in chapter 2, and 4 were gifts from Professor Richard Bucala of The Picower Institute for Medical Research, Manhasset, New York, USA. There is no accepted standard 'unit' of MIF bioactivity against which our samples can be tested. The presence of biological activity of MIF and anti-MIF mAb was tested in FLS proliferation assays before they were used in any further experiments.

B-cell contamination is an important consideration when working with FLS. As a result FLS cultures were stained for CD45 and measured by flow cytometry at passage 3. FLS were CD45 negative. All experiments were performed on cells after passage 3.

Thymidine incorporation is a measure of DNA synthesis rather than proliferation. Cell counts were not performed on cultures treated with MIF. Morphological changes were not observed in FLS cultures treated with MIF. However, recently published work (leech et atl, *Arthritis Rheum* 2003) demonstrated that cell counts of MIF -/- cells growing from primary tissue explants were significantly lower than from WT tissues, suggesting that the observation made with thymidine incorporation was reflective of proliferation.

In itself, MIF regulation of FLS DNA synthesis, as measured by tritiated thymidine incorporation is not novel, and has been shown by numerous groups in various cell types. This thesis confirms MIF regulates FLS DNA synthesis. In addition, this thesis demonstrates that MIF utilizes the ERK1/2 MAPK pathway but not the p38 MAPK pathway to regulate FLS DNA synthesis.

Chaper 3

In chapter 3, I have shown that MIF knockout cells are hyporesponsive to serum factors and cytokines. However, due to the lack of biologically active MIF, I was unable to show that addition of exogenous MIF could restore cells to a normal level responsiveness. The lack of such data limits our understanding of the mechanisms by which MIF regulates the

level of MAPK activation by serum factors and cytokines. It is unknown if the hyporesponsive state of the MIF knockout cells is a result of the absence of MIF or disruption of other unknown genes. Therefore addition of exogenous MIF to the knockout cells would prove that MIF is needed for maximum activation of MAPK by cytokines.
The genotype of MIF knockout and wild type mice was checked using PCR. The status of cells derived form MIF knockout and wild type mice were also checked by PCR, as follows. Genomic DNA was extracted from mouse tail tips or cultured dermal fibroblast. The genomic DNA was diluted 1:20 and 1µl of DNA was added to 5pmol/µl of primers. The NEO, WT or control primer pairs were used. The NEO primer pairs produced a 500bp product, the WT primer pair produced a 189bp product and the control primer pair produced a 1021bp product.
Figure 3.6.5
This is a representative Gel of the PCR products produced when test the genotype of MIF KO and WT mice. The same figure also shows the primer sequences used to check the genotype.

Genotyping for Wt and MIF KO

Primer Sequence: WT Forward: 5' AGA CCA CGT GCT TAG CTG AG 3'
Reversed: 5' GCA TCG CTA CCG GTG GAT AA 3'

MIF KO Forward: 5' AAT GAA CAA GAT GGA TTG CAC 3'
Reversed: 5' CGT CCA GAT CAT CCT GAT C 3'

Chapter 4
In chapter 4, I demonstrated that MIF can down-regulate p53 and p53-mediated events in FLS. To confirm that MIF regulates p53, further experiment need to be carried out such as: a) to knockdown p53 in FLS and show that this has similar effect to addition of MIF. b) To knockdown p53 in FLS and show that addition of MIF has no additive effect due to the lack of p53. c) To over express p53 in FLS and show that addition of MIF has reduced or no affect.

Erratum
Typographical errors

Page 6. 1.2.3.1 Line 5 "active" should read "activate".
Page 155. Line 4 delete "to" after the word "may".
Page 157. Line 4 "blockadge" should read "blockade".

References to Addendae
The following references were repeated and should be deleted.
Bacher, et al. Molecular medicine **4**: 217-230
Bozza, et al Journal of Experimental Medicine **189**: 341-346
Firestein, et al. Journal of Immunology **144**: 3347-3353
Firestein, and Zvaifler Arthritis and Rheumatism **30**: 864-871
Lacey, et al Arthritis and Rheumatism **48**(1): 103-9
Lafyatis, et al. Journal of Clinical Investigation **83**: 1267-1276
Mitchell, et al. Journal of Immunology **154**: 3863-3870
Onodera, et al. Journal of Biological Chemistry **277**(10): 7865-74
Santos, et al. Clin Exp Immunol **123**(2): 309-14
Sun, et al. Proceedings of the National Academy of Sciences USA **93**: 5191-5196

The following references should read:
Chesney, J., C. Metz, M. Bacher, T. Peng, A. Meinhardt and R. Bucala (1999). "An essential role for macrophage migration inhibitory factor (MIF) in the angiogenesis and the growth of a murine lymphoma." Mol Med Mar **5**(3) 181-191.

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Haynes, D. R., T. N. Crotti, M. Loric, G. I. Bain, G. J. Atkins and D. M. Findlay (2001). "Osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritic joint." Rheumatology (Oxford) **40**(6): 623-30.
Horwood, N. J., V. Kartsogiannis, J. M. Quinn, E. Romas, T. J. Martin and M. T. Gillespie (1999). "Activated T lymphocytes support osteoclast formation in vitro." Biochem Biophys Res Commun **265**(1): 144-50.
Romas, E., O. Bakharevski, D. K. Hards, V. Kartsogiannis, J. M. Quinn, P. F. Ryan, T. J. Martin and M. T. Gillespie (2000). "Expression of osteoclast differentiation factor at sites of bone erosion in collagen-induced arthritis." Arthritis Rheum **43**(4): 821-6.

NFκB Independent pathway activation of rheumatoid arthritis FLS by macrophage migration inhibitory factor (MIF).

A thesis submitted for the degree of Doctor of Philosophy

To the

Faculty of Medicine, Monash University

By

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December 2003

Summary

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease whose manifestations are characterised by inflammation, synovial hyperplasia and altered immune response. It is a disease of unknown aetiology. However, its pathology is well described, consisting of synovial inflammation, leukocyte infiltration, destruction of cartilage and bone, and synoviocyte expansion due to dysregulated proliferation and incomplete apoptosis. Numerous cells, cytokines, chemokines, proteins, proteases and enzymes modulate disease severity and progression. This thesis however, focuses on one particular cell type, fibroblast-like synoviocytes (FLS), and the influences of the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF).

MIF is expressed in many tissues and is up regulated in a number of inflammatory diseases. MIF has been identified as a glucocorticoid induced pro-inflammatory cytokine, with the unique ability to reverse the immuno-suppressive effects of glucocorticoids. Interestingly, MIF derived from FLS can induce monocyte tumour necrosis factor α (TNFα), indicating an important role for MIF in RA pathogenesis. More recently, it has been demonstrated that MIF is involved in interlukin-1β (IL-1β) induction of eicosanoid generation in FLS. In addition, MIF plays a fundamental role in lipopolysaccaride (LPS) signaling, as MIF deficient mice are hypo-responsive to LPS due to reduced expression of the LPS receptor TLR-4, and in the adaptive immune response.

Studies presented in the first part of this thesis examine the role of signal transduction pathways in MIF induced FLS proliferation. In chapter 2, mitogen activated protein kinase (MAPK) antagonists were utilized to examine the effects of the extracellular-signal regulated kinases (ERK1/2) MAPK pathway and p38

MAPK pathways on MIF-induced RA FLS proliferation. The findings presented in this chapter demonstrate that MIF activates ERK1/2 and p38 MAPK in RA FLS, while antagonism of the ERK pathway but not the p38 pathway blocks MIF induced FLS proliferation.

In chapter 3 the influence of endogenous MIF on MAPK signaling was investigated utilizing MIF deficient and wild type (wt) mouse dermal fibroblasts. The findings presented in this chapter suggest that endogenous MIF is necessary for complete MAPK signaling by IL-1β and TNFα, or that the presence of MIF is important for amplifying IL-1β and TNFα signaling via MAPK. In addition MIF is required for fibroblast proliferation in response to IL-1β and TNFα.

Studies presented in chapter 4 investigate the ability of MIF to down regulate the tumor suppressor protein p53 and protect FLS from p53 mediated apoptosis. The findings presented in this chapter suggest that exogenous and endogenous MIF can down regulate p53 protein levels. MIF can also protect FLS from p53 mediated apoptosis. These results suggest that MIF plays a pivotal role in protecting host cells at sites of inflammation by modulating p53 protein levels and that dysregulation of MIF control of p53 could have deleterious consequences.

Studies presented in chapter 5 investigate the activation of the MAPK pathways in relation to RA FLS proliferation and apoptosis. The findings presented in this chapter indicate that ERK1/2 and c-Jun N-terminal kinase (JNK1/2) pathway activation is necessary for RA FLS proliferation, however only the JNK pathway has a pro-survival function in RA FLS. These results suggest that MAPK influence multiple cellular processes and play an important role in synovial hyperplasia in RA.

In conclusion, the studies presented in this thesis expand knowledge of the control of fibroblast proliferation and apoptosis by the interactions between MIF, MAPK and p53.

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.

(Sig[redacted]

Derek Lacey

Publications arising from work presented in this thesis

Articles in refereed journals

1. **Derek Lacey***, Annaleise Sampey*, Robert Mitchell, Richard Bucala, Leilani Santos, Michelle Leech, Eric F Morand. "Control of fibroblast-like synoviocyte proliferation by macrophage migration inhibitory factor (MIF)" Arthritis & Rheumatism 2003 Jan 48(1): 103-9
2. **Derek Lacey***, Michelle Leech*, Jin Rong Xue, Leilani Santos, Paul Hutchinson, Ernst Wolvetang, John R. David, Richard Bucala, Eric F Morand. "Macrophage migration inhibitory factor (MIF) regulates p53 in inflammatory arthritis" Arthritis & Rheumatism 2003 Jul 48 (7):1881-1889
3. Michelle Leech*, Elliot Tarranto*, Jin Rong Xue, **Derek Lacey**, Paul Hutchinson, Malcolm Smith, Eric F Morand "Detection of the p53 regulator MDM2 in Rheumatoid Arthritis" Submitted to J Rheumatol
4. Leilani Santos, **Derek Lacey**, Yuan Yang, Michelle Leech, Eric F Morand.

   "Activation of p38 MAP kinase in synovial cells by macrophage migration inhibitory factor (MIF)" Submitted to J Rheumatol
5. **Derek Lacey**, Annaleise Sampey, Leilani Santos, Georgia Milenkovski, Paul Hutchinson, Eric F Morand. "Control of proliferation and apoptosis of human rheumatoid arthritis synoviocytes by MAP Kinases." Submitted to Arthritis & Rheumatism

* Denotes dual first authorship

Presentations at meetings of learned societies

1. Morand EF, Sampey AV, **Lacey D**, Hall PH, Bucala R. NF-κB-independent activation of rheumatoid arthritis synovial cells by MIF. Oral presentation at American College of Rheumatology Annual Scientific meeting 2000.

2. Morand EF, Sampey AV, **Lacey D**, Hall PH, Bucala R. NF-κB-independent activation of rheumatoid arthritis synovial cells by MIF. Oral presentation Inflammation Research Association Biennial Scientific meeting 2000.

3. **Lacey D**, Sampey AV, Mitchell R, Bucala R, Santos L, Leech M, Morand EF. Control of fibroblast-like synoviocyte proliferation by macrophage migration inhibitory factor (MIF). Poster presentation at Australian Rheumatology Association Scientific Conference 2001.

4. **Lacey D**, Sampey AV, Mitchell R, Bucala R, Santos L, Leech M, Morand EF. Control of fibroblast-like synoviocyte proliferation by macrophage migration inhibitory factor (MIF). Poster presentation 11th International Conference on Second Messengers and Phosphoproteins 2001.

5. **Derek Lacey**, Michelle Leech, Yang Y-H, Paul Hutchinson, Ernst Wolvetang, John R. David, Richard Bucala, Eric F Morand. MIF involvement in RA synoviocyte proliferation and apoptosis. Oral presentation at American College of Rheumatology Annual Scientific meeting 2001.

6. **Derek Lacey**, Michelle Leech, Yang Y-H, Paul Hutchinson, Ernst Wolvetang, John R. David, Richard Bucala, Eric F Morand. MIF involvement in RA synoviocyte proliferation and apoptosis. Oral presentation New Zealand Rheumatology Association/Australian Rheumatology Association Combined Scientific Conference 2002 (Finalist for Young Investigators Award).

7. Morand EF, Sampey AV, Santos L, Milenkovski G, Hutchinson P, **Lacey D**. Control of proliferation and apoptosis of human rheumatoid arthritis synoviocytes by MAPK. Oral presentation at The European League Against Rheumatism annual congress of rheumatology 2003.

8. Morand EF, Sampey AV, Santos L, Milenkovski G, Hutchinson P, **Lacey D**. Control of proliferation and apoptosis of human rheumatoid arthritis synoviocytes by MAPK. Poster presentation at New Zealand Rheumatology Association/Australian Rheumatology Association Combined Scientific Conference 2003.

9. **Lacey D**, Santos L, Milenkovski G, Hutchinson P, Sampey AV, Leech M, Morand EF. Control of proliferation and apoptosis of human rheumatoid arthritis synoviocytes by MAPK. Poster presentation at American College of Rheumatology Annual Scientific meeting 2003.

Abbreviations

AA	Arachidonic acid
AA	Adjuvant arthritis
AIA	Antigen induced arthritis
ACTH	Pituitary adrenocorticotrophic hormone
AP-1	Activator protein 1
BSA	Bovine serum albumin
CIA	Collagen-induced arthritis
COX	Cyclooxygenase
DNA	Deoxyribose nucleic acid
DTH	Delayed type hypersensitivity
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FLS	Fibroblast-like synoviocytes
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GR	Glucocorticoid receptor
HLA	Histocompatibility antigen
HPA	hypothalamo-pituitary-adrenal
ICAM-1	intracellular adhesion molecule-1
IL	Interleukin
iNOS	inducible nitric oxide synthase
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide

mAb	Monoclonal antibody
MAPK	Membrane activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MDF	Mouse dermal fibroblasts
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MMP	Matrix metalloproteinases
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis
PCNA	Proliferation cell nuclear antigen
PLA_2	Phospholipase A_2
PGE_2	Prostaglandin E_2
PMN	Polymorphonuclear neutrophils
RA	Rheumatoid arthritis
ROS	Reactive oxide species
SCID	Severe combined immunodeficient
TIMP	Tissue inhibitors of metalloproteinase
TNFα	Tumor necrosis factor-α
wt	Wild type
VCAM-1	Vascular cell adhesion molecule-1

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I would like to thank my parents and family for their support and encouragement over the years, not just in my PhD but throughout my entire life. Finally, I would like to thank my beautiful wife Sally, who has supported and encouraged me and made everything possible.

Chapter One:

Activation of rheumatoid arthritis fibroblast-like synoviocytes by Macrophage Migration Inhibitory Factor (MIF):

A review of the literature

1.1 Overview

In chapter 1 I will review that literature which concerns the pathogenesis of rheumatoid arthritis (RA) and the influence of pro-inflammatory mediators that regulate mechanisms relevant to RA hyperplasia. The structure and function of synovium and the involvement of cellular and soluble mediators in RA pathogenesis will be outlined. Particular emphasis on mechanisms influencing fibroblast-like synoviocytes (FLS) hyperplasia including extracellular and intracellular proteins will be detailed. The involvement of tumour suppressor protein p53 and signal transduction pathways involved in synoviocyte hyperplasia will be discussed. The structure, function, distribution, and intracellular signalling mechanisms of the pro-inflammatory cytokine macrophage migration inhibitory factor (MIF), including its role in health and disease, will be reviewed.

1.2 Rheumatoid Arthritis

1.2.1 Pathology and Pathogenesis

Rheumatoid arthritis (RA) is a chronic systemic disease that affects approximately 1% of the population, with a higher incidence in females. Although the aetiology remains unknown, RA results in inflammation and deformity preferentially involving the metacarpal and interphalangeal joints of the hands and feet. Extra-articular manifestations, chronic pain, and progressive physical impairment all contribute to decreased social interaction and life expectancy. The pathology of RA is increasingly well understood, is characterised by synovial hyperplasia, inflammation and altered systemic immune responses. Chronic inflammation is associated with dysregulated proliferation and apoptosis of resident synovial cells, and infiltration of activated leukocytes. Destruction of articular cartilage and subchondral bone, and subsequent erosion of joints, is mediated by invasive synovial tissue.

The sections to follow will outline the structure and function of synovium, and the involvement of cellular and soluble components of the immune system in the pathogenesis of RA. Due to the wide range and complexity of the immune responses observed in RA, emphasis will be given to aspects relevant to the studies presented in this thesis.

1.2.2 Normal Synovium

In normal joints, synovium covers all surfaces within the joint, except for contact areas of cartilage (Edwards 1994). Synovium secretes a thin viscous film, rich in hyaluronic acid, which is known as synovial fluid. Synovial fluid is required for lubrication (Krane et al. 1990) and the delivery of nutrients to articular cartilage

(Edwards 1994). Normal synovium is relatively acellular, and cells are predominantly separated by interstitium, with little direct cell to cell contact.

Two distinct layers have been identified in normal synovium. The intima, or lining layer, comprises cells designated Type A (macrophage-like) or Type B (fibroblast-like) synoviocytes. Macrophage-like synoviocytes are similar to macrophages resident in other tissues, and have a high content of cytoplasmic organelles and non-specific esterase activity. They also express macrophage differentiation markers, including CD14, CD68, HLA-DR, and Fc receptors (Edwards 1995). The major resident cells of synovium are fibroblast-like synoviocytes (FLS), which have fewer organelles and more extensive endoplasmic reticulum. FLS are presumed to be mesenchymal in origin, and exhibit bipolar fibroblast morphology when grown *in vitro* (Wilkinson et al. 1992). FLS have low MHC class II expression and do not express leukocyte phenotype antigens; however they do express adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), β1 integrins, and CD44 (Firestein 1996). One of the few characteristics distinguishing FLS from other stromal fibroblast is that FLS express uridine diphosphoglucose dehydrogenase (UDPDG) enzymatic activity (Firestein 1994).

The other distinct section of normal synovium is the sublining or subintimal layer, which comprises mainly of fat cells, fibroblasts, occasional lymphocytes, and macrophages. The subintimal layer has an extracellular matrix containing collagen, fibronectin and proteoglycans (Firestein 1994), and is supported by a rich bed of microvesssels. Lymphatic vessels have also been identified, which are required for the clearance of synovial proteins delivered via the microvasculature.

The endothelial cells of the microvasculature are also an important component of normal synovium. The microvasculature can directly participate in the development of synovial inflammation due to its interaction with blood-derived immune cells and surrounding cells.

1.2.3 Inflammatory synovium in RA

Synovial tissue undergoes dramatic changes in RA, with the earliest described changes involving inflammation of synovial microvasculature, oedema, and increased vascularity (Zvaifler 1983). Subsequently, expansion of FLS and macrophages leads to hyperplasia, and an inflamed synovial intima that is between six and eight cells thick (Firestein 1996). The inflamed subintimal region has an abundance of immune cells, in particular macrophages, T lymphocytes, plasma cells, dendritic cells, activated fibroblasts and mast cells (Harris 1990; Firestein 1996). T lymphocytes infiltrate the sublining and form small aggregates of T cells, with more diffuse infiltrates in between (Firestein 1994). It is common to have diffuse lymphocytes and pseudofolliclies, however lymphoid aggregates also form with germinal centres resembling those of lymphoid follicles (Firestein 1994).

Expansion of blood vessels, capillaries, and micro capillary venules also occurs in the subintima region. A characteristic of RA is the highly vascularised inflamed synovium that resembles granulation tissue and is located at the synovial interface with cartilage and bone (Zvaifler et al. 1994). This is known as pannus. The pannus consists mainly of macrophages and fibroblasts, which are thought to be invasive synovial lining cells (Chu et al. 1992; Zvaifler et al. 1994). The cells within the pannus are responsible for the active invasion of cartilage and bone, and ultimately, joint destruction. These cells also release cytokines and

prostaglandins, which perpetuate the inflammatory process, as well as matrix metalloproteinases (MMPs), that perpetrate tissue damage. The contribution of individual components in rheumatoid synovial inflammation will be outlined in sections 1.2.3-1.2.6.

1.2.3 Cells involved in RA synovial pathology

1.2.3.1 Fibroblast-like synoviocyte.

The role of FLS in the initiation of RA disease is unclear; however, their involvement in the pathology and perpetuation of the disease is increasingly well established. FLS have been found in the most destructive areas of joint damage, which has lead to numerous investigations into their role in RA pathogenesis. There is increasing knowledge about FLS ability to active or recruit immune cells and actively participate in joint destruction.

FLS have the morphology of fibroblasts, and have the machinery to synthesize and secrete a vast array of inflammatory products implicated in RA pathogenesis. FLS isolated from synovium have a dendritic or stellate morphology; they are non-phagocytic and lack leukocyte or macrophage surface antigens, which distinguish them from antigen presenting cells such as dendritic cells. FLS do express a variety of surface adhesion receptors such as ICAM-1, VCAM-1, CD44 (the hyaluronic acid receptor), β1 and β3 integrins (Johnson, M.D. et al. 1993; Szekanecz et al. 1994; Nikkari et al. 1995). Another aid to FLS identification is the intracellular expression of UDPGD, which confers their ability to synthesize hyaluronan (Pitsillides et al. 1993).

The expansion of FLS in synovial hyperplasia is important in the generation of pannus and hence joint destruction; the mechanism behind this synovial lining

expansion remains controversial. Early studies reported a lack of mitoses and little thymidine incorporation by synovial lining cells (Nykanen et al. 1986). Also, studies using immunohistochemical staining showed an absence of proliferation markers (Lalor et al. 1987). Both of these studies suggested that proliferation was not the driving force behind synovial hyperplasia. More recently however, the expression of proliferative markers such as PCNA/cyclin, c-myc, and zinc finger transcription factor Z-225/Egr-1, have been demonstrated in FLS (Aicher et al. 1994; Qu et al. 1994b). In contrast, it has been shown that the relative expression of c-myc is similar in reactive arthritis and RA synovial specimens, and c-myc is also expressed in osteoarthritis (OA) (Pelletier et al. 1993; Ritchlin et al. 1994; Roivainen et al. 1996).

FLS from RA patients show an increased expression of proto-oncogenes and transcription factors such as c-myc, myb, ras, c-fos, jun-b (Case et al. 1989; Trabandt et al. 1990; Trabandt et al. 1991; Ritchlin et al. 1994; Muller-Ladner et al. 1995; Muller-Ladner et al. 1998), while cells in the superficial lining layer are positive for proliferation cell nuclear antigen (PCNA) (Qu et al. 1994b). FLS derived from RA synovial tissue display an autonomous invasive and proliferative phenotype (Zvaifler et al. 1994; Firestein 1996). In culture, RA FLS grow at a faster rate than control cells, can escape contact inhibition, and grow in an anchorage-independent manner (Lafyatis et al. 1989a). In contrast to osteoarthritis FLS, RA FLS are capable of autonomous invasion of human cartilage in an engrafted co-culture system in the SCID mouse (Muller-Ladner et al. 1991). These characteristics have been described as indicating that RA FLS have a "transformed" phenotype.

FLS proliferate *in vitro*, however by passage 10-12 growth gradually diminishes and cells become somewhat senescent. During long term culture with addition of no other stimuli, RA FLS can constitutively secrete a number of cytokines and growth factors, such as IL-6, transforming growth factor (TGF) β, macrophage migration inhibitory factor (MIF) and fibroblast growth factor (FGF) (Arend, W.P. et al. 1990; Firestein, G.S. et al. 1990; Sano et al. 1990; Alvaro-Gracia et al. 1991; Ben-Av et al. 1995; Leech et al. 1998b; Leech et al. 1999). The level of constitutive cytokine production by RA FLS diminishes over several passages, but the RA phenotype of FLS can be restored by stimulation with IL-1 or TNF (Dayer et al. 1986; Alvaro-Gracia et al. 1990).

A number of studies have shown evidence of defects in apoptosis in RA synovium (Firestein et al. 1995; Sugiyama et al. 1996; Catrina et al. 2002), suggesting that dysregulated apoptosis may contribute to synovial hyperplasia. Several pro- and anti-apoptotic proteins have been identified in RA tissue (Sugiyama et al. 1996; Catrina et al. 2002); however, no clear role in RA pathogenesis has yet been established for these proteins. This area of research is also explored in section 1.3.

Early studies into apoptosis in RA pannus suggested a high level of apoptosis, using DNA strand break labelling techniques (Firestein et al. 1995). More recently it has been demonstrated that there is little apoptosis in RA synovium (Nakajima et al. 1995). Although there is a relatively high degree of DNA strand breaks in RA FLS, there may be an inability of RA FLS to complete apoptosis. It has been shown that only 20% of RA FLS are sensitive to Fas-induced apoptosis (Kobayashi et al. 1999). It has further been suggested that RA FLS resistance to Fas-induced apoptosis is due to the expression of anti-apoptotic molecules (Okura et al. 1996). Sentrin, also called SUMO-1, has been speculated as a molecule that

arguably the most important family of proteases in RA, consisting of at least 13 structurally related members (Woessner 1991). A number of studies have identified the synovial lining as the major source of MMPs in RA. Experiments employing *in situ* hybridization localized collagenase and stromelysin mRNA almost exclusively to FLS (McCachren et al. 1990; Firestein et al. 1991). Tissue inhibitors of matrix metalloproteinases (TIMP), the natural inhibitors of MMP, are also expressed in both RA and OA synovium. The ratio between collagenase or stromelysin mRNA compared with TIMP is significantly higher in RA than in OA, which is consistent with the greater level of joint destruction in RA. Aggrecanases are also important matrix degrading enzymes, which can cleave aggrecan, an important constituent of articular cartilage. Aggrecanase-1 and -2 mRNA expression has been detected in both RA and OA FLS (Yamanishi et al. 2002a). More recently it has been shown that aggrecanase-1 mRNA expression in RA FLS is increased in response to TGF-β stimulation, as measured by real-time PCR (Yamanishi et al. 2002a).

The 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

Along with FLS, macrophages/monocytes have been identified as being major contributors to RA pathogenesis. Evidence of their contribution in RA includes the detection of vast amounts of macrophage cytokine products, in particular TNFα and IL-1β, in RA synovial tissue and fluid. Approximately 20% of RA synovial cells express macrophage surface markers, such as CD14, CD 68 and

may play a role in RA FLS apoptosis resistance. Sentrin is an ubiquitin-like protein which has been shown to interact with the signalling-component forms of Fas/Apo-1 and TNF receptor-1. It is through this interaction that sentrin may protect cells against Fas and TNF mediated apoptosis (Okura et al. 1996). In a comparison of cultured RA FLS, there was greater than 30 times increase in sentrin mRNA than that observed in osteoarthritis FLS and normal FLS (Franz et al. 2000). Also, sentrin expression was found predominantly in the lining layer of RA synovium (Franz et al. 2000).

One of the more interesting discoveries in recent years has been the involvement of tumour suppressor genes in RA. The description of somatic mutations of p53 in RA FLS could explain the aggressive and invasive phenotype of RA FLS (Firestein et al. 1997). Interestingly, the somatic mutations observed are similar to those seen in various cancers. Experiments where p53 was functionally inactivated in normal FLS resulted in a RA FLS-like phenotype (Firestein et al. 1997), thereby providing support for p53 inactivation as a contributing factor in RA. In contrast, a separate study in patients in another part of the world reported that no mutations in p53 were detected (Kullmann et al. 1999). p53 inactivation may therefore be a result of RA processes such as the high genotoxic condition within the joint, or it could be an indicator of disease severity. The role of p53 in RA will be further explored in section 1.3.

FLS are major protagonists in the matrix remodelling and destruction that typifies RA. Through stimulation by inflammatory cytokines such as IL-1, MIF and TNF, FLS produce copious amounts of proteolytic enzymes, such as aggrecanases, cathepsins, and proteases like matrix metalloproteinases (MMPs) (Kumkumian et al. 1989; MacNaul et al. 1990; Sandy et al. 1992; Keyszer et al. 1998). MMPs are

CD11b (Firestein, G. S. et al. 1990). Synovial macrophages also express CD40, CD54, CD80, CD86. ICAM-3 and MHC class II molecules, all of which indicate that synovial macrophages may recognize and present antigens (El-Gabalaway et al. 1994; Ranheim et al. 1994; Liu et al. 1996).

The macrophage population in RA tissue is comprised of both lining layer macrophage like synoviocytes and macrophages in the sublining layer. The expansion of the macrophage population seen in RA could conceivably be due to either proliferation of resident macrophages or infiltration of peripheral blood macrophages, which are attracted to the joint once inflammation is initiated. *In situ* proliferation of macrophages has, however, not been observed in human RA.

Macrophages accumulate in the intimal and sub-intimal layers and the cartilage-pannus junction in RA synovium. Like the FLS, macrophages in RA pannus produce MMP's such as collagenase, elastase, stromelysin and gelatinase, although their cartilage degrading potential is less than that of FLS (Jensen et al. 1991; Tetlow et al. 1995). This suggests that FLS are the primary effector cell in bone and cartilage destruction, and that macrophages play an amplifying role in RA pathogenesis through cytokine production. This amplifying role of macrophages was demonstrated by Janusz *et al* in an *in vitro* cartilage degradation system. They showed that an *in vitro* co-culture of mouse fibroblasts and macrophages resulted in greater cartilage destruction than was observed by either cell type alone (Janusz et al. 1993).

In RA, macrophages produce a vast array of products that can activate and stimulate other cells, especially FLS. IL-1β and TNFα are arguably the two most important macrophage products. In addition, macrophages show increased expression of other cytokine genes such as IL-6, IL-8, MIF, GM-CSF and G-CSF

(Firestein, G.S. et al. 1990) (Seitz et al. 1985; Hahn et al. 1993; Leech et al. 1999). Activated RA macrophages are also an important source of chemokines such as monocyte chemoattractant protein-1 (MCP-1), reactive oxygen species, and plasminogen (Hamilton et al. 1991; Akahoshi et al. 1993; Kunkel et al. 1996). A number of other important molecules produced by macrophages include patelet-derived growth factor which stimulates fibroblast growth factor, which in turn may contribute to the transformed phenotype of FLS (Lafyatis et al. 1989b). Macrophages can also promote angiogenesis through the production of IL-1 and up regulation of adhesion molecules VCAM-1 and E-selectin (Koch et al. 1992; Koch et al. 1995).

1.2.3.3 T cells

T cells constitute approximately 40% of synovial cells. T cells can be found in the synovial sublining layer, organised as discrete lymphoid aggregates or as a diffuse infiltrate. The lining layer is devoid of T cells, while the synovial fluid contains abundant T cells. The ratio of CD4 and CD8 T cells in the synovial fluid is approximately equal. In the sublining synovium, the CD4 helper subset predominates, with a CD4/CD8 ratio of between 4:1 and 14:1 (Firestein 1994).

The role of T cells in RA disease pathogenesis has been a point of much discussion over the last two decades. Originally, T cells were thought to have a prominent role in RA due to their role in antigen-specific T cell mediated diseases. Initial studies showed relatively high levels of the T cell cytokines IL-2 and IFNγ, however this was with the use of relatively non-specific biological assays (Hooks et al. 1979; Wilkins et al. 1983). Studies using more specific reagents showed that very little IFNγ was present in synovial fluid, but that synovial T cells were

capable of IFNγ production upon stimulation (Firestein, G.S. et al. 1987). It was shown that the level of T cell cytokines such as INFγ in the RA joint was 100 times lower than that in known antigen-specific T cell mediated disease such as chronic tuberculosis (Barnes et al. 1990). A relative lack of other T cell products such as IL-4 and TNFβ was also shown (Miossec et al. 1990; Fox 1997). T cells can potentially produce TNFα, GM-CSF and IL-6, all of which are highly expressed in RA joints. Studies using *in situ* hybridization and isolated cell populations from RA synovium have shown that monocytes and FLS are chiefly responsible for the production of these cytokines (Firestein, G. S. et al. 1987b; Firestein et al. 1988; Firestein, G. S. et al. 1990).

T cells can be divided into CD4 helper and CD8 cytotoxic T cells, T helper (Th) cells can be further divided into Th1 or Th2 cells subsets depending on their cytokine profiles (Mosmann et al. 1989). Th1 cells produce mainly IFNγ, IL-2 and IL-17, while Th2 cells produce IL-4, IL-5, IL-10 and IL-13. Th1 cells regulate delayed type hypersensitivity, whereas Th2 cells are involved in antibody isotype switching and allergic response (Mosmann et al. 1996). One of the important features of the Th1 and Th2 paradigm is that each can suppress the activity of the other. This is consistent with certain animal models of autoimmunity, wherein Th1 cytokines are involved in pathogenesis, such as extrinsic allergic encephalomyelitis, whilst Th2 cytokines correlate with protection from disease (Miossec et al. 1997). Th2 cytokines IL-4, IL-10 and IL-13 have been shown to have an anti-inflammatory effect on RA synoviocytes (Morita et al. 2001). Synovial macrophages could influence the Th1/Th2 bias by the production of IL-12, which can direct T cell differentiation towards a Th1 phenotype (Bucht et al.

1996). This suggests that an inadequate Th2 response in RA may contribute to RA pathogenesis.

The direct involvement of T cells in RA disease initiation remains controversial. Studies in animal models of inflammatory arthritis show that early stages of arthritis such as joint swelling and tenderness are accompanied by hyperplasia of the lining and vasodilatation but no infiltration of lymphocytes or leukocytes (Marinova-Mutafchieva et al. 2002). T cell independent arthritis has been demonstrated in genetic models of arthritis. IL-1 receptor antagonist (IL-1Ra) knockout mice on a BALB/c background develop spontaneous chronic inflammation and destructive arthritis (Horai et al. 2000). Severe combined immuno-deficient (SCID) mice that have RA FLS engrafted into the joint develop an aggressive, destructive arthritis (Muller-Ladner et al. 1991). Synovitis has also been described in the absence of T cells in MRL/1 and c-fos transgenic mouse models (O'Sullivan et al. 1985; Shiozawa et al. 1992). T cell targeted therapies such as lymphoid irradiation and thoracic duct drainage have no significant effect on RA clinical manifestations. Interestingly though, patients with quiescent RA can flare if administered human recombinant IL-2 for the treatment of malignant disease suggesting that reactivation of T cells can perpetuate disease (Lavelle-Jones et al. 1990).

IL-17, a Th1 cytokine which is found in RA synovium (Chabaud et al. 1999), has similar functions to IL-1 and TNF, such as enhancing cytokine and MMP production by FLS (Chabaud et al. 2000). IL-15, a macrophage product, shares similar biological properties and common receptor signaling to IL-2 (Kirman et al. 1998). IL-15 is readily detectable in RA joints, and activates T cells to induce macrophage TNFα release via a cell contact-dependent mechanism (McInnes et al.

1997; McInnes et al. 1998). The role of cytokines and other soluble factors are important in RA, but so too is direct cell to cell contact. Studies have shown that direct T cell and fibroblast or T cell and macrophage contact can enhance cytokine, MMP and prostanoid production (Dayer et al. 1999). The importance of cell to cell contact could explain a role for T cells even in their hyporesponsive state in RA.

1.2.3.4 Neutrophils

Polymorphonuclear neutrophils (PMN) constitute the majority of leukocytes in human peripheral blood. Although there are relatively few PMN found in synovial tissue, they are abundant in the synovial fluid (Leirisalo-Repo 1994). There is also a correlation between neutrophil activity and disease development, suggesting a role for neutrophils in RA (Leirisalo-Repo et al. 1993). Neutrophils can be activated by a number of products in synovial fluid such as GM-CSF, IL-8 and immune complexes, resulting in the release of myeloperoxidase, MMPs and oxygen radicals. In addition, neutrophil chemotactic responses and generation of oxygen radicals are associated with erosions in early RA (Leirisalo-Repo et al. 1993). Pre-treatment of cartilage with neutrophil proteases has been shown to enhance RA FLS adhesion (McCurdy et al. 1995). It has also been shown that neutrophils can aid inflammation through production of cytokines including IL-1β, IL-6 and TNFα, chemokines IL-8 and MIP-1α, and growth factors GM-CSF and G-CSF (Cassatella 1995). Furthermore, it has been suggested that neutrophils can act as accessory cells by upregulation of MHC class II molecules in response to IFNγ and G-CSF (Gosselin et al. 1993). The involvement of neutrophils has been demonstrated in animal models such as rat adjuvant-induced arthritis, where

it has been shown that depletion of neutrophils prevents joint inflammation (Santos et al. 1997).

1.2.3.5 Other cells

Dendritic cells have been found in synovial effusions of patients with RA, however they are less readily identifiable in synovial tissue (Zvaifler et al. 1985). Dendritic cells are professional antigen-presenting cells, express MHC class II, are non-phagocytic and are characterised by multiple long dendritic processes. They are distinct from macrophages as they lack Fc receptor and surface CD 14 (Tsai et al. 1988). It has also been shown that dendritic cells lack conventional cytoplasmic enzymes such as myeloperoxidase and non-specific esterase (Firestein, G. S. et al. 1987a). Thomas *et al* have described antigen-presenting CD14/CD33 positive dendritic cells in rheumatoid synovium (Thomas et al. 1994).

B cells and plasma cells can be readily detected in the subintimal region of RA synovium. Rheumatoid factor and type II collagen antibodies, both of which are produced by B cells, are readily detectable in patients with RA (Firestein et al. 1994; Lydyard et al. 1994). A significant sub population of B cells that express CD5 can be found in RA patients. This phenomenon of elevated circulating CD5 positive cells has been observed in a number of autoimmune diseases, and may be associated with the generation of autoantibodies, including rheumatoid factor (Firestein et al. 1994).

Mast cells secrete eicosanoids, MMPs, vasoactive and chemotactic molecules (Firestein et al. 1994). Although there are only small numbers of mast cells in RA synovium, there are significantly more than compared to control tissue (Godfrey

et al. 1984). Whether mast cells in RA are a result of inflammation or contribute to the disease remains to be resolved.

1.2.4 Cytokines

Understanding the processes of cell to cell communication in RA is of utmost importance. Pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, IL-8 and GM-CSF are expressed throughout the RA synovium at an mRNA and protein level (Feldmann et al. 1996; Szekanecz et al. 1998). Other cytokines, such as those produced by T cells including IL-2, IL-3, IL-4 and IFNγ are much less abundant (Firestein, G. S. et al. 1990). Two of the most important pro-inflammatory cytokines found in RA are TNFα and IL-1β. These cytokines have an overlap in their effect in RA, including inducing cytokine production, PGE_2 generation, cartilage destruction, bone resorption and angiogenesis.

The importance of TNFα in RA pathogenesis is revealed in its effect in RA tissue. TNF can induce the production of PGE2 and collagenase as well as stimulate fibroblast proliferation (Dayer et al. 1985). The blockade of TNF in RA mononuclear cell cultures results in significant inhibition of IL-1β, IL-6, IL-8 and GM-CSF release (Maini et al. 1995). Studies in animal models of arthritis have further highlighted the importance of TNFα. The intra-articular administration of TNFα either before or after induction of collagen-induced arthritis resulted in accelerated onset or increase severity of disease (Brahn et al. 1992; Cooper et al. 1992). More interestingly, the administration of monoclonal antibodies against TNFα or recombinant human TNF receptors in the same model of arthritis resulted in attenuation of severity and joint destruction (Thorbecke et al. 1992; Williams et al. 1992; Wooley et al. 1993). The generation of transgenic mice with

dysfunctional TNFα overexpression was associated with spontaneous destructive arthritis, which was completely prevented by anti-TNFα monoclonal antibodies (Keffer et al. 1991). This evidence for TNFα as an important cytokine in RA led to the development of TNFα targeted therapies which have shown efficacy in RA patients (Moreland et al. 1997; Maini et al. 1998), and are now established in clinical practice.

IL-1 is also important in RA pathogenesis, with its ability to induce PGE2, IL-6 and IL-8 production, induce RA FLS proliferation, and upregulate MMP expression (Balavoine et al. 1986; Rupp et al. 1986; Guerne et al. 1989; Kumkumian et al. 1989). Studies in animal models of arthritis have shown the importance of IL-1, with its blockade preventing inflammation and cartilage destruction in antigen-induced arthritis (Tyler et al. 1988). IL-1 receptor antagonist (IL-1Ra) inhibited IL-1 induced collagenase and PGE2 production by RA synovial cells (Arend, W. P. et al. 1990; Seckinger et al. 1990). IL-1Ra also ameliorates inflammation caused by IL-1 in streptococcal cell wall-induced arthritis in rats (Schwab et al. 1991). IL-1 specific therapies are also of proven clinical benefit in RA.

IL-6 is extensively expressed in RA synovium, and is positively regulated by both IL-1β and TNFα (Wong et al. 1988; Firestein, G. S. et al. 1990). IL-6 can induce RA FLS proliferation (Mihara et al. 1995), while in animals IL-6 dependent collagen-induced arthritis can be ameliorated through IL-6 receptor blockage (Takagi et al. 1998). Mice deficient in IL-6 showed attenuated radiologic destruction in collagen-induced arthritis (Sasai et al. 1999).

The pro-inflammatory cytokine macrophage migration inhibitory factor (MIF) has a broad range of effects in RA including induction of TNFα (Leech et al. 1999).

The involvement of MIF in RA will be discussed extensively in section 1.6 of this thesis. A complete analysis of data arising from the study of other cytokines relevant to RA is beyond the scope of this thesis.

1.2.5 Other soluble mediators

1.2.5.1 Phospholipase A2, Cyclooxygenase and Eicosanoids

Eicosanoids are a family of biological active lipid compounds that are believed to have a net pro-inflammatory effect in RA. They are formed by oxidative metabolism of polyunsaturated fatty acids and include arachidonic acid metabolites, prostaglandins, leukotrienes and platelet-activating factor (PAF).

Phospholipase A2 (PLA_2) is an enzyme family that regulates lipid mediator generation resulting from cell activation. The major isotypes of PLA_2 are secretory and cystosolic PLA_2. Serum levels of PLA_2 have been found to correlate with disease severity in RA patients (Pruzanski et al. 1994). The main source of PLA_2 in RA is cytokine activated resident synovial cells and infiltrating leukocytes (Angel et al. 1993; Terry et al. 1999). The principle function of PLA_2 is believed to be the release of arachidonic acid from phospholipids in cellular membranes. PLA_2 has also been shown to be involved in signal transduction. In animal models of adjuvant arthritis, existing paw swelling can be exacerbated by injection of recombinant PLA_2; injection into normal paws, however, had no effect (Murakami et al. 1990).

Arachidonic acid has a number of functions relevant to RA, including stimulation of bone resorption, enhancement of neutrophil oxidative function, potentiation of T cell activation and mediation of MAPK activation (Englberger et al. 1987; Ellies et al. 1993; Hernandez et al. 1998; Siese et al. 1999). Cyclooxygenase and

lipooxygenase oxidatively metabolise arachidonic acid to yield a family of eicosanoids including prostaglandins and leukotrienes. Many of these eicosanoids have pro-inflammatory properties and are also known as lipid mediators of inflammation. Numerous inflammatory stimuli can activate cells to produce eicosanoids, with PGE_2 being the main eicosanoid generated in RA. RA synoviocytes produce large amounts of PGE_2, and it is believed that the functions of PGE_2 include increasing vascular permeability and the stimulation of osteoclast bone resorption. PGE_2 can in contrast inhibit many pro-inflammatory activities of neutrophils, and suppress macrophage and monocyte IL-1β and TNFα production (Needleman et al. 1997).

1.2.5.2 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) such as collagenase, stromelysin and gelatinase are believed to be important agents of cartilage and bone damage in RA. FLS and chondrocytes release MMPs in response to cytokines and growth factors. In the normal joint, MMPs are responsible for normal cartilage matrix turnover and are directly inhibited by tissue inhibitors of metalloproteinase (TIMP). Imbalance between MMPs and TIMPs is associated with cartilage damage and joint destruction in RA. Increases in stromelysin and collagenase have been detected in synovial fluid and synoviocyte supernatants from RA patients (Wernicke et al. 1996). Abundant expression has also been seen at the cartilage-pannus junction and in synovial tissue from animal models of arthritis (Wolfe et al. 1993; Konttinen et al. 1998). Protein and mRNA levels of stromelysin are higher in RA synovium compared to OA synovium (Sawai et al.

1996). Inhibitors of MMPs have shown to be protective against cartilage and bone destruction in an animal model of arthritis (Conway et al. 1995).

1.2.5.3 Inducible nitric oxide synthase (iNOS) and nitric oxide (NO)

Nitric oxide (NO) is a gaseous free radical which is generated by the oxidation of L-arginine by nitric oxide synthase (NOS). There are three distinct isomeric forms of the NOS enzyme that have been identified. The calcium dependent constitutive form (cNOS) is expressed by endothelial and neuronal cells. The NO product from cNOS is released at low levels, and is believed to be involved in intracellular signalling and may exert an anti-inflammatory role by inhibiting cyclooxygenase activity (Flower 1999).

The calcium independent form is called inducible NOS (iNOS), and its expression can be mediated by cytokines and lipopolysaccharide (LPS) in many cell types including macrophages and neutrophils. Expression of iNOS leads to abundant and prolonged production of NO, which amplifies inflammatory processes. Increased expression of NO has 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). In addition, RA synovial tissue exhibits increased NO production, iNOS protein and mRNA expression (Sakurai et al. 1995). NO has been shown to influence a number of important processes in the joint, including inhibiting collagen and proteoglycan synthesis as well as the activation of MMPs (Taskiran et al. 1994; Murrell et al. 1995). NO also decreases the expression of IL-1Ra and increases susceptibility to injury by other oxidants like hydrogen peroxide (Pelletier et al. 1996). In patients with RA, serum and peripheral blood monocyte nitrite concentrations correlate with disease severity (St Clair et al.

1996). These findings suggest that NO plays a role in pathogenesis of RA; however FLS are not believed to contribute significantly to NO production in RA.

1.2.5.4 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are generated by activated macrophages and neutrophils in the inflamed joint and include the superoxide anion, hydrogen peroxide and hydroxyl radicals (Halliwell 1995). Hypoxia-reperfusion injury results in ROS generation that is thought to lead to degradation of collagen and proteoglycans in RA (Halliwell 1995). TNFα can also directly stimulate neutrophils and chondrocytes to produce ROS and thereby augment joint destruction (Miesel et al. 1996). Low levels of ROS generation by neutrophils correlates with a lack of erosion in early RA. Like NO, ROS can act as an intracellular signalling molecule which mediates the biological effects of cytokines (Lo et al. 1995; Lo et al. 1998).

1.3 p53

The tumour suppressor p53 is a nuclear protein, with a relatively short half-life in normal unstressed cells. The half-life of p53 increases dramatically after DNA damage (Price et al. 1993; Maki et al. 1996). There are a number of stimuli which can cause DNA damage and subsequent increase in p53 protein levels, such as ionizing radiation, ultraviolet radiation, hypoxia, and redox stress. Activation and accumulation of p53 leads to cell cycle arrest, which allows for DNA repair or apoptosis. Although all of these DNA damaging agents are important, in the context of RA, oxidative stress and hypoxia are most relevant. As mentioned previously, there are increased levels of ROS, NO and iNOS in RA synovium. It

is believed that the increased expression of these inflammatory molecules modulates p53 expression in RA, and possibly generates p53 mutations in long standing disease (Yamanishi et al. 2002c).

Increased levels of p53 can result in G_1 growth arrest, through p53 dependent transcription of multiple genes that mediate cell cycle arrest. The cyclin dependent kinase (Cdk) enzyme inhibitor, $p21^{WAF1/CIP-1}$ can inhibit DNA replication by binding to proliferating cell nuclear antigen (PCNA), without inhibiting the ability of PCNA to mediate DNA repair (Warbrick et al. 1995). p21 can also inhibit cyclin E/cdk2 and cyclinA/cdk2 kinases from promoting cell cycle progression (el-Deiry et al. 1993; Waldman et al. 1995). Growth arrest and DNA damage induced protein45 (GADD45) is regulated by p53 and interacts with p21 and PCNA (Hall et al. 1995; Kearsey et al. 1995). Another important function of p53 is the ability to repress the transcription of a number of cellular genes such as PCNA, *c-fos*, *c-jun*, IL-6, Rb and bcl-2 (Caelles et al. 1994).

Transcription of p53 target genes is also necessary for inducing apoptosis, including Fas/APO1, BAX and other p53 induced genes (PIGs) (Miyashita et al. 1994; Owen-Schaub et al. 1995; Polyak et al. 1997). BAX can antagonize the anti-apoptotic activity of bcl-2 (White 1996). Interestingly, p53 can also down regulate bcl-2, thereby promoting apoptosis by down-regulation of an anti-apoptotic protein (White 1996).

In addition, p53 can induce transcription of its own negative regulator, mouse double minute 2 (mdm2) or its human homolog hdm2 (Picksley et al. 1993). Mdm2 has been shown to bind to and inactivate p53 protein, consequently leading to p53 ubiquitin-dependent proteolysis (Oliner et al. 1993). The interaction between mdm2 and p53 keeps the growth suppressive functions of p53 in check

during the normal cell cycle. However, during cellular stress, p53 is activated and disassociates from mdm2, and regulates expression of genes involved in growth arrest and/or apoptosis. A full elucidation of the activation/regulation of p53 and its interactions with cellular proteins is beyond the scope of this thesis; the following paragraphs outline the findings relevant to p53 and RA.

1.3.1 p53 involvement in RA

The observed incomplete and dysregulated apoptosis of RA FLS has lead to increased investigation of cell cycle and apoptosis related proteins, such as the p53 protein. Investigations into p53 expression in synovium have found that there is prominent p53 expression in RA synovium (Firestein et al. 1996; Sugiyama et al. 1996; Nickels et al. 1997). Specifically, synovial tissue from RA, reactive arthritis and osteoarthritis patient, display p53 expression in the intimal lining layer, sublining, lymphocyte aggregates and on endothelium, as measured by immunohistochemical staining for p53 (Tak et al. 1999). Greater p53 expression was detected in synovial tissue from RA patients, compared to reactive arthritis or osteoarthritis patients, as measured by Western blotting (Tak et al. 1999). Although not statistically significant, when synovial p53 expression was compared to patients with early RA disease, patients with longstanding disease had approximately double the level of p53 expression, as measured by Western blotting (Tak et al. 1999). Western blot analysis demonstrated that RA FLS expressed significantly more p53 than OA FLS or dermal fibroblasts (Firestein et al. 1996).

In a study comparing wild type (wt) and mutant p53 in RA FLS, cells that expressed greater than 95% wt p53 were transfected with a retroviral vector

encoding the human papilloma virus 18 E6 gene, which binds to and inactivates p53 (Aupperle et al. 1998). This therefore, allowed distinction between the roles mutant p53 (non-functional) and wt p53 play in RA FLS. Inactivation of p53 in RA FLS resulted in an increased invasive potential compared to parental RA FLS, as measured by migration through a polycarbonate filter into cartilage extract (Aupperle et al. 1998). Increased resistance to the apoptotic stimuli H_2O_2 and sodium nitroprusside was observed in E6 transfected RA FLS, compared to parental RA FLS, as measured by DNA fragmentation and detection of hypodiploid cells. Additionally, loss of p53 function in RA FLS resulted in increased anchorage-independent growth and increased cell proliferation (Aupperle et al. 1998).

Investigations into p53 mutations in RA FLS have found conflicting results. Initial studies identified p53 mutations in 40% of RA synovial tissue tested, by RNA mismatch detection analysis (Firestein et al. 1997). A study of RA FLS from Japanese patients found p53 mutations in four out of nine FLS lines tested, using single-strand conformational polymorphism analysis (Inazuka et al. 2000). Analysis of DNA from synovial tissue of 20 RA patients identified p53 point mutations in 15% of samples, but none in the 3 OA synovial tissues, as measured by single-strand conformational polymorphism (Reme et al. 1998). In contrast, analysis of RA FLS from 10 patients from Germany did not detect any p53 mutations, as analysed using single-strand conformational polymorphism, non-isotopic RNAse cleavage assay and base excision sequence scanning T-scan (Kullmann et al. 1999). In the same study, p53 mutations were detected in RA FLS from North American patients. More recently, RA synovial tissue has been micro-dissected and analysed for p53 mutations, with the aim of locating the

region or regions within the synovium which are prone to p53 mutation (Yamanishi et al. 2002c). Synovial intimal lining was identified to contain the majority of the p53 transitional mutations. IL-6 mRNA expression was found to be significantly greater in tissue that contained p53 mutations compared to tissue that contained wt p53 from the same RA patient (Yamanishi et al. 2002c).

It is believed that p53 mutations occur later in disease, due to the genotoxic condition within the inflamed joint, but it is not believed that they cause RA. In addition, the type of p53 mutation commonly found in RA is characteristic of the type caused by oxidative stress (Firestein et al. 1997; Reme et al. 1998; Inazuka et al. 2000; Yamanishi et al. 2002c). As p53 is not an oncogene, mutations in p53 do not result in malignant transformation. Mutation of endogenous FLS p53 did not cause morphological changes in FLS (Aupperle et al. 1998).

Animal models of arthritis have been used to explore the involvement of p53 and arthritis. In a rat adjuvant arthritis (AA) model, p53 expression increased gradually over the duration of the disease as measured by Western blotting and immunohistochemistry (Tak et al. 2000). Apoptosis as measured by TUNEL staining in rat AA joints was found to be preceded by increased p53 expression (Tak et al. 2000). Studies of the collagen-induced arthritis model found that synovial cells from mice that develop arthritis have increased p53 expression and apoptosis (Yamanishi et al. 2002b). The same model was induced in p53-/- mice, and it was found that p53-/- mice developed disease earlier and had increased disease severity compared to wt mice, as measured by clinical and histological scoring. Interestingly, expression of collagenase-3, IL-1 and IL-6 was higher in p53-/- arthritic joints compared to wt arthritic joints, as measured by Northern blotting and ELISA (Yamanishi et al. 2002b). The function of p53 was also

examined in a SCID mouse *in vivo* model of cartilage invasion, where RA FLS are co-implanted with human cartilage under the renal capsule of SCID mice (Pap et al. 2001). In this study, endogenous p53 was inactivated in RA FLS, by transfection of the human papilloma virus type 18 E6 protein. RA FLS with inactive p53 had significantly increased invasion characteristics compared with parental FLS or vector alone transduced FLS, as measured by histological evaluation of invasion and cartilage degradation (Pap et al. 2001).

The use of p53 as a therapeutic agent has also been explored both *in vitro* and *in vivo* by Yao *et al.* Functional p53 was overexpressed in RA and OA FLS and in the joints of rabbits using an adenoviral delivery system. Overexpression of p53 in RA and OA FLS induced significant apoptosis, with no difference between RA and OA observed (Yao et al. 2001). A significant increase in synovial apoptosis was observed in the joints of rabbits with experimental arthritis and overexpression of p53 when compared to control joints, as measured by TUNEL staining (Yao et al. 2001). Interestingly, p53 overexpression also reduced the level of leukocyte infiltration 24hrs after adenoviral injection into the joint (Yao et al. 2001). The mechanism by which p53 reduced inflammation within the joint remains unclear.

1.4 Mitogen-activated protein kinases

Signal transduction pathways are the communication lines by which external cellular signals influence nuclear events. Mitogen-activated protein kinases (MAPK) are a family of proteins that constitute one element of a number of signal transduction pathways. Protein kinases are ubiquitous enzymes that are able to modulate the activities of other proteins by adding a phosphate group to either a

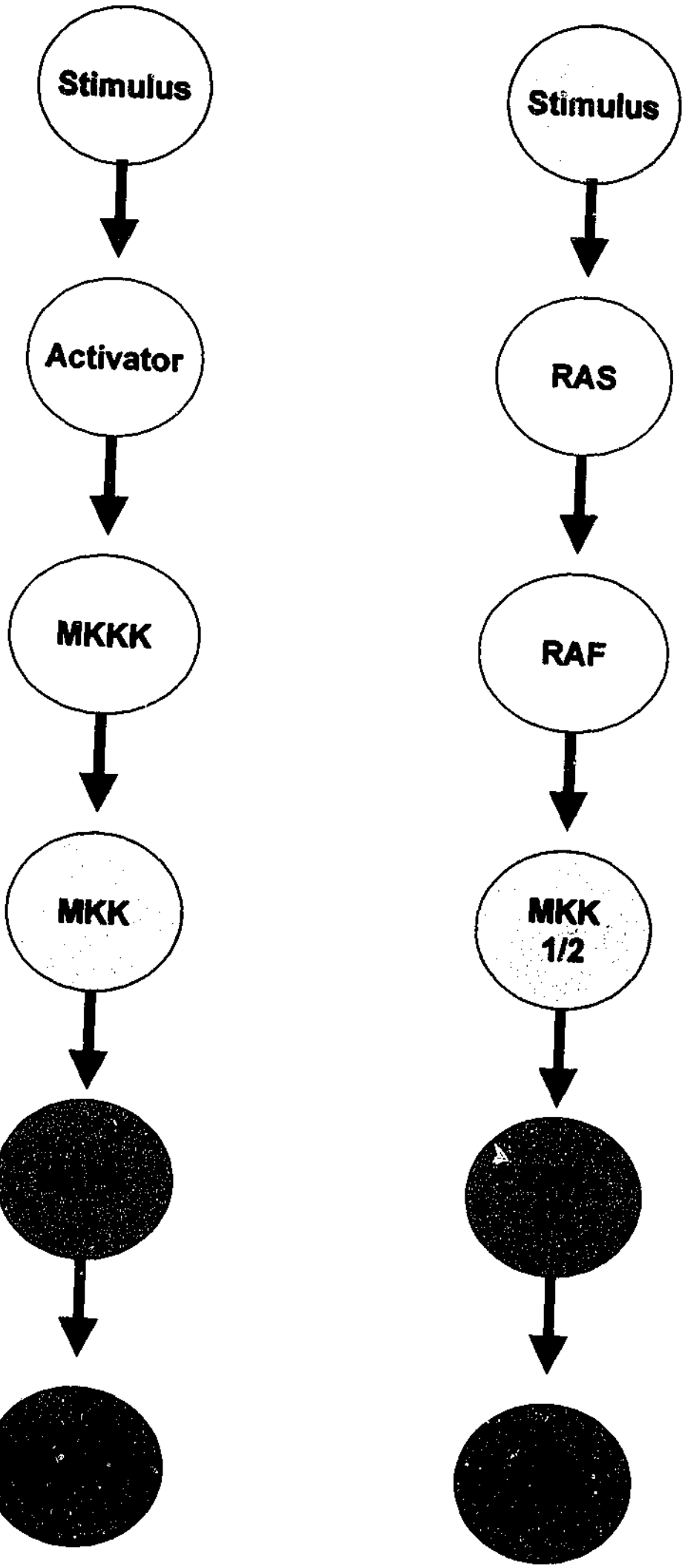
tyrosine, serine or threonine amino acids. This activation is called phosphorylation. MAPK pathways connect cell surface receptors to key transcription factors within the cell. They also respond to chemical and physical stresses, thereby controlling cell survival and adaptation to environmental stress. MAPK pathway activity is regulated through three-tiered cascades, composed of a MAPK, which is activated by a MAPK kinase (MAPKK, MKK, or MEK), which is in turn activated by a MAPKK kinase (MAPKKK, MEK kinase or MEKK). Many MAPK activate specific effector kinases or MAPK-activated protein kinases (MAPKAPK), which in turn are inactivated by MAPK phosphatases (MKP), thereby providing a negative feedback loop. MAPK are also inactivated by MKPs.

Currently, four distinct mammalian groups of MAPK have been identified; the extracellular-signal regulated kinases (ERK1/2), the c-Jun N-terminal kinases (JNK1/2/3), the p38 protein kinases (p38α/β/γ/δ) and ERK5. Each group of kinases are activated by specific up stream kinases, as illustrated in Figure 1.4.

MAPKKs: MEK1/2

Figure 1.4

Simplified diagram of MAPK pathway activation.


Stimulus
Activator
MKKK
MKK
Stimulus
RAS
RAF
MKK
1/2

activates ERK1/2, MKK3/6 activates p38s, MKK4/7 (JNKK1/2) activates JNKs and MEK5 activates ERK5. However each MAPKK can be activated by more than one MAPKKK, thereby increasing the diversity and complexity of MAPK signalling. This classification is a general rule of thumb, and although there is crossover between MAPKKs. Individual MAPK modules generally signal independently of each other, thereby generating distinct physiologic responses to specific stimuli.

The signalling mechanism and proteins involved in the MAPK cascades are complex and numerous. In order for a signal to traverse from the cell surface to the nucleus a number of specific events and protein interactions must occur. Briefly, the MKKK can be activated by a number of activators, such as receptor coupled G-proteins. Once phosphorylated, MKKK bind to and activate their specific substrates (MKK) via an enzymatic reaction. Phosphorylated MKK will then bind to and activate their specific substrate (MAPK) via an enzymatic reaction. Phosphorylated MAPK will be either transported to the nucleus and bind to transcription factors, or bind to and activate specific kinases in the cytoplasm, or may execute other as yet unknown functions. Specificity is maintained for each target substrate through specific binding motifs, which are only exposed when each member of the cascade is activated. Each MAPK signalling module also needs scaffolding and adaptor proteins which are necessary for MKKK-MKK-MAPK interactions and signal transduction. These accessory proteins organise MAPK pathway signalling, by binding and sequestering select components of specific MAPK modules (Pawson et al. 1997). This function enables a coordinated and efficient activation of MAPK components in response to specific stimuli. Scaffolding proteins such as JNK interacting protein 1 (JIP1) and MEK

partner 1 (MP1) add to MAPK module specificity. JIP1 selectively binds to JNK, MKK7, and MLK3 thus aiding signal transduction and signal specificity within the JNK MAPK module (Whitmarsh et al. 1998). MP1 bind to and enhances the activation of MEK1 and ERK1 but not their close family members MEK2 and ERK2 (Schaeffer et al. 1998).

1.4.1.1 ERK MAPK

The extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway preferentially regulates meiosis, mitosis and post-mitotic functions in differentiated cells. The ERK1/2 pathways is activated by numerous stimuli, including growth factors, cytokines, virus infection, ligands binding to G-coupled receptors, transforming agents and carcinogens. The ERK1/2 MAPK are part of a three tiered kinase phospho-relay module which includes the MKKK c-Raf1, B-Raf, or A-Raf, which can activate the MKK MEK1/2, which subsequently activates the MAPK ERK1/2. The MKKKs in the ERK1/2 pathway are predominantly activated by the proto-oncogene Ras. Many human tumours contain mutated Ras, which persistently activates the ERK1/2 pathway, thereby contributing to increased proliferation in those tumours.

In resting/unstimulated cells, inactive ERK is associated with MEK and remains in the cytoplasm (Fukuda et al. 1997). Once activated by an upstream MKK, ERK is phosphorylated and undergoes conformational change exposing a binding motif. Phosphorylated ERK dimerizes, which is necessary for active translocation to the nucleus, where it can bind to specific transcription factors (Khokhlatchev et al. 1998). Activated ERK has a number of targets including the transcription factors Elk-1, c-Jun, c-Fos, c-Myc, estrogen receptor and NF-IL6, as well as other kinases

such as MAPK signal-integrating kinase 1 and 2 (MNK1/2), MAPKAP kinase 2 and 3, and mitogen- and stress-activated protein kinase 1 (MSK1) to name a few (Lewis et al. 1998). A selective MEK1/2 antagonist has been created (PD98059) which binds to MEK1/2 and prevents ERK1/2 activation (Alessi et al. 1995).

1.4.1.2 JNK MAPK

JNK, also known as stress activated protein kinases (SAPK) were cloned independently by two groups: Kyriakis *et al* and Derijard *et al* (Derijard et al. 1994; Kyriakis et al. 1994). There are at least three genes encoding JNK, known as JNK1, JNK2 and JNK3 (Gupta et al. 1996). JNK1 and JNK2 are widely expressed in many cell types; however JNK3 expression is restricted to neuronal cells, heart and testis (Gupta et al. 1996). JNK was originally found to be activated in response to heat shock, ionizing radiation, oxidant stress, DNA damaging chemicals, reperfusion injury, mechanical shear stress, cycloheximide and anisomycin (Derijard et al. 1994; Kyriakis et al. 1994; Pombo et al. 1994; Kyriakis et al. 1996). More recently, it has been shown that many pro-inflammatory cytokines activate the JNK pathway, including TNF, IL-1, CD40 ligand, CD27 ligand, Fas ligand, receptor activator of NF-κB, (RANK) ligand to name a few (Bird et al. 1994; Berberich et al. 1996; Kyriakis et al. 1996; Akiba et al. 1998). Like ERK and p38, activated JNK binds components of the AP-1 transcription factor, c-Jun and JunD, while it also binds ATF2 and Elk1 (Gupta et al. 1996).

1.4.1.3 p38 MAPK

p38 was originally described as a 38kDa polypeptide phosphorylated in response to endotoxic shock and osmotic pressure. Since then, four p38 isoforms have been identified, known as p38α, p38β, p38γ and p38δ. The importance of p38 was highlighted when it was shown that p38 was activated in response to IL-1, and compounds that inhibited p38 activity inhibit the transcriptional induction of TNF and IL-1 during endotoxic shock (Lee et al. 1994). The compound (SB203580) worked by binding to and inhibiting a subset of p38s from activating AP-1 transcription of IL-1 and TNF genes (Lee et al. 1994). However, SB203580 only blocks the p38α and p38β isoforms while the p38γ and p38δ isoforms are unaffected (Goedert et al. 1997). p38 is activated by inflammatory cytokines and environmental stress, with a very similar activation pattern to JNK (Kyriakis et al. 1996).

1.4.1.4 MAPK phosphatases

MAPK activity is controlled by induction of feedback inhibition pathways, phosphatases that inactivate MAPK. Specific phosphatases inactivate/dephosphorylate specific MAPK or MKK family members. MAPK phosphatase (MPK) 3 selectively inactivates ERK while phosphatase M3/6 inactivates JNK (Muda et al. 1996). Other phosphatases however, have multiple substrates. For example, MKP-1 and MKP-2 dephosphorylate ERK, JNK and p38 MAPK to various degrees (Chu et al. 1996). An important feature of phosphatases to consider is their cellular location; MKP-1 is localized to the nucleus (Brondello et al. 1995) whereas MKP-3 is cytoplasmic (Groom et al. 1996). Therefore, MKP-

1 will switch off MAPK signalling within the nucleus, but not in the cytoplasm where MAPK have been shown to have additional functions, for example activated p38 MAPK has been shown to stabilize COX2 mRNA or activate other kinases. Other phosphatases function at the level of MKK or MKKK such as PP1 and PP2A which are thought to dephosphorylate MEK and Raf (Casillas et al. 1993). Phosphatases play an important role in regulating the duration of MAPK signal; however the phosphorylation feedback mechanisms still remain unclear.

1.4.2 MAPK involvement in RA

The JNK and p38 pathways were initially believed to be responsible for mediating responses to inflammatory cytokines, osmotic shock and agents that induce apoptosis (Karin 1995), while the ERK pathway was thought to be responsible for transducing growth factor-dependent proliferation signals (Gille et al. 1995). This assertion was made because of the ability of p38 and JNK to transduce signals resulting in cytokine gene transcription, whereas ERK phosphorylates and activates Elk-1, which trans-activates the serum response element and thereby promotes growth and differentiation (Gille et al. 1995). It is now clear that the roles of MAPK are not as discrete as previously hypothesised. Cytokines such as IL-1 and TNF can activate all three MAPK pathways (Barchowsky et al. 2000), and all three MAPK can activate Elk-1, which mediates growth factor responses by activating the c-fos promoter (Whitmarsh et al. 1995; Whitmarsh et al. 1997). The focus of this thesis is not on the biochemical properties of ERK, p38 or JNK, or their interactions with other intracellular proteins. This thesis focuses on the activation and antagonism of these MAPK in response to activation of RA FLS. I

will briefly outline the function and involvement of three MAPK pathways, ERK1/2, p38 and JNK in RA.

Investigations into MAPK activation in RA and OA synovial tissue have found evidence of ERK, p38 and JNK activation in synovial tissue from RA patients as measured by Western blot and immunohistochemistry (Schett et al. 2000). Specifically, ERK activation was localised around synovial microvessels, p38 activation was detected in the synovial lining layer and in synovial endothelial cells, and JNK activation was detected around and within mononuclear cell infiltrates, as measured by immunohistology (Schett et al. 2000). In the same study, activation of MAPK in RA FLS by cytokines was also measured. The pro-inflammatory cytokines IL-1, TNFα and IL-6 were shown to activate all three MAPK. Interestingly, IFNγ and the anti-inflammatory cytokines IL-4, IL-10 and TGFβ, failed to activate MAPK in RA FLS. TNFα was shown to activate MAPK in a dose and time dependent manner, and induce nuclear translocation of activated MAPK (Schett et al. 2000). Another study found that although inactive MAPK are constitutively expressed in both RA and OA FLS, their activation profile is different upon cytokine stimulation. When FLS were stimulated with IL-1, ERK1/2 and p38 were both activated in RA and OA FLS; however JNK was only activated in RA and not OA FLS after IL-1 stimulation (Han et al. 1999). Similarly, TNFα was shown to activate RA but not OA FLS JNK. Additionally, the JNK2 isoform was shown to be the major JNK MAPK family member expressed by FLS as measured by RT-PCR (Han et al. 1999). The relevance of JNK activity in RA FLS was explored thorough antagonism of the JNK and p38 pathway and analysis of collagenase gene expression. When p38 pathway was blocked with low dose SB203580, it failed to inhibit IL-1 induced collagenase

expression in RA FLS. In contrast, when p38, JNK and c-raf pathways were blocked with high dose SB203580, IL-1 induced collagenase mRNA accumulation and AP-1 binding were inhibited (Han et al. 1999). As multiple pathways were blocked in this study it is difficult to dissect whether one pathway is more important than another, or whether blockade of multiple pathways are needed.

More recently, a specific JNK MAPK pathway inhibitor (SP600125) has become available which has aided in answering these questions. Han *et al* have shown that SP600125 blocks IL-1 induced c-Jun phosphorylation and AP-1 binding in RA FLS (Han, Z. et al. 2001). Moreover, it was also shown that antagonism of the JNK pathway by SP600125 blocked IL-1 induced collagenase gene expression; which was confirmed in JNK1 and JNK2 KO FLS (Han, Z. et al. 2001). Use of SP600125 in a rat adjuvant-induced arthritis model has shown therapeutic benefit. Specifically, when the JNK inhibitor SP600125 was administered to arthritic rats, a modest decrease in paw swelling was observed compared to control animals (Han, Z. et al. 2001). Additionally, histological evaluation showed a reduction in synovial inflammation in SP600125 treated rats. Radiographic analysis demonstrated reduced bone and cartilage destruction in SP600125 treat rats compared to controls (Han, Z. et al. 2001). Animals treated with SP600125 also had significantly lower collagenase mRNA and AP-1 binding compared to control animals (Han, Z. et al. 2001). As JNK2 is the major isoform expressed in the RA FLS, investigations into its role in joint destruction have been explored using JNK-2 KO mice. In a passive collagen induced arthritis model, *Jnk2-/-* mice had marginally more severe clinical arthritis, however, histological scores for synovial inflammation were not significantly different than wt mice (Han et al. 2002). A

difference in joint damage was detected, however, with *Jnk2-/-* mice exhibiting significantly less joint damage as detected by Safranin O staining (Han et al. 2002).

The use of the p38 inhibitor SB203580 has also demonstrated therapeutic effects in animal models of arthritis and p38 inhibitors are currently in clinical trials in RA. Specifically, SB203580 was evaluated in two arthritic models, murine collagen-induced arthritis and rat adjuvant-induced arthritis (Badger et al. 1996). Paw inflammation and serum amyloid protein levels were significantly reduced in collagen-induced arthritis mice after treatment with SB203580 compared to controls. SB203580 demonstrated disease modifying activity in rat adjuvant-induced arthritis by improving bone mineral density and histological evaluation (Badger et al. 1996). Such has been the success of p38 targeted drugs in preclinical studies that p38 inhibitor Vertex 745 has entered phase II trials (Haddad 2001).

An additional function of p38 has been alluded to recently in a number of studies, that is, the stabilization of mRNA. Stimulation of MAPK-AP2 kinase in RA FLS by IL-1β was completely suppressed by SB203580 (Miyazawa et al. 1998). This was associated with a dose dependent decrease in IL-6 protein production and gene expression (Miyazawa et al. 1998). Similarly, SB203580 was found to inhibit IL-1α- and TNFα-induced IL-6 and IL-11 production by RA FLS (Taki et al. 1998). SB203580 significantly reduced IL-1β-induced IL-6 mRNA expression without affecting the rate of IL-6 gene expression in RA FLS (Miyazawa et al. 1998). These results indicate that the activation of p38 MAPK can influence the mRNA stability of pro-inflammatory molecules. This mechanism thereby enables cytokines that do not affect gene transcription of IL-6, for example, to influence

IL-6 protein levels through activation of p38 MAPK and subsequently IL-6 mRNA stability. Similar observations have been made in regards to p38 and COX2 mRNA stabilisation in RA FLS.

1.5 Glucocorticoids

Glucocorticoids are widely prescribed in the treatment of RA, and have had a profound effect in alleviating the symptoms of arthritis in the 50 year since their discovery. The impact of exogenous and endogenous glucocorticoid hormones in modulating the immune response and subsequently restoring homeostasis is well documented. One of the initial observed anti-inflammatory effects was glucocorticoids' ability to inhibit the generation of eicosanoids mediators. Since then, it has been shown that glucocorticoids inhibit a wide range of pro-inflammatory and immuno-regulatory cytokines, including IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-11, IL-12, IL-15, IL-16, IFN-γ, TNFα and several colony stimulating factors (Almawi et al. 2002). The mechanisms by which glucocorticoids exert their effect have only recently been discovered with the aid of modern molecular techniques. A full elucidation of the interactions between glucocorticoids and the immune response is beyond the scope of this thesis. Due to the unique relationship between glucocorticoids and MIF, however, I will briefly outline the intracellular signalling mechanisms of glucocorticoids.

1.5.1 Intracellular signalling mechanisms of glucocorticoids

Glucocorticoids exert their anti-inflammatory effects via a variety of mechanisms which include receptor mediated regulation of gene expression, and non-genomic

effects on multiple transcription factors and signal transduction pathways. The prelude to the majority of these effects is the penetration of glucocorticoid into the cell and binding of glucocorticoids to the cytoplasmic glucocorticoid receptor (GR). The GR is a transcription factor that undergoes a conformational change when bound to glucocorticoids (hormone-receptor complex), which exposes a nuclear transport sequence. It is subsequently transported to the nucleus, where it binds to DNA sequences known as glucocorticoid response elements (GRE). It is through this binding to DNA that glucocorticoids are able to positively regulate a wide variety of genes. Glucocorticoids induce the expression of a number of potentially anti-inflammatory proteins including annexin I, IL-1RA, and IκB.

The hormone-receptor complex has also been shown to inhibit inflammation in a non-genomic manner by interfering with the transcriptional activity of the transcription factors CREB (Stauber et al. 1992), AP-1 (Paliogianni et al. 1993) and NF-κB (Unlap et al. 1995). It is thought that this transcriptional modulation accounts for the broad spectrum of effects glucocorticoids have on inflammatory processes. Collagenase and stromelysin genes both have AP-1 binding motifs in their promoter regions and interference with AP-1 transcriptional activity leads to a decrease in their expression (Benbow et al. 1997). Similarly, interference with NF-κB transcription can effect a wide variety of immuno-regulatory genes including IL-1β, IL-2, IL-6, IL-8, TNFα, GM-CSF and a number of adhesion molecules (Baeuerle 1998; Roebuck et al. 1999). Chemokines such as MCP-1 and RANTES which are important in RA also have NF-κB binding motifs in their promoter regions (Roebuck et al. 1999). The finding that the glucocorticoid hormone receptor complex can directly decrease NF-κB mediated transcription by

binding to the p50 and p65 subunits (Scheinman et al. 1995) is important as many cytokines exert their pro-inflammatory effects by activating NF-κB.

Although it was generally thought that transcriptional inhibition of pro-inflammatory genes was the predominant mechanism by which glucocorticoids exerted their anti-inflammatory effects; this hypothesis is under review due to the recent discovery of two novel glucocorticoid induced genes. These two novel genes are glucocorticoid-induced leucine zipper (GILZ) and MAPK phosphatase-1 (MKP-1). GILZ and MKP-1 modulate crosstalk between the glucocorticoid pathway and the MAPK pathway (D'Adamio et al. 1997; Lasa et al. 2002). GILZ can directly inhibit transcription by binding to AP-1 or NFκB (D'Adamio et al. 1997). GILZ can also inhibit ERK signalling by sequestering the MAPKKK Raf (Widen et al. 2000; Ayroldi et al. 2001). MKP-1 preferentially inactivates JNK and p38, but can also inactivate ERK (Franklin et al. 1997). Dexamethasone can induce MKP-1 expression within one hour, and this expression is sustained for at least 12 hours in cells such as osteoblasts (Engelbrecht et al. 2003). The finding that both GILZ and MPK-1 are induced by glucocorticoids provides another insight into the potential mechanisms by which glucocorticoids modulate inflammation. Little is known about the effects of glucocorticoids on these molecules in RA.

1.6 Macrophage migration inhibitory factor (MIF)

1.6.1 History

Macrophage migration inhibitory factor (MIF) was first described in 1966 by Bloom and Bennett as a non-dialyzable, T cell derived substance that inhibited the

random migration of cultured macrophages and was involved in delayed-type-hypersensitivity (Bloom et al. 1966; David 1966). It was the first soluble lymphokine to be identified, and was subsequently named macrophage migration inhibitory factor (Nathan et al. 1973). In addition it was discovered that MIF activated macrophages and enhanced their turmicidal and parasitical activities (Churchill et al. 1975). However the discovery of other factors that were also able to inhibit macrophage migration such as IFNγ and IL-4, delayed the characterisation and cloning of the MIF molecule for many years.

1.6.2 Structure and function

MIF is a 12.5kDa protein, first successfully cloned in 1989 from a human T cell line (Weiser et al. 1989). Mouse MIF was cloned from cDNA of the AtT-20 anterior pituitary cell line (Mitchell et al. 1995b). Mouse and rat MIF were found to exhibit 90% homology over 115 amino acids with human MIF (Mitchell et al. 1995b). The MIF gene was found to be small (<1kb) and to consist of 3 exons that are separated by two small introns of 100-200 bp (Mitchell et al. 1995b).

X-ray crystallographic analysis has shown that MIF is an α/β structure, which exists as a homotrimer with dimensions of approximately 35×50×50Å (Sun, H. et al. 1996). The monomer subunit contains two anti-parallel α-helices that pack against a four stranded β-sheet. The homotrimer forms a barrel containing a solvent accessible channel, which is formed by six α-helices surrounding three β-sheets. This channel is lined by hydrophilic molecules and is positively charged, suggesting that it may interact with negatively charged molecules (Calandra et al. 1997).

The structure of MIF is unique, and no significant sequence homologies have been found between MIF and known proteins. The α/β-structural motif of MIF with the central channel running through the middle of the molecule distinguishes MIF from other known mediators and reveals that MIF does not belong to any known cytokine family. It has been suggested that MIF interacts with an intracellular protein Jab1, which is a co-activator of AP-1 transcription, after endocytosis (Kleemann et al. 2002). Bernhagen *et al* showed that MIF was like IL-1 in that both the human and mouse forms of MIF lack a N-terminal leader sequence, and is therefore released form the cell via a non-conventional protein secretion pathway (Bernhagen et al. 1994).

Unlike other cytokines, MIF has enzymatic activity (Rosengren et al. 1996). Structural comparison between MIF and bacterial enzymes revealed that it shared almost identical subunit topology with the *E.coli* enzyme 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI), which catalyses the isomerization of unsaturated ketones (Sugimoto et al. 1996; Sun, H.W. et al. 1996). Although the biological relevance of the enzymatic activity is unknown, MIF catalyses a tautisomerisation reaction *in vitro*, which involves the conversion of D-dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Rosengren et al. 1996). There is no physiologically relevant substrate for the catalytic activity of MIF, and site-directed mutations studies of the catalytic site of MIF have suggested that MIF does not require a functional catalytic site to exert its biological effects (Hermanowski-Vosatka et al. 1999). This is controversial however, as another study using the same technique showed the opposite, that MIF enzymatic activity and biological activity are linked (Swope et al. 1998). In a separate study where small molecule inhibitors of MIF were designed to bind to

the catalytically active site, it was shown that both enzymatic activity and biological activity were blocked (Dios et al. 2002). The same study demonstrated that the small molecule inhibitor of MIF also blocked MIF binding to its unidentified receptor. The MIF receptor has since been identified as CD74, which is part of the MHC class II invariant chain (Leng et al. 2003).

Significant conservation is observed in the potential regulatory motifs in the upstream regions of the mouse and human *Mif* genes. Mouse MIF mRNA is 0.6 kB (Mitchell et al. 1995a), while human mRNA is 0.8 kB (Paralkar et al. 1994). The human MIF gene is located on chromosome 19, with its promoter region containing multiple Sp-1 and CRE sites (Paralkar et al. 1994). Human MIF mRNA, like mouse MIF mRNA, is highly expressed in many tissues, including brain, kidney and liver (Paralkar et al. 1994). In the mouse, the MIF gene is located on chromosome 10. MIF promoter region contains consensus sequences for transcription factors and other enhancer/regulatory binding domains include a cytokine-1 site (CK1), a nuclear factor-κB site (NFκB), and an AP-2 site (Mitchell et al. 1995a). A potential negative glucocorticoid responsive element (nGRE) has been identified in the upstream region of the mouse MIF gene, along with sites for interaction with the proto-oncogene c-fos, a Sp-1 site and a cAMP responsive element (CRE) (Mitchell et al. 1995a). As the MIF promoter has both NFκB and CRE regulatory sequences it has cytokine and endocrine hormone characteristics.

1.6.3 Tissue distribution and cellular sources of MIF

1.6.3.1 T cells

MIF was first described as T cell product in 1966, and confirmed as a T cell cytokine involved in delayed type hypersensitivity in 1996 (Bernhagen et al. 1996). Understanding of the relationship between MIF and T cells has advanced since the cloning and identification of the MIF molecule. It is now known there is a pre-formed store of MIF in resting T cells (Calandra et al. 1994). Human peripheral blood and mouse splenic T cells express MIF mRNA, and upon stimulation with anti-CD3 antibody or superantigen, MIF secretion is induced. Both anti-CD3 and superantigen induce IL-2 secretion and T cell proliferation, which was reduced by treatment with specific anti-MIF antibodies. Studies into the adaptive immune response suggested that although both Th1 and Th2 clones produce MIF, Th2 clones predominantly produce MIF (Bacher et al. 1996). This is of interest in light of the finding that *in vivo* treatment of mice with anti-MIF antibodies inhibits antigen-driven T cell proliferation and reduces antigen specific immunoglobulin (Ig) G production. In collagen induced arthritis, it was shown that treatment with anti-MIF antibody resulted in a decrease in IgG2a, rather than IgG1 antibody subclass, which is consistent with decreases in Th1 responsiveness (Mikulowska et al. 1997a). This evidence suggests a role for MIF in development of Th1 driven antibody production. IL-10, a Th2-derived cytokine, inhibits the synthesis of MIF and abolishes recombinant MIF-mediated inhibition of human monocyte migration (Wu et al. 1993). Further, MIF can induce macrophages to produce IL-12, thereby suggesting that MIF may play an important role in the Th1 immune response (de Jong et al. 2001). Overall, the available evidence supports the participation of MIF in Th1 mediated immune events.

MIF secretion by T cells is also induced by glucocorticoids in a dose dependent fashion and MIF has been shown to override glucocorticoid-mediated suppression

of T cell proliferation (Bacher et al. 1996), suggesting autocrine/paracrine feedback control of cell activation by MIF in response to glucocorticoids.

1.6.3.2 Monocytes/Macrophages

Monocytes and macrophages were originally considered to be the target for T cell derived MIF. It is now known that macrophages are an important source of MIF, with high levels of MIF mRNA and protein constitutively expressed in unstimulated macrophages (Calandra et al. 1994). This preformed store of MIF is released from macrophages upon stimulation by a number of pro-inflammatory stimuli, including endotoxins, exotoxins and cytokines such as INFγ, TNFα and IL-1β (Calandra et al. 1994). Interestingly, the concentration of LPS required for inducing MIF mRNA and protein is significantly lower than that required to induce TNFα (Bernhagen et al. 1993). MIF is released from cytoplasmic stores prior to the synthesis of new protein, suggesting that it may be an early response cytokine. MIF production seems to be tightly regulated, as it follows a bell shaped curve in response to LPS, decreasing at higher levels (Bernhagen et al. 1993).

Glucocorticoids can induce MIF secretion from macrophages, and MIF is the only known macrophage cytokine to be induced by glucocorticoids (Calandra et al. 1995). It was shown it the same study that MIF overrides, in a dose-dependent manner, the glucocorticoid inhibition of macrophage cytokines TNFα, IL-1β, IL-6 and IL-8. These findings support the hypothesis that MIF has glucocorticoid counter-regulatory properties.

MIF influences a number of pro-inflammatory macrophage functions. One of the earliest reported functions of MIF on macrophages was to increase intracellular killing and H_2O_2 generation (Nathan et al. 1973). At high concentrations, MIF can

induce TNFα secretion and synergises with IFNγ to increase NO production (Cunha et al. 1993; Bernhagen et al. 1994). MIF has been shown to enhance phagocytosis of foreign particles by macrophages in an autocrine and paracrine fashion (Onodera et al. 1997). Treatment of murine macrophages with rMIF confers protection from *Leishmaniasis major* infection, which is mediated by increased iNOS and is dependent on endogenous TNFα (Juttner et al. 1998). Initial work by Churchill *et al* which identified the capacity of T cell supernatants to induce tumoricidal activity in cultured macrophages was later reproduced in studies where rMIF-activated human macrophages killed tumor cells (Churchill et al. 1975; Pozzi et al. 1992).

1.6.3.3 Eosinophils

Circulating human eosinophils are another source of pre-formed MIF (Rossi et al. 1998). PMA stimulation of eosinophils resulted in release of significant quantities of MIF in a dose and time dependant manner. This PMA induced release of MIF was blocked by protein synthesis inhibition using cycloheximide and protein kinase C inhibition. IL-5 and C5a have also been shown to stimulate MIF release from eosinophils (Rossi et al. 1998). Although the effect of MIF on eosinophil function remains unknown, it has been reported that MIF is over expressed in eosinophils of asthma patients (Yamaguchi et al. 2000).

1.6.3.4 Other cellular sources of MIF

MIF has been found in number of other cell types, both at the protein and mRNA level. These include human vascular endothelial cells, which express MIF in their cytoplasm, and upon stimulation with LPS dramatically increase MIF expression

in dose-dependent fashion (Nishihira et al. 1998). Similar to macrophages, at high concentrations of LPS vascular endothelial cells showed a decrease in MIF mRNA in a bell shaped dose response. This suggests that MIF is tightly regulated and there are protective mechanisms that may limit a deleterious response in life-threatening situations, such as endotoxic shock.

1.6.3.5 Tissue expression of MIF

An Epstein-Barr virus-transformed human B-cell line has been identified as a source of large amounts of MIF (Wymann et al. 1999). In another B cell line transfected with MIF antisense resulted in protection from surface immunoglobulin mediated growth arrest and apoptosis (Takahashi et al. 1999).

Abundant MIF expression has been described in the rat brain, in the neurons of the cortex, hypothalamus, hippocampus, cerebellum and pons (Bacher et al. 1998b). MIF immunoreactivity was prominent in hippocampal structures which have been shown previously to be involved in glucocorticoid-induced tissue damage within the hippocampus (Bacher et al. 1998a). MIF expression at the site of glucocorticoid action may reflect a glucocorticoid counter-regulatory role in the hippocampus. In the same study an intracisternal LPS injection increased MIF release and MIF mRNA in a distribution that co-localized with TNFα, IL-1β and IL-6 (Bacher et al. 1998a). MIF has been suggested to participate in a detoxification pathway for catecholamine products and could therefore have a protective role in neural tissues (Matsunaga et al. 1999).

MIF has also been detected diffusely throughout the brain (Matsunaga et al. 1999). As monocytes and tissue macrophages are important sources of MIF, it is thought that glial cells maybe responsible for the extra neural staining, as previous

work has demonstrated MIF expression in bovine subependymal glial cells (Nishibori et al. 1996). Peripheral nerves have also been identified to have MIF expression (Nishio et al. 1999). MIF mRNA and protein was detected in the rat sciatic nerves, with Schwann cells identified as sources of MIF protein. The same study postulated a role for MIF in nerve regeneration as MIF levels increase dramatically after nerve transection and diminished several hours after injury.

MIF expression has been reported in cornea and retina and is localised to the basal cells of corneal epithelium and endothelial cells (Matsuda et al. 1996a; Matsuda et al. 1996b). Increased expression of MIF mRNA was detected during corneal wound healing after penetration injury. MIF levels in the contralateral eye remained unchanged but the concentrations of MIF in the aqueous humor were elevated in both injured and contralateral eyes (Matsuda et al. 1997). It has been demonstrated that normal aqueous humor contains MIF which has an important protective function (Apte et al. 1998). This study demonstrated that MIF inhibits the NK-mediated lysis of corneal endothelial cells, which due to their lack of MHC class I are potentially vulnerable to NK-mediated lysis. The protective effect of MIF was neutralized by anti-MIF antibodies (Abs). MIF mediated its protective effects by inhibiting the release of perforin granules by NK cell (Apte et al. 1998). This result indicates that MIF has functions other than its known pro-inflammatory functions.

Reproductive tissue such as granulosa cells from human ovaries expresses MIF mRNA and protein (Wada et al. 1997). The concentration of MIF in follicular fluid is inversely proportional to follicular size, suggesting that MIF is involved in the regulation of oocyte development (Wada et al. 1997). Correspondingly, MIF mRNA and protein has been reported in Leydig cells of adult rat testis and

testicular fluid (Meinhardt et al. 1996). Treatment of adult rat seminiferous tubules and purified Leydig cells with rMIF resulted in a dose dependent decrease in the secretion of inhibin (Meinhardt et al. 1996). These results suggest that MIF may play an important role in regulating testicular function.

In mice, MIF has been detected in ovaries, oviduct and uterus during pre-implantation and all stages of estrus cycle (Suzuki, H. et al. 1996). Additionally, MIF mRNA is expressed in ovulated oocytes, zygotes, 2-cell and 8-cell embryos and blastocytes (Suzuki, H. et al. 1996). Even though MIF is ubiquitously expressed in reproductive tissue, its function is uncertain as MIF knockout mice have normal fertility with predictable litter sizes for both heterozygous and homozygous mating (Bozza et al. 1999a).

MIF expression has been detected in human kidney, with predominant expression in renal epithelial cells and to a lesser extent in Bowman's capsular epithelial cells (Imamura et al. 1996). Human cultured keratinocytes and surgically obtained human epidermis express MIF. Murine osteoblasts and an osteoblastic cell line express MIF, suggesting that it is involved in bone metabolism (Onodera et al. 1996). MIF is expressed in normal lung parenchymal tissue (Kayser et al. 1993). There is a lack of evidence of MIF expression in normal synovial tissue, however MIF has been detected in pseudo-synovial tissue associated with loosening of prosthetic joints (Suzuki, K. et al. 1996). The expression of human MIF in RA synovial tissue and other diseases will be discussed in section 1.6.5.

1.6.4 MIF Knockout mouse

Bozza *et al* generated a mouse strain lacking the MIF gene by targeted disruption of the MIF gene in mouse embryonic stem cells (Bozza et al. 1999b). The MIF -/-

mice develop normally in relation to size, behaviour and fertility. MIF protein and mRNA were not detected in homozygotes. Organs including kidney, liver, spleen, thymus, adrenal, lung, brain, heart and intestine were histopathologically normal. Splenic and thymus-derived lymphocyte populations were normal on flow-cytometric analysis. Normal neutrophil accumulation was observed in thiglycollate elicited peritoneal exudates. In contrast to these findings, MIF knockout mice show resistance to endotoxic shock lethality and reduced production of TNFα, IL-1β and IL-6 upon challenge with endotoxin (Bozza et al. 1999a). Macrophages derived from MIF-/- mice exhibited diminished TNFα production, but surprisingly exhibited increased production of nitric oxide (NO), after stimulation with LPS and IFNγ.

More recently investigations into MIF-/- mice have discovered that MIF plays an important role in the control of the expression of Toll-like receptor 4 (TLR-4) (Roger et al. 2001). MIF-deficient macrophages were shown to have reduced cell surface expression of TLR-4, which was due to impaired PU.1 transcription activity, which is known to regulate *Tlr4* gene expression. This observation is believed to explain at least in part the reported alterations in LPS response in MIF-/- mice. Elsewhere in this thesis I shall document novel observations in regard to the function of cells derived from MIF-/- mice.

1.6.5 MIF involvement in animal models

1.6.5.1 Delayed-type hypersensitivity

The involvement of MIF in delayed-type hypersensitivity (DTH) has been elucidated. MIF mRNA and protein are abundantly expressed in DTH skin lesions in mice (Bernhagen et al. 1996). Despite MIF originally being described as a T

cell cytokine, macrophages were identified as the predominant cellular source of MIF in DTH. The administration of anti-MIF Abs significantly inhibited the development of DTH in terms of cellular infiltration, vascular dilatation and dermal expansion (Bernhagen et al. 1996; Santos, L. et al. 2001). These results highlight the importance of MIF in the DTH reaction and that macrophages are an important *in vivo* source of MIF.

1.6.5.2 Endotoxemia and sepsis

The involvement of MIF in endotoxemia is well documented. The administration of LPS to animals results in the release of preformed intracellular stores of MIF and an increase in serum MIF levels. LPS also stimulates MIF mRNA, and intracellular stores of MIF are restored within 24hrs (Bacher et al. 1997). The same study suggested that a major source of serum MIF is the pituitary gland, based on LPS administered to hypophysectomised, T cell deficient and control mice. It has been reported that administration of anti-MIF Abs in an endotoxemia model results in reduced lethality in mice (Bernhagen et al. 1993; Calandra et al. 2000). Additionally, when rMIF was co-injected with LPS, an increase in lethality was observed. In patients suffering form severe sepsis or septic shock, high concentrations of MIF have been detected in the plasma (Calandra et al. 2000).

MIF knockout mice have been reported to be resistant to the lethal effect of high dose LPS and *Staphylococcus aureus* enterotoxin B (SEB) with lower plasma levels of TNFα, but levels of IL-6 and IL-10 were unchanged. The reduction in TNFα reported in MIF-/- mice suggests MIF may act upstream from TNFα, i.e. as a TNF inducer. MIF-/- mice were shown to be sensitive to the lethal effects of low dose LPS by first sensitizing MIF-/- mice to the effects of TNFα using

galactosamine. However, MIF-/- remained resistant to the lethal effects of SEB even in the setting of galactosamine sensitization (Bozza et al. 1999a). Interestingly, anti-MIF antibodies have been shown to protect 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 both CLP and *Escherichia coli*, even when treatment was started up to 8 hours after CLP. Conversely, co-injection of recombinant MIF and *E. coli* markedly increased the lethality of peritonitis (Calandra et al. 2000). These studies suggest a critical role for MIF in the pathogenesis of septic shock.

In contrast, Honma *et al.* have suggested that MIF does not play a critical role in LPS-induced endotoxaemia. They observed no significant difference in LPS lethality or TNFα formation between MIF-/- or normal mice (Honma et al. 2000). More recent results have shown however, that macrophages from MIF-/- mice are hyporesponsive to LPS and Gram-negative bacteria, as shown by a profound reduction in the activity of NFκB and the production of TNFα (Roger et al. 2001). This reduction is due to a down regulation of Toll-like receptor 4 (TLR4), the signal-transducing molecule of the LPS receptor complex, and is associated with decreased activity of transcription factor PU.1, which is required for optimal transcription of the Tlr4 gene in myeloid cells (Roger et al. 2001). Studies in the candidate's laboratory have confirmed the hypo-responsiveness of MIF-/- splenocytes to LPS as measured by TNF release (Santos unpublished observations). These findings identify an important role for MIF in innate immunity and provide a molecular basis for the resistance of MIF-deficient mice to endotoxic shock.

1.6.5.3 Arthritis

Macrophages have been implicated as the initiating antigen-presenting cell in collagen-induced arthritis (CIA) (Michaelsson et al. 1995). It is for this reason that the role of MIF was examined in a murine model of CIA. Administration of anti-MIF Abs prior to immunisation resulted in delayed onset and lower frequency of arthritis (Mikulowska et al. 1997b). This was associated with decreased IgG_{2a} response to type II collagen with no difference observed in IgG_1 production. This result suggested that MIF blockade may be associated with a decrease in Th1 responsiveness. However, anti-MIF treatment increased the overall T cell proliferative response to type II collagen (Mikulowska et al. 1997b).

In rat adjuvant arthritis (AA) it was reported that MIF was detected in AA synovial tissue, predominantly in synovial lining cells, including macrophages and FLS (Leech et al. 1998a). The level of MIF was increased in established AA sera and cultured synovial macrophages. Treatment with anti-MIF Ab resulted in a profound, dose dependent inhibition of AA in terms of clinical score, paw swelling and synovial lavage leukocyte count, as well as histological severity (Leech et al. 1998a). Treatment with anti-MIF mAb also prevented the lethal effects of adrenalectomy in rats developing AA (Leech et al. 2000). Additionally, in a murine antigen-induced arthritis (AIA) model, anti-MIF mAbs significantly inhibited arthritis and DTH (Santos, L. et al. 2001). In the same study it was shown that treatment with the glucocorticoid dexamethasone (DEX) induced a significant dose-dependent inhibition of AIA, while MIF treatment reversed the therapeutic effect of DEX on AIA (Santos, L. et al. 2001). A study of antigen induced arthritis in MIF KO mice has shown that MIF-/- mice have less severe arthritis than wild type mice as measured by histology and Safranin O staining

(Leech et al. 2003). The decrease in arthritis severity seen in MIF-/- mice was associated with an increase in p53 levels and apoptosis in synovium (Leech et al. 2003).

1.6.5.4 Glomerulonephritis

MIF is constitutively expressed in both glomerular and tubular epithelial cells of normal kidneys. In rat crescentic anti-glomerular basement membrane glomerulonephritis, MIF expression is up-regulated in glomerular and tubular epithelial cells, and is correlated with macrophage accumulation (Lan et al. 1996). Macrophages, T cells and fibroblasts within the glomerulus express MIF as detected by *in situ* hybridisation (Lan et al. 1996). MIF was subsequently shown to have a pathogenic role in immunological-induced kidney disease in rats. Rats treated with anti-MIF Abs demonstrated a significant decrease in leukocyte infiltration, histological damage and proteinuria, however no effect on antibody response or deposition within the kidney was observed (Lan et al. 1997a). Additionally, anti-MIF treatment reversed rat crescentic glomerulonephritis and was associated with increased plasma corticosterone levels (Yang et al. 1998).

Although MIF can induce the expression of TNFα, a study utilizing soluble TNFα receptors suggested that TNFα up regulates renal MIF expression in rat crescentic glomerulonephritis (Lan et al. 1997b). In this study, TNFα receptor treatment almost completely abrogated MIF mRNA and protein expression in established rat crescentic glomerulonephritis, while also inhibiting glomerular and interstitial macrophage infiltration, suppressed renal injury and biological damage. These findings suggest that TNFα or TNFα mediated events up-regulate MIF production in immunologically induced renal disease (Lan et al. 1997b). MIF has also been

detected in other models of kidney disease/damage. In a model of lipid-induced glomerular injury, MIF mRNA expression was up regulated in glomerular cells prior to macrophage infiltration (Miyazaki et al. 1997). In a rat model of acute uric acid nephropathy, tubules within the areas of granuloma showed a six-fold increase in MIF mRNA, compared with uninvolved areas, as measured by *in situ* hybridization. Moreover, the areas of increased MIF mRNA expression correlated with sites of dense accumulation of macrophages and T cells (Kim et al. 2000). Both these studies provide evidence that local MIF production influences pathogenic processes in the kidney.

1.6.5.5 Lung injury

In a rat model of acute lung injury, LPS induces neutrophil migration into alveoli and this is significantly attenuated by pre-treatment with anti-MIF Abs. Anti-MIF treatment was also associated with a reduction in the level of the chemokine, macrophage inflammatory protein-a (MIP-1α) (Makita et al. 1998). In a mouse model of bleomycin (BLM)-induced lung injury and fibrosis, treatment with the anti-MIF Abs significantly reduced mortality at 14 days and histopathological lung injury score at 10 days. However, anti-MIF Ab treatment did not affect either the content of lung hydroxyproline or the histopathological lung fibrosis score at 21 days after BLM (Tanino et al. 2002). These studies provide evidence for the role of MIF in acute and chronic lung injury.

1.6.5.6 Models of neoplasia

MIF has been implicated in tumour angiogenesis. Treatment of a murine model of B cell lymphoma with anti-MIF mAb significantly reduced the growth and

vascularization of the lymphoma (Chesney et al. 1999; Chesney et al. 2000). MIF protein and mRNA were also highly expressed in a murine colon carcinoma cell line. Transfection of these cells with an antisense MIF plasmid suppressed their proliferation significantly (Takahashi et al. 1998). Furthermore, antisense MIF prevents anti-IgM mediated growth arrest and apoptosis of a murine B cell line by regulating cell cycle progression (Takahashi et al. 1999). Tumor-bearing mice treated with anti-MIF Ab exhibited suppression of tumor induced angiogenesis (Shimizu et al. 1999a). These findings highlight the involvement of MIF in cell proliferation and angiogenesis.

1.6.5.7 Models of colitis

Elevated levels of MIF have been detected in the sera and in cells from colonic mucosa of patients with ulcerative colitis (Murakami et al. 2001). It has been demonstrated that MIF is required for the development of colitis, as MIF-/- mice failed to develop colitis, in a murine model of dextran sulfate sodium-induced colitis (de Jong et al. 2001). The disease phenotype could be restored in MIF-/- mice by reconstitution with wt immune cells (de Jong et al. 2001). In a separate study, anti-MIF Ab treatment of mice with dextran sulfate sodium-induced colitis reduced disease severity (Ohkawara et al. 2002). Specifically, anti-MIF Ab treatment significantly reduced diarrhea and rectal bleeding in mice with dextran sulfate sodium-induced colitis compared with controls (Ohkawara et al. 2002). Anti-MIF Ab treatment also increased the mouse survival rate in a trinitrobenzenesulfonic acid model of colitis (Ohkawara et al. 2002). These findings suggest that MIF is involved in the inflammatory processes in colitis.

1.6.5.8 Models of multiple sclerosis

MIF has been shown to play an important role in the disease severity of experimental autoimmune encephalitis (EAE). SJL mice with acute EAE treated with anti-MIF Ab have decreased disease severity and accelerated recovery from the symptoms of EAE (Denkinger et al. 2003). Specifically, anti-MIF treatment inhibits autoreactive T cells from reaching their neuroantigens with the central nervous system (CNS) (Denkinger et al. 2003). This result indicates that MIF is involved in the clinical symptoms of EAE and MIF targeted treatments may provide a novel therapy for the treatment of multiple sclerosis.

1.6.6 MIF in human disease

The actions of MIF like those of other cytokines such as TNFα, span both the innate and adaptive immune response. As a result MIF has been implicated in many human diseases as outlined in Table 1. However, a complete review of MIF involvement in diseases other than RA is beyond the scope of this thesis.

Table 1: List of diseases MIF has been implicated in and their relevant reference.

Disease	**Reference:**
Atherosclerosis	(Burger-Kentischer et al. 2002)
Cancers	(Pozzi et al. 1992; Meyersiegler et al. 1996; Shimizu et al. 1999a; Ogawa et al. 2000; Akbar et al. 2001; Markert et al. 2001; Meyer-Siegler 2001; Bando et al. 2002; Bin et al. 2002; Isidori et al. 2002; Meyer-Siegler et al. 2002; Munaut et al. 2002; Sasaki et al. 2002)

Diseases of the Eye	(Matsuda et al. 1996b; Wen et al. 1996; Mitamura et al. 2000; Taguchi et al. 2001; Kotake et al. 2002; Takase et al. 2002)
Diseases of the Skin	(Shimizu et al. 1996; Abe et al. 1999; Shimizu et al. 1999b; Steinhoff et al. 1999; Thakur et al. 2001; Shimizu et al. 2002)
Endometriosis	(Yang et al. 2000; Akoum et al. 2002; Kats et al. 2002a; Kats et al. 2002b)
Human lung disease	(Donelly et al. 1997; O'Leary et al. 1997; Saboroe et al. 2000; Guo et al. 2002; Otsubo et al. 2002)
Infectious diseases: Bacterial, viral and parasitic	(Weiser et al. 1991; Joshi et al. 2000; Jaworski et al. 2001; Tan et al. 2001; Arndt et al. 2002; Koebernick et al. 2002; Maaser et al. 2002; Yamada et al. 2002)
Liver disease	(Gando et al. 2001a; Kumagi et al. 2001)
Myocardial ischemia	(Takahashi et al. 2001)
Multiple sclerosis	(Niino et al. 2000; Nishihira et al. 2001)
Renal disease	(Boyce et al. 1986; Imamura et al. 1996; Lan et al. 2000; Brown et al. 2001; Matsumoto et al. 2001; Brown et al. 2002)
Sepsis	(Bucala 1994b; Bucala 1994a; Nishihira et al. 1996; Beishuizen et al. 2001; Froidevaux et al. 2001; Gando et al. 2001b; Lehmann et al. 2001)
Systemic lupus erythematosus	(Rovensky et al. 1975)
Ulcerative colitis, Crohn's	(de Jong et al. 2001; Murakami et al. 2001;

disease	Maaser et al. 2002; Murakami et al. 2002; Ohkawara et al. 2002)

1.6.7 MIF involvement in RA

MIF expression was first identified in RA synovial tissue in 1999 (Leech et al. 1999). RA synovial macrophages and FLS express MIF, while unstimulated cultured RA FLS constitutively express MIF mRNA and release abundant MIF (Leech et al. 1999). RA patients have significantly elevated levels of MIF in their serum and synovial fluid compared to controls (Leech et al. 1999; Onodera et al. 1999). Cultured RA FLS express greater amounts of MIF protein than OA FLS. There is also an association between RA disease activity, as measured by serum C-reactive protein, and synovial MIF content in patients with RA (Morand et al. 2002).

Exposure of FLS to IL-1β, TNFα or IFN-γ did not increase MIF expression. Biphasic regulation of MIF was observed in FLS by glucocorticoids; low concentrations of glucocorticoids induced synovial MIF expression in cultured FLS, while high concentrations inhibited it (Leech et al. 1999).

The pro-inflammatory function of MIF has been directly evaluated in RA synoviocyte-conditioned medium (Leech et al. 1999). RA synoviocyte-conditioned medium stimulated monocytes to release TNF, and this was abrogated by anti-MIF Ab. It has been shown that MIF stimulates macrophages to release TNF, IL-1, IL-6 and IL-8 (Calandra et al. 1995; Donnelly et al. 1997). Additionally, MIF has been reported to upregulate IL-1β mRNA in FLS (Onodera et al. 2000). These results suggest that MIF can act as a regulator of synovial cytokine expression in RA, particularly with regards to IL-1 and TNF.

Recent evidence also suggests that MIF directly activates RA FLS expression of PLA_2 and COX2 and the release of PGE_2. MIF is essential for the upregulation of these molecules by IL-1β, as anti-MIF Ab blocks IL-1β induction of PLA_2 and

COX2 mRNA in FLS (Sampey et al. 2001). This effect of anti-MIF mAb was associated with significant reduction in both $cPLA_2$-mediated arachidonic acid release and COX2 activity (Sampey et al. 2001).

MIF has also been implicated in the destruction of cartilage and bone, MMP-1 and MMP-3 have been shown to be upregulated by MIF in FLS (Onodera et al. 2000). Additionally, this MMP expression was shown to be independent of IL-1, as co-treatment with IL-1 receptor antagonist did not inhibit MIF induced expression of MMPs. Also of note, studies in rat calvarial osteoblasts have shown that MIF can upregulate MMP-13 and MMP-9 mRNA, with proportionally less TIMP-1 detected (Onodera et al. 2002).

Advances in genetics have aided the understanding of disease pathogenesis. Recently, Donn *et al* have identified a single-nucleotide polymorphism in the *Mif* gene (G- to C- transition at position -173) which confers an increased risk of juvenile idiopathic arthritis (JIA) (Donn et al. 2001). In a study of 88 UK Caucasian JIA patients it was also found that the MIF-173* G to C variant resulted in altered expression of MIF in a cell type-specific manner (Donn et al. 2002). Also, serum levels of MIF were significantly higher in individuals who carried a MIF-173*C allele (Donn et al. 2002). A separate study has shown a novel CATT-tetranucleotide repeat polymorphism at position -794 of the human *Mif* gene and shows that it functionally affects the activity of the MIF promoter in gene reporter assays (Baugh et al. 2002). The presence of the low expressing, 5-CATT repeat allele correlated with low disease severity in a cohort of rheumatoid arthritis patients (Baugh et al. 2002). Both of these studies suggest that MIF expression may be important in disease severity.

1.6.8 MIF and intracellular signalling

1.6.8.1 MIF and NF-κB

Cytokines elicit their effects on cells involved in RA by binding to cellular receptors and activating one or more intracellular signal transduction pathways, such as the NF-κB or MAPK pathways (Firestein et al. 1999). MIF is distinct from other cytokines implicated in RA in that it does not activate the NF-κB pathway. In study examining the ability of MIF to activate NF-κB, it was found that MIF does not affect the activity of the inhibitor of NF-κB kinase (IKK), as measured by Western blotting of the inhibitor of NF-κB (IκB) protein (Daun 2000). Similarly, MIF treatment of RA FLS does not activate NF-κB as measured by NF-κB translocation to the nucleus (Lacey, D. et al. 2003). In the same study, antagonism of the NF-κB pathway with the specific NF-κB antagonist SN50 did not significantly inhibit MIF activation of RA FLS. MIF antisense transfected cells and control transfected cells had identical NF-κB reporter gene expression after treatment with TNFα (Kleemann et al. 2000). Similarly, co-treatment of RA FLS with IL-1β and anti-MIF mAb failed to inhibit IL-1-induced NF-κB nuclear translocation, despite inhibiting IL-1-induced cell activation (Lacey, D. et al. 2003). The observation of reduced NF-κB activation in MIF-/- cells in response to LPS reported by *Roger et al.* reflects the reduced TLR4 expression in those cells and does not constitute evidence that MIF directly activates NF-κB (Roger et al. 2001).

1.6.8.2 MIF and AP-1

MIF can upregulate components of the AP-1 transcription factor that in turn affect the regulation of genes important in cartilage degradation. Onodera *et al* have

shown MIF can upregulate MMP-1 and MMP-3 mRNA in RA FLS as measured by Northern blot analyses and enzyme-linked immunosorbent assay (ELISA). The same study demonstrated that the upregulation of both MMPs was preceded by upregulation of *c-jun* and *c-fos* mRNA (Onodera et al. 2000). In a separate study it was shown that MIF upregulation of MMP-9 and MMP13 mRNA in rat osteoblasts was also preceded by upregulation of *c-jun* and *c-fos* mRNA (Onodera et al. 2002). Antagonism of the ERK MAPK pathway by PD98059 suppressed MIF induced MMP13, c-jun and c-fos mRNA expression (Onodera et al. 2002). Interestingly, MIF stimulated phosphorylation of Ras and ERK1/2, but not p38, JNK or c-Jun in osteoblasts.

1.6.8.3 MIF and MAPK

The ability of MIF to activate the MAPK pathway is evident in a number of studies. Specifically, MIF can induce a unique sustained pattern of activation of the ERK1/2 pathway in NIH/3T3 fibroblast cell line (Mitchell et al. 1999). In the same study it was shown that MIF activation of ERK was dependent on protein kinase A activity, and MIF regulated $cPLA_2$ via a ERK-dependent pathway. MIF increased proliferation of NIH/3T3 cells as measured by thymidine incorporation, which was associated with a sustained pattern of ERK activation (Mitchell et al. 1999). In addition, serum treatment of NIH/3T3 cells results in MIF release and an increase in proliferation, while co-treatment with anti-MIF Ab decreases proliferation and ERK1/2 phosphorylation. The same study demonstrated an increase in $cPLA_2$ activity by MIF as measured by arachidonic acid release. By using specific MAPK antagonists, it was demonstrated that MIF utilized the ERK1/2 pathway, but not the p38 pathway to increase $cPLA_2$ activity. Similarly,

MIF increases $cPLA_2$ activity and COX2 expression, as measured by arachidonic acid release, prostaglandin E2 ELISA, and RT-PCR in RA FLS (Sampey et al. 2001). The same study showed that treatment with anti-MIF mAb significantly reduced IL-1β-induced PLA_2 and COX2 expression in RA FLS. More recently, MIF-induced expression of COX2 in FLS has been shown to transduce its signal via the p38 MAPK pathway and not the ERK1/2 pathway, in studies using the specific MAPK antagonists SB203580 and PD98059 respectively (Santos unpublished observations). This contrast to the results of Mitchell *et al* on NIH/3T3 cells may reflect the utilization of different signalling pathways in different cell types in the regulation of different genes by MIF.

Investigation into adhesion-dependent signalling by MIF has found that MIF secretion is induced by cell adhesion to fibronectin (Liao 2002). Adhesion-mediated MIF secretion results in integrin-dependent activation of MAPK. Neutralization of MIF with anti-MIF Ab reduced integrin-dependent ERK1/2 activation, as measured by Western blotting. MIF-/- mouse embryonic fibroblasts (MEFs) have reduced integrin-induced ERK1/2 activation compared to wt MEFs, as measured by Western blotting and ERK kinase enzymatic assay (Liao 2002). In addition, MIF-/- MEFs displayed reduced proliferation compared with wt MEFs. Immortalised MIF-/- fibroblasts had an impaired ability to accumulate cyclin D1 in response to growth factor compared to immortalised wt fibroblasts. Additionally, immortalized MIF-/- fibroblasts were resistant to H-ras^{v12} transformation (Liao 2002).

A study into MIF effects on angiogenesis confirmed the involvement of MAPK in MIF signalling (Amin et al. 2003). Specifically, MIF activation of ERK1/2 and phosphatidylinositol 3-kinase human (PI3K) in dermal microvascular endothelial

cells (HMVEC) is essential for MIF-dependent migration, however, Src and p38 are not required (Amin et al. 2003). Furthermore, antagonism of the ERK1/2 pathway with dominant negative mutants of MEK1 and antisense oligonucleotides to ERK1/2 and Elk-1 attenuated MIF-induced activation of downstream elements of the angiogenesis-related signalling cascade (Amin et al. 2003). The same study demonstrated that MIF abrogates human endothelial cell apoptosis via a PI3K/Akt pathway and not via the ERK1/2 pathway.

1.6.8.4 MIF receptor

Recently, the MIF receptor has been discovered to be CD74, the cell surface form of the class II-associated invariant chain (Leng et al. 2003). Upon binding to CD74, MIF was rapidly internalized and activated the ERK1/2 signalling pathway. The evidence confirming CD74 as the MIF receptor is comprehensive. Leng *et al* demonstrated that MIF bound to CD74 by "pull-down" experiments and detecting MIF in CD74 protein immunoprecipitates by Western blotting. MIF and CD74 expression were co-localised by confocal microscopy. Anti-CD74 Ab inhibited MIF induced ERK1/2 activation in CCL210 lung fibroblasts. Additionally, MIF failed to stimulate ERK1/2 and PGE2 in CD74-/- macrophages, but not wt macrophages (Leng et al. 2003).

Due to the known role of ERK1/2 in cellular proliferative responses, MIF activation of ERK1/2 could underpin the pro-proliferative effect of MIF. This hypothesis was the basis for the work carried out in chapter 2.

1.6.8.5 MIF and p53

The role of MIF has recently been expanded with its identification as being the only pro-inflammatory cytokine capable of functionally inactivating the tumor suppressor protein p53 (Hudson et al. 1999). It has been suggested by Mitchell *et al* that the mechanism by which MIF exerts its effect on p53 is via COX2. MIF deficient peritoneal macrophages that were primed *in vivo* with endotoxin were significantly less viable and had increased apoptosis when compared to wt cells, as measured by DNA fragmentation (Mitchell et al. 2002). Treatment with rMIF or overexpression of MIF in RAW 264.7 cells protected them from apoptosis induced by the NO donor, sodium nitroprusside (SNP). Additionally, both exogenous and endogenous MIF suppressed NO-induced p53 accumulation, and this correlated with an increase in COX2 expression (Mitchell et al. 2002). Inhibition of COX2 with indomethacin abolished the protective effects of MIF. The increased apoptosis in MIF-/- macrophages was shown to be p53 dependent, as over-expression of a dominant mutant form of p53, restored normal pro-inflammatory function (Mitchell et al. 2002). Two other studies support the theory that MIF protects cells from oxidative stress induced apoptosis via a p53 mechanism (Hudson et al. 1999; Nguyen et al. 2003).

Functional inactivation of endogenous p53 in RA FLS has been reported to lead to enhanced proliferation, anchorage-independent growth, invasiveness into cartilage extracts and impaired apoptosis (Firestein et al. 1996; Aupperle et al. 1998). Given the overexpression of MIF in RA, its ability to functionally inactivate p53, as well as the importance of p53 in RA, I investigated the influence of MIF on p53 and apoptosis in RA FLS. The results are outlined in chapter 4.

1.6.8.6 MIF and Jab1

The evidence that MIF does not activate the NF-κB pathway indicates that MIF utilizes other signal transduction pathways. It has been suggested that MIF has an intracellular function. Specifically, it has been shown that MIF interacts with an intracellular protein, Jab1 (Kleemann et al. 2000). Jab1 is a co-activator of AP-1 transcription and promotes degradation of the cyclin kinase inhibitor $p27^{kip1}$. By over expressing MIF-EGFP, or treating HeLa cells with FLUOS-MIF, both endogenous and exogenous MIF was shown to localize to the cytosol and bind to Jab1. The specific interaction of MIF and Jab1 resulted in inhibition of Jab1 mediated AP-1 activity in 293T cells, but the MIF/Jab1 interaction did not affect NF-κB as measured by reporter gene activity. In addition, transient overexpression of Jab1 in 293T cells enhanced JNK phosphorylation of glutathione S-transferase (GST)-cJun, while addition of exogenous MIF inhibited JNK activity. Interestingly, neither Jab1 nor MIF affected the level of JNK phosphorylation (Kleemann et al. 2000). The same study demonstrated that MIF increased $p27^{Kip1}$ levels via a Jab1 dependent mechanism. Specifically, MIF induced $p27^{Kip1}$ levels in NIH/3T3 cells in a dose-dependent manner; however, this effect was shown to be Jab1 dependent, as rMIF had no affect on $p27^{Kip1}$ levels in cells transfected with an antisense Jab1 construct (Kleemann et al. 2000). The observations by Kleemann *et al.* do not account for the increased MAPK activation induced by MIF. Bucala *et al.* suggested that the apparent suppressive effects of intracellular MIF could be part of a counter regulatory loop, preventing unopposed activation of AP-1 in the setting of hyper induced MIF expression (Bucala 2000). Intracellular MIF has been shown to protect CHO cells from intracellular, but not extracellular dopamine toxicity; however the mechanism for this protection remain unknown (Weingarten et al. 2001).

1.7 Conclusion

RA is a disease characterised by cellular activation, cytokine production, and an imbalance between proliferation and apoptosis of resident synovial cells. Increased understanding of the role of individual cytokines and signal transduction pathways has led to novel treatments for human RA. There are significant gaps in knowledge in relation to the activation of MAPK in RA by MIF, the effect of MIF on p53 in RA and the role of MAPK in the regulation of RA cell proliferation and apoptosis. The studies presented in this thesis were designed to address these issues.

Chapter Two:

Control of Fibroblast-like synoviocyte proliferation by Macrophage Migration Inhibitory Factor (MIF)

2.1 Chapter Summary

The hyperplasia of fibroblast-like synoviocytes (FLS) is considered essential to the evolution of joint destruction in rheumatoid arthritis (RA), but the mechanisms underlying FLS hyperplasia remain poorly understood. Macrophage migration inhibitory factor (MIF) is a cytokine which has recently been shown to exert pro-inflammatory effects on RA FLS. The mechanisms of activation of FLS by MIF are unknown, and effects of MIF on synovial cell proliferation have not been reported. MAPKs are signal transduction pathways reported to be involved in synovial joint inflammation.

In the studies described in this chapter, human RA FLS were treated with recombinant MIF and proliferation was measured by thymidine incorporation. Specific MAPK inhibitors were employed to antagonise the ERK and p38 pathways in MIF treated RA FLS. MAPK activation was measured by Western blotting. MIF activation of MAPK was antagonised with the use of specific inhibitors to the ERK pathway (PD98059) or the p38 pathway (SB203580). MIF involvement in cytokine induced proliferation was examined with the use of anti-MIF antibody in IL-1β and TNFα stimulated FLS.

FLS proliferation was significantly increased by MIF. MIF induced phosphorylation of ERK and p38 MAPK. ERK pathway antagonism, but not p38 pathway antagonism, prevented the effect of MIF on FLS proliferation. IL-1β and TNFα stimulated FLS proliferation was abrogated by anti-MIF antibody treatment.

These data suggest that although MIF activates the ERK and the p38 pathways, MIF regulates RA synovial hyperplasia by utilizing the ERK pathway. These data suggest an important therapeutic potential for MIF antagonism in RA.

2.2 Introduction

The synovial lesion of rheumatoid arthritis (RA) is characterised by the presence of chronic inflammation and synovial hyperplasia. While cells of bone marrow origin including macrophages and CD4+ T cells are increased in RA synovium, there is no evidence that these cells proliferate within the synovium. By contrast, evidence of *in situ* proliferation of fibroblast-like synoviocytes (FLS) suggests that these cells are locally expanded (Lafyatis et al. 1989a; Qu et al. 1994a). The hyperplasia of FLS is regarded as being of pivotal importance for the development of pannus, the expanded destructive synovial tissue responsible for cartilage and bone erosion in RA (Zvaifler et al. 1994). Pathways controlling the proliferation of FLS are therefore of considerable interest. For example, interleukin IL-1β stimulates the proliferation of FLS *in vitro* (Inoue et al. 2001), and is known to act via signal transduction pathways including nuclear factor κB (NF-κB) and MAPK (Foxwell et al. 1998; Schett et al. 2000).

As reviewed in chapter 1, macrophage migration inhibitory factor (MIF) is increasingly recognised as an important regulatory cytokine in immune and inflammatory responses. MIF is a product of activated macrophages, T-cells and endothelial cells, and upregulates the pro-inflammatory activity of these cells (Calandra et al. 1994; Calandra et al. 1998; Chesney et al. 1999). In RA synovium, MIF is expressed by FLS as well as macrophages, and FLS-derived MIF upregulates monocyte tumor necrosis factor (TNF) release, suggesting MIF

as an upstream member of the network of cytokines operative in RA (Leech et al. 1999). Recent evidence also suggests that MIF directly activates RA FLS expression of phospholipase A2 and cyclooxygenase (COX) 2, and is essential for the upregulation of these molecules by IL-1β (Sampey et al. 2001). Indirect evidence for a role of MIF in synovial proliferation is found in rodent models of RA, in which monoclonal antibody (mAb) MIF antagonism profoundly inhibits disease severity and synovial hypercellularity (Mikulowska et al. 1997b; Leech et al. 1998a; Santos, L.L. et al. 2001). In this chapter, I sought to test the hypothesis that MIF is an important regulator of synovial hyperplasia, by investigating the effects of MIF on FLS proliferation.

Cytokines elicit their effects on cells involved in RA by binding to cellular receptors and activating one or more intracellular signal transduction pathways, especially the NF-κB or MAPK pathways (Firestein et al. 1999). As outlined in section 1.6.6, MIF can influence a number of signal transduction pathways. Signal transduction networks transmit extracellular signals from the cell surface to the nucleus, enabling the cell to mount an appropriate response. MAPK pathways are three-tired signaling modules which transmit extracellular signals. Currently, four distinct mammalian groups of MAPK have been identified; the extracellular-signal regulated kinases (ERK1/2), the c-Jun N-terminal kinases (JNK1/2/3), the p38 protein kinases (p38α/β/γ/δ) and ERK5.

The ability of MIF to activate MAPK pathways is evident in a number of studies. MIF can induce sustained ERK1/2 pathway activation pattern in NIH/3T3 fibroblasts (Mitchell et al. 1999). In the same study it was shown that MIF activation of ERK was dependent on protein kinase A activity, and MIF regulated $cPLA_2$ via a ERK-dependent pathway. MIF increased proliferation of NIH/3T3

cells as measured by thymidine incorporation, which was associated with a sustained pattern of ERK activation (Mitchell et al. 1999). In addition, serum treatment of NIH/3T3 cells results in MIF release and an increase in proliferation, while co-treatment with anti-MIF Ab decreased proliferation and ERK1/2 phosphorylation. By using specific MAPK antagonists, it was demonstrated that MIF utilized the ERK1/2 pathway, but not the p38 pathway to increase $cPLA_2$ activity. More recently, MIF-induced expression of COX2 in FLS has been shown to depend on the p38 MAPK pathway and not the ERK1/2 pathway, in studies using the MAPK antagonists SB203580 and PD98059 (Santos unpublished observations).

MIF is different from IL-1 and TNF in that it does not activate the NF-κB pathway in FLS. A study examining MIF's ability to activate NF-κB reported that MIF does not affect the activity of the inhibitor of NF-κB kinase (IKK), as measured by Western blotting of the inhibitor of NF-κB (IκB) protein (Daun 2000). Similarly, MIF treatment of RA FLS does not activate NF-κB as measured by NF-κB translocation to the nucleus (Lacey, D. et al. 2003). In the same study antagonism of the NF-κB pathway with the specific NF-κB antagonist SN50 did not significantly inhibit MIF activation of RA FLS. MIF antisense transfected cells and control transfected cells had identical NF-κB reporter gene expression after treatment with TNFα (Kleemann et al. 2000). Similarly, co-treatment of RA FLS with IL-1β and anti-MIF mAb failed to inhibit NF-κB nuclear translocation (Lacey, D. et al. 2003). The evidence that MIF does not activate the NF-κB pathway indicates that MIF utilizes other signal transduction pathways. I therefore demonstrated in the following studies the effects of MIF on synoviocyte MAPK activation as a possible mechanism by which MIF induces RA FLS proliferation.

2.3 Methods

2.3.1 Isolation and culture of fibroblast-like synoviocytes

Fibroblast-like synoviocytes (FLS) were obtained from synovium of rheumatoid arthritis (RA) patients undergoing joint replacement surgery. All patients satisfied the American College of Rheumatology criteria for the classification of RA (Arnett et al. 1988). FLS were isolated using enzyme digestion and cultured in RPMI/10% fetal calf serum (FCS, ICN, Melbourne Australia). A single cell suspension was obtained by digesting minced synovial tissue with 2.4 mg/ml dispase (grade II, 5 U/mg; Boehringer Mannheim, Melbourne, Australia), 1 mg/ml collagenase (type II; Sigma, Melbourne, Australia) and DNase (type I; Boehringer Mannheim). FLS were propagated in 10 cm culture plates in RPMI (ICN Biomedicals, Cincinatti OH)/10 % FCS (Trace Biosciences Pty Ltd, Melbourne, Australia) at 37°C in a 5% CO_2 humidified incubator. Cells were used between passages 4 and 9. In each group of experiments, n refers to the number of individual human RA donor FLS used.

2.3.2 Measurement of fibroblast-like synoviocyte proliferation

To determine the effect of MIF on FLS proliferation, DNA synthesis was measured by [^{3}H] thymidine incorporation. FLS were seeded overnight at $1x10^5$ cells per well in 24 well tissue culture plates in RPMI/10% FCS. Cell growth was synchronised by culturing FLS in RPMI/0.1% bovine serum albumin (BSA, Sigma) for 24hr. FLS were stimulated with recombinant human MIF (100 ng/ml),

and treated with specific MAPK inhibitors PD98059 (50μM) or SB203580 (5μM) or vehicle (DMSO) for 72hr. FLS were separately stimulated with IL-1β (0.1ng/ml) or TNFα (1ng/ml), and anti-MIF antibody or isotype control antibody for 72hr, cells were pulsed for the last 18hr with 1μCi/ml [^{3}H]thymidine (Amersham International, Sydney, Australia). Triplicate cultures were used for each determination. FLS were detached using Trypsin-EDTA, harvested using a cell harvester (Packard, CT, USA), and the radioactivity incorporated into DNA determined using a Wallac 1409 liquid scintillation counter (Pharmacia, Turku, Finland). The activation of ERK was antagonised with an inhibitor of MEK2, 50μM PD98059 (Alexis Biochemicals, San Diego, CA). The activation of p38 MAPK was antagonised with 5μM SB203580 (kindly provided by Dr Alison Badger, SmithKline Beecham Pharmaceuticals, King of Prussia, PA).

2.3.3 Analysis of MAPK activation

The phosphorylation of ERK, and p38 was assessed using Western blotting with mAb and polyclonal Ab specific for the phosphorylated (activated) and total MAPK, as described (Mitchell et al. 1999). Cells were seeded at 5×10^5 per well in a 6 well plate and cultured for 24hr in serum free medium (RPMI alone). Cells were treated with MIF for 15, 30, or 60min. Cells were disrupted by repeated aspiration through a 21-gauge needle. After incubation on ice for 10 min and micro centrifugation at 12000 rpm for 15 min (4 °C), the lysates were stored at -80 °C. Protein concentration was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL). Equal amounts (50μg) of cellular proteins were fractionated on 10% SDS-polyacrylamide electrophoresis gels and transferred to

polyvinylidene difluoride membranes (Millipore, Bedford, MA). Western blotting was performed using antibodies directed against phospho-p44/42 (ERK), phospho-p38, total p44/42 (ERK), and total p38 according to the manufacturer's instructions (Cell Signaling Laboratories, Beverly, MA).

2.3.4 Statistical Analysis

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

2.4 Results

2.4.1 Effect of MIF on FLS proliferation

Constitutive proliferation of serum-exposed FLS was readily detected (mean ±sem cpm for FLS with and without MIF). FLS proliferation was significantly increased by MIF (100 ng/ml) ($p < 0.02$) (Figure 2.6.1).

2.4.2 Effect of MIF on ERK MAPK

The involvement of ERK in the activation of 3T3 murine fibroblasts by MIF has been reported (Mitchell et al. 1999), but no study has examined the effect of MIF on the activation of ERK MAPK in human FLS cells. MIF induced the phosphorylation of ERK in RA FLS in a time dependent fashion (Figure 2.6.2).

2.4.3 Effect of MIF on p38 MAPK

MIF induced the phosphorylation of p38 in RA FLS in a time-dependent fashion (Figure 2.6.3).

2.4.4 Effect of MAPK inhibition on MIF-induced FLS proliferation

Antagonism of ERK phosphorylation using the specific inhibitor PD98059 completely inhibited the stimulatory effect of MIF on FLS proliferation ($p<0.05$) (Figure 2.6.4). PD 98059 also inhibited constitutive proliferation of RA FLS, consistent with the role of ERK in the response to serum stimulation (see chapter 5). In contrast, inhibition of p38 MAPK activity using the inhibitor SB203580 had no effect on MIF-induced or basal proliferation (Figure 2.6.4).

2.4.5 Effect of endogenous MIF on cytokine induced proliferation

FLS proliferation was increase significantly by treatment with 0.1ng/ml IL-1β ($p<0.02$) and 1ng/ml TNFα ($p<0.05$). Neutralization of endogenous MIF with anti-MIF Abs resulted in abrogation of cytokine induced proliferation (Figure 2.6.5).

2.5 Discussion

In recent years, the expression of many cytokines has been reported in RA synovium. Partly as a result of the successful application of specific treatment strategies in humans, IL-1β and TNF have been regarded as critical cytokines in the evolution of RA. Both these cytokines contribute to inflammation in the synovial lesion, but effective inhibition of these cytokines still leaves significant inflammation and joint destruction in many patients with RA, suggesting the presence of other critical factors (Joosten et al. 1999; Campbell et al. 2001).

The biological functions of MIF in the immune system include activation of monocyte TNF and innate immunity (Bernhagen et al. 1993; Calandra et al. 1995), and an essential role in T cell activation and models of T cell-directed immunity (Bacher et al. 1996; Bernhagen et al. 1996). This range of functions suggests the involvement of MIF in diseases such as RA, where activation of both innate and adaptive immunity may be involved. Considerable evidence directly supports the hypothesis that MIF is involved in RA. The contribution of MIF to arthritis has been demonstrated in numerous animal models of RA, including rat adjuvant arthritis (Leech et al. 1998a), murine collagen-induced arthritis (Mikulowska et al. 1997b), and murine antigen-induced arthritis (Santos, L.L. et al. 2001). MIF is expressed abundantly in human RA synovial FLS, macrophages, and to a lesser extent T cells, and is overexpressed in RA compared to control tissues and cells (Leech et al. 1999; Onodera et al. 1999). Induction of monocyte cell TNF release by FLS-derived MIF suggests an important role for FLS-derived MIF in RA pathology (Leech et al. 1999).

Mitchell *et al* has demonstrated an increase in $cPLA_2$ activity induced by MIF as measured by arachidonic acid release in NIH/3T3 cells and that MIF utilized the ERK1/2 pathway but not the p38 pathway in eliciting this response (Mitchell et al. 1999). MIF has been shown to induce RA FLS $cPLA_2$ and cyclooxygenase- 2 (COX2) expression as measured by arachidonic acid release, prostaglandin E2 ELISA, and RT-PCR (Sampey et al. 2001). MIF has been demonstrated to be essential for the activating effects of IL-1β on FLS, in that inhibition of MIF using a mAb prevented IL-1β activation of FLS $cPLA_2$ and COX2 (Sampey et al. 2001). MIF has also recently been reported to induce FLS matrix metalloproteinase activity (Onodera et al. 2000). Onodera *et al* demonstrated that the upregulation of MMP-1 and MMP-3 were preceded by upregulation of *c-jun* and *c-fos* mRNA (Onodera et al. 2000). In a separate study it was shown that MIF upregulation of MMP-9 and MMP13 mRNA in rat osteoblasts, was also preceded by upregulation of *c-jun* and *c-fos* mRNA (Onodera et al. 2002). Antagonism of the ERK MAPK pathway by PD98059 suppressed MIF induced MMP13, c-jun and c-fos mRNA expression (Onodera et al. 2002). Interestingly, MIF stimulated phosphorylation of Ras and ERK1/2, but not p38, JNK or c-Jun in rat osteoblasts.

Activation of inflammation is of considerable importance in RA, but the progressive destruction of articular cartilage is reliant on the evolution of hyperplastic and invasive synovial tissue. Hyperplasia of FLS may be dependent upon a combination of dysregulated proliferation and apoptosis (Qu et al. 1994a; Zvaifler et al. 1994; Firestein 1996). Factors considered important for the induction of proliferation include pro-inflammatory cytokines such as IL-1β, acting at least in part through the NF-κB and MAPK signal transduction pathways (Morita et al. 1998; Aupperle et al. 1999; Schett et al. 2000; Inoue et al. 2001).

MIF has been reported to be involved in the control of cell proliferation in contexts other than RA, including tumours and cell lines (Takahashi et al. 1998; Chesney et al. 1999; Mitchell et al. 1999). Moreover, MIF has been identified as capable of inducing phosphorylation of ERK MAPK in murine fibroblasts, and of overriding p53-dependent cell cycle control (Hudson et al. 1999; Mitchell et al. 1999). MIF induces proliferation of NIH/3T3 cells as measured by thymidine incorporation, associated with a sustained pattern of ERK activation (Mitchell et al. 1999). In addition, serum treatment of NIH/3T3 cells results in MIF release and an increase in proliferation, while co-treatment with anti-MIF Ab decreases proliferation and ERK1/2 phosphorylation (Mitchell et al. 1999). Investigation into adhesion-dependent signalling by MIF has found that MIF secretion is induced by cell adhesion to fibronectin, and this results in integrin-dependent activation of ERK1/2 which can be reduced with anti-MIF Abs (Liao 2002). In a separate study, MIF activation of ERK1/2 and phosphatidylinositol 3-kinase human (PI3K) in dermal microvascular endothelial cells (HMVEC) is essential for MIF-dependent migration, however, Src and p38 are not required (Amin et al. 2003). Furthermore, antagonism of the ERK1/2 pathway with dominant negative mutants of MEK1 and antisense oligonucleotides to ERK1/2 and Elk-1 attenuated MIF-induced activation of downstream elements of the angiogenesis-related signalling cascade (Amin et al. 2003). The same study demonstrated that MIF inhibits human endothelial cell apoptosis via a PI3K/Akt pathway and not via the ERK1/2 pathway.

The data presented in this chapter demonstrates the ability of MIF to stimulate the proliferation of cultured FLS derived from human RA tissues. The concentration range of MIF that induced proliferation is similar to that which induces FLS

$cPLA_2$ and COX2 expression (Sampey et al. 2001). Moreover, the ability of IL-1β and TNFα to induce FLS proliferation was shown to be dependent on MIF, in that neutralisation of MIF with a mAb prevented activation of FLS proliferation by these stimuli. These data suggest that, in addition to the important effects of MIF on T cell and monocyte activation, and induction of inflammatory eicosanoid synthesis by FLS, MIF is involved in the control of FLS proliferation. Reduced synovial cellularity was a feature of synovial tissue from rats and mice receiving anti-MIF mAb during the evolution of arthritis, although factors other than proliferation may have contributed to this effect (Leech et al. 1998a; Santos, L.L. et al. 2001). Given that FLS expansion is necessary for the generation of pannus, and given evidence of the ability of MIF to induce FLS metalloproteinase expression (Onodera et al. 2000), these results suggest a critical contribution of MIF to the pathology of RA joint damage. The profound inhibitory effect of anti-MIF mAb on IL-1β- and TNFα-induced proliferation also suggests an important role of MIF. In addition to directly regulating genes involved in the synovial inflammatory response, MIF appears to provide an essential enabling or permissive function in synoviocyte activation.

The mechanisms of action of MIF have not been fully elucidated. Activation of synovial cells has variously been reported to be dependent upon NF-κB and/or MAPK pathways (Taki et al. 1998; Aupperle et al. 1999; Badger et al. 2000; Barchowsky et al. 2000; Schett et al. 2000; Han, C.W. et al. 2001; Inoue et al. 2001). MIF differs from other cytokines in that it does not activate the NF-κB pathway. The evidence that MIF does not activate the NF-κB pathway suggests that MIF utilizes other signal transduction pathways. In this chapter I describe the ability of MIF to activate RA FLS ERK1/2 and p38 MAPK (Figures 2.6.2-3), and

show that induction of FLS proliferation by MIF is dependent on ERK1/2 MAPK but not p38 (Figures 2.6.4).* Constitutive expression of activated ERK1/2 MAPK has been reported in RA tissues and cultured RA FLS, and the upregulation of ERK phosphorylation by other cytokines has been identified (Schett et al. 2000). The current finding that ERK pathway inhibition inhibited constitutive (serum-induced) RA FLS proliferation is consistent with these observations and will be explored in more detail in chapter 5.

Interpretation of the effects of ERK pathway inhibition on MIF-induced cell activation is difficult given the effects on constitutive proliferation. It is clear, however, that MIF-induced proliferation requires the activation of ERK but not p38 to proceed. The p38 pathway is well known for its involvement in regulating pro-inflammatory gene expression and cellular response to pro-inflammatory molecules. There is little evidence that the p38 pathway regulates proliferation and it is therefore not surprising that inhibition of the p38 pathway failed to inhibit RA FLS proliferation. In studies in the candidate's laboratory, MIF has been shown to utilize the p38 pathway and not the ERK pathway to induce RA FLS COX2 expression (Santos, unpublished observations). MAPK including ERK can influence gene expression without influencing NF-κB nuclear translocation and DNA binding (Vanden Berghe et al. 1998). My data suggest such a pathway is operative in MIF activation of FLS proliferation. The influence of MAPK in the activation of RA FLS will be further discussed in chapter 5.

In conclusion, I have shown that MIF has significant effects on FLS proliferation *in vitro*, through activation of ERK MAPK. These data suggest that MIF may be a

* At the time this investigation was undertaken the specific JNK inhibitor SP600125 was not available. I subsequently obtained SP600125 which I utilized in work detailed in chapter 5, however, at that time I no longer had access to bio-active MIF

significant contributor to synovial hyperplasia in RA. Taken together with the effects of MIF on TNF and eicosanoid production, these data suggest that targeting MIF in the treatment of RA could have significant anti-inflammatory and disease-modifying effects.

The relative contribution of proliferation and apoptosis to the hyperplasia of FLS in the RA synovial lesion are somewhat controversial, with several scientists proposing that dysregulated apoptosis rather than proliferation is responsible. In subsequent chapters I present data examining the potential for MIF to influence synovial hyperplasia via inhibitory effects on apoptosis.

2.6 Figures

Figure 2.6.1

Effects of MIF on FLS proliferation

RA FLS were treated with MIF (100 ng/ml) for 72hr and labelled with 1μCi/ml [^{3}H]thymidine for the last 18hr. FLS proliferation was determined from triplicate cultures and expressed as percentage of baseline proliferation Mean ± SEM of 4 separate experiments. *$p< 0.05$.

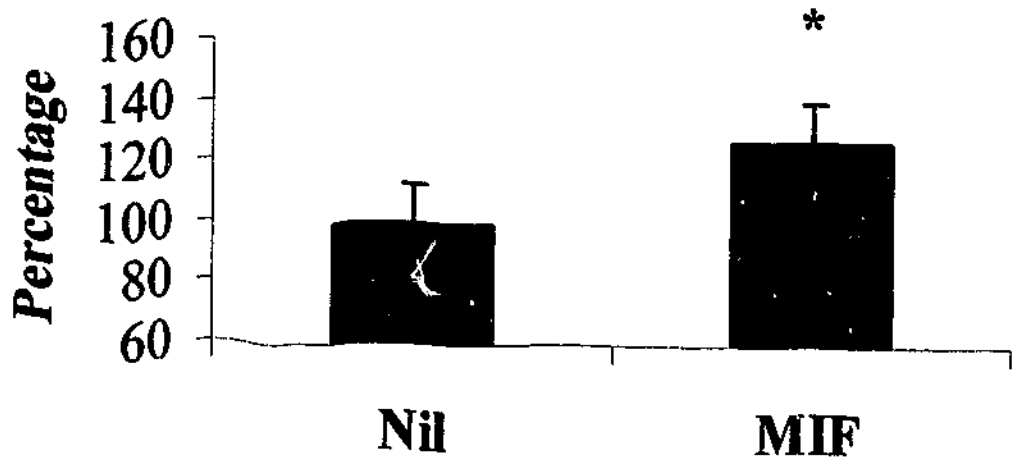

Percentage
160
140
120
100
80
60
*
Nil
MIF

Figure 2.6.2

Induction of ERK MAPK phosphorylation by MIF in RA FLS

RA FLS were rendered quiescent by overnight culture in serum free medium. Cells were stimulated for up to 30min with recombinant human MIF (100ng/ml). Lysates were examined for MAPK activation by Western blotting with a phospho-specific ERK MAPK monoclonal antibody. Total ERK specific antibody was used to verify equal protein loading. Results are 1 representative example of 4 independent experiments, with cells from 4 different RA tissue donors. MIF induced phosphorylation of ERK MAPK in FLS.

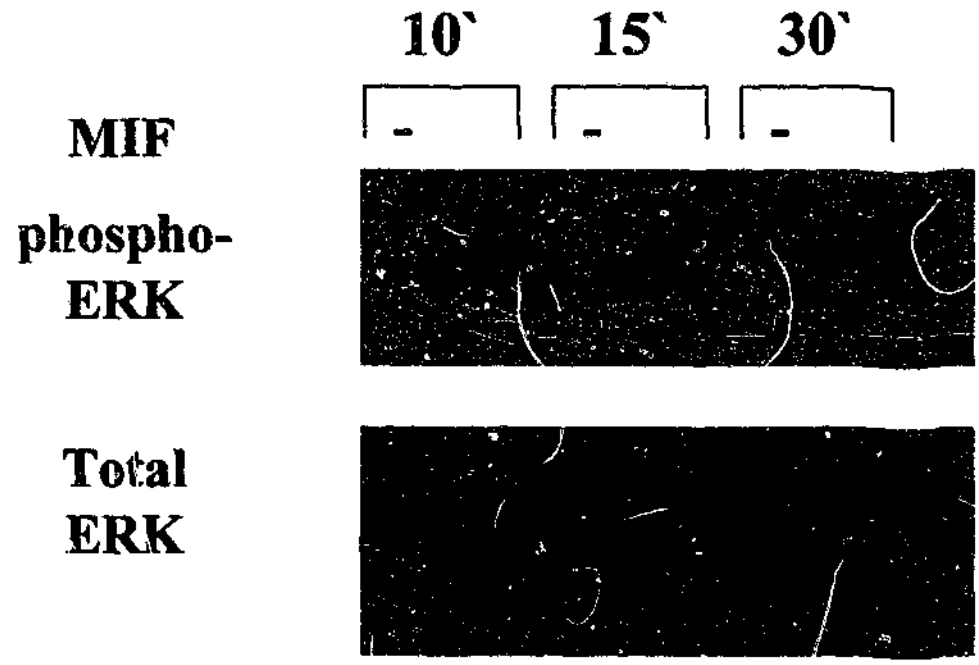

10`
15`
30`
-
-
-
MIF
phospho-
ERK
Total
ERK

Figure 2.6.3

Induction of p38 MAPK phosphorylation by MIF in RA FLS

RA FLS were rendered quiescent by overnight culture in serum free medium. Cells were stimulated for up to 30min with recombinant human MIF (100ng/ml). Lysates were examined for MAPK activation by Western blotting with a phospho-specific p38 MAPK monoclonal antibody. Total p38 specific antibody was used to verify equal protein loading. Results are 1 representative example of 4 independent experiments, with cells from 4 different RA tissue donors. MIF induced phosphorylation of p38 MAPK in FLS.

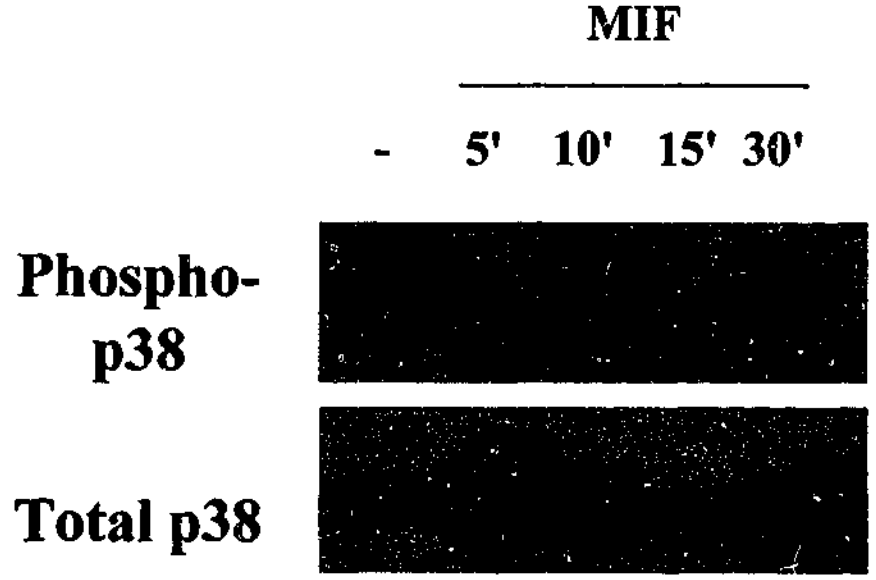

MIF
-
5'
10'
15'
30'
Phospho-p38
Total p38

Figure 2.6.4

Effects of MAPK inhibition on MIF-induced FLS proliferation

RA FLS were treated with MIF (100ng/ml) with or without the addition of the ERK MAPK inhibitor PD98059 (50μM) or the p38 MAPK inhibitor SB203580 (5μM) for 72hr, and labelled with 1μCi/ml [^{3}H] thymidine for the last 18hr. FLS proliferation was determined from triplicate cultures and expressed as percentage of baseline proliferation Inhibition of ERK substantially inhibited FLS proliferation, whereas inhibition of p38 MAP kinase had no significant effect. Mean ± SEM of 4 separate experiments (*$p< 0.01$, **$p<0.05$).

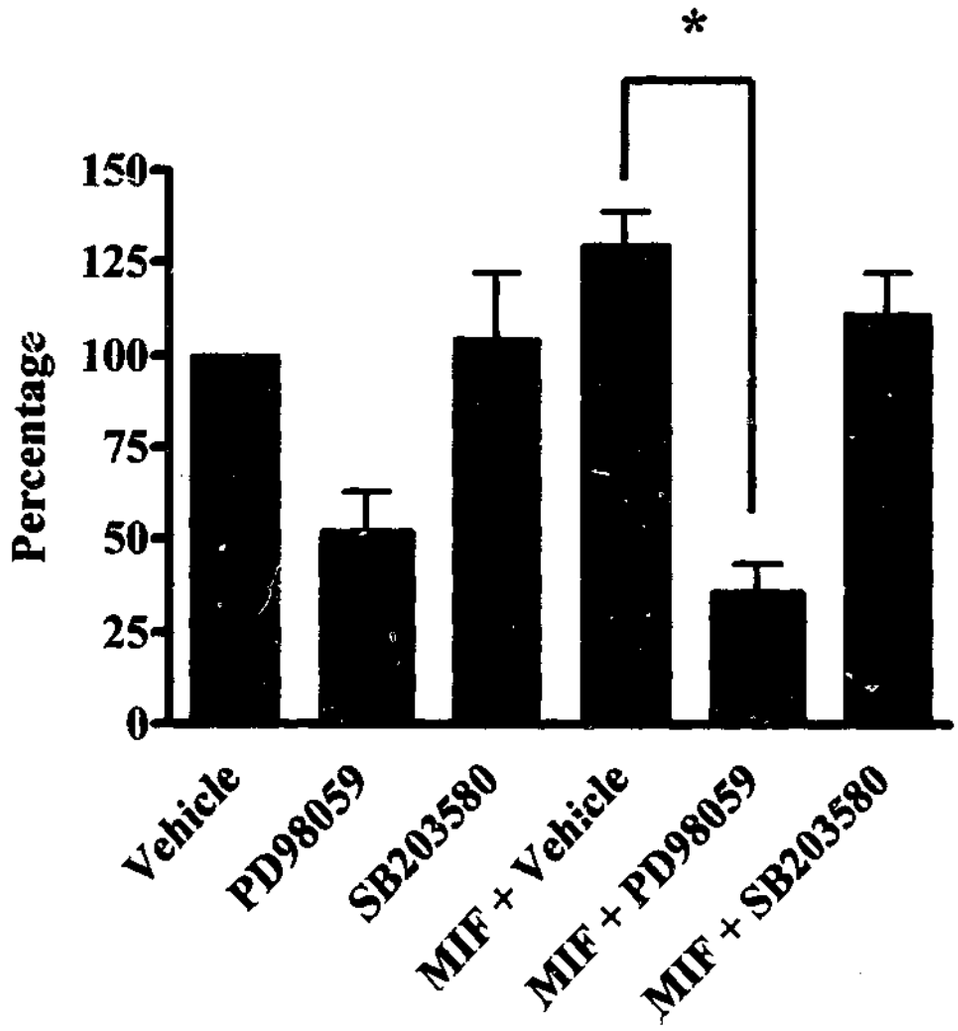
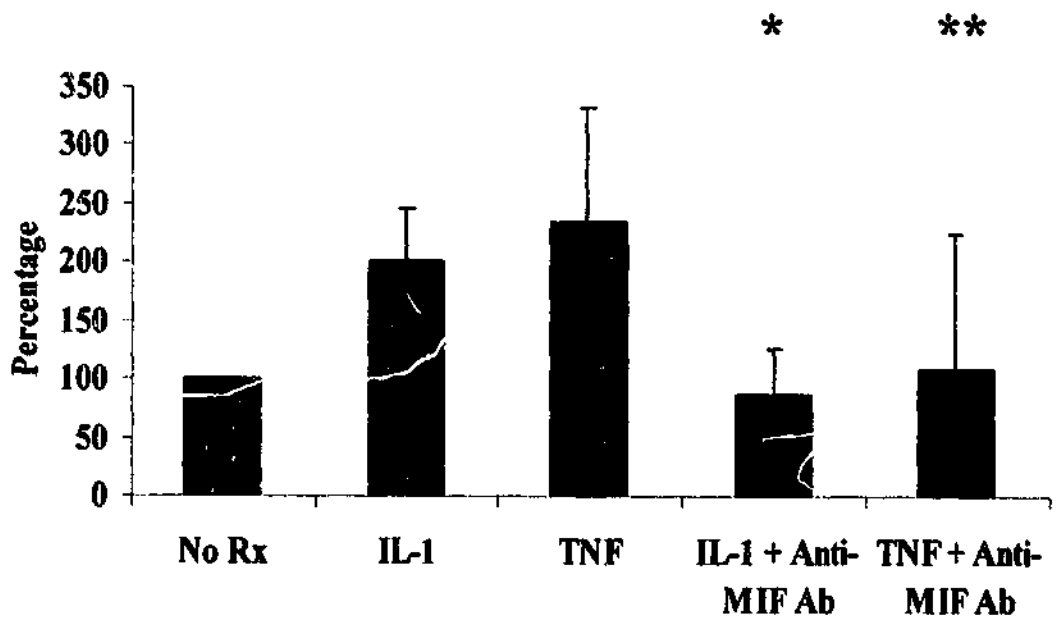

*
150
125
100
75
50
25
0
Percentage
Vehicle
PD98059
SB203580
MIF + Vehicle
MIF + PD98059
MIF + SB203580

Figure 2.6.5

Effect of MIF mAb on cytokine stimulated FLS proliferation

FLS were treated with IL-1β (0.1ng/ml), TNF (1ng/ml), and with anti-MIF mAb (200μg/ml. FLS proliferation was determined from triplicate cultures and expressed as [^{3}H] thymidine incorporation (cpm). Values are the mean ± SEM of 4 separate experiments. IL-1β and TNFα significantly increased FLS proliferation (*$p<0.02$, **$p < 0.05$). In contrast, in the presence of anti-MIF mAb, the effect of IL-1β and TNF on FLS proliferation was abrogated.


*
**
350
300
250
200
150
100
50
0
Percentage
No Rx
IL-1
TNF
IL-1 + Anti-MIF Ab
TNF + Anti-MIF Ab

Chapter Three:

Role of endogenous MIF in dermal fibroblast activation

3.1 Chapter Summary

This chapter examines the role of endogenous MIF in regulating fibroblast activation. It has been shown that exogenous MIF can induce activation of MAPK. Given the role of ERK MAPK in cellular proliferative responses, the activation of ERK by MIF may underpin the pro-proliferative effect of MIF.

Given the requirement of MIF for cytokine induced proliferation as shown in chapter 2, I hypothesised that endogenous MIF is required for complete cellular activation by IL-1β and TNFα. MIF involvement in IL-1β- and TNFα-induced activation was investigated with the use of MIF-/- and wild type (wt) cells. Dermal fibroblast proliferation and MAPK activation in MIF-/- and wt cells was measured and compared following cytokine stimulation.

Dermal fibroblast proliferation was measured by [3H]thymidine incorporation. MAPK were measured by Western blotting.

MIF-/- dermal fibroblast demonstrated lower levels of proliferation, and MAPK expression when compared with wt dermal fibroblasts. Similar to the result shown in chapter 2, MIF-/- dermal fibroblasts were hypo-responsive to IL-1β and TNFα, with lower levels of proliferation and MAPK activation observed.

These results demonstrate that endogenous MIF is involved in the activation of all 3 MAPK by cytokines, and confirms the observation MIF plays an important role in fibroblast proliferation.

3.2 Introduction

Many cytokines that are operative in RA act through both the NFκB and MAPK intracellular signalling pathways (Taki et al. 1998; Firestein et al. 1999; Barchowsky et al. 2000). Current evidence suggests that MIF differs from other cytokines, as evidence of direct activation of the NFκB is lacking (Daun 2000; Kleemann et al. 2000; Lacey, D. et al. 2003). MIF does not directly affect the activation of the inhibitor of NF-κB kinase (IKK) as measured by Western blotting of the inhibitor of NF-κB (IκB) protein (Daun 2000). Similarly, MIF treatment of RA FLS does not activate NF-κB as measured by NF-κB translocation to the nucleus (Lacey, D. et al. 2003). In the same study antagonism of the NF-κB pathway with the specific NF-κB antagonist SN50 did not significantly inhibit MIF activation of RA FLS. MIF antisense transfected cells and control transfected cells had identical NF-κB reporter gene expression after treatment with TNF (Kleemann et al. 2000). Similarly, co-treatment of RA FLS with IL-1β and anti-MIF mAb failed to inhibit NF-κB nuclear translocation (Lacey, D. et al. 2003). The observation of reduced NFκB activation in MIF-/- cells in response to LPS reported by Roger *et al.* reflects the reduced TLR4 expression in these cells, and does not constitute evidence that MIF activates NFκB (Roger et al. 2001). The evidence that MIF does not activate the NF-κB pathway indicates that MIF utilizes other signal transduction pathways, such as the MAPK pathways.

MIF has been shown to induce uniquely sustained activation of ERK1/2 MAPK (Mitchell et al. 1999), which has been suggested to underpin the pro-proliferative effect of MIF, given ERK involvement in cell proliferation. Evidence presented in chapter 2 of this thesis further supports MIF involvement in proliferation and

activation of ERK1/2. MIF has been shown to increase MMP13 mRNA levels in rat osteoblasts via the Src-related tyrosine kinase- Ras-, ERK1/2-, and AP-1 dependent pathways. However, MIF failed to activate JNK or p38 in rat osteoblasts (Onodera et al. 2001). Similarly, pharmacological inhibitors of MIF tautomerase activity have been shown to inhibit ERK1/2 activity (Dios et al. 2002). Recombinant MIF has also been shown to activate ERK1/2 in neonatal rat cardiomyocytes, while anti-MIF suppresses ERK1/2 activation by oxidative stress (Fukuzawa et al. 2002).

MIF-/- mice develop normally in relation to size, behaviour and fertility (Bozza et al. 1999a). Macrophages derived from MIF-/- mice have diminished TNFα production and surprisingly exhibited increased production of nitric oxide (NO), after stimulation with LPS and IFNγ (Bozza et al. 1999a). MIF knockout mice show resistance to septic shock lethality and reduced production of TNFα, IL-1β and IL-6 upon challenge with endotoxin (Bozza et al. 1999a). More recently, investigations into MIF-/- mice have discovered that MIF plays an important role in the control of the expression of Toll-like receptor 4 (TLR-4) (Roger et al. 2001). MIF-deficient macrophages were shown to have reduced cell surface expression, which was due to impaired PU.1 transcription activity, which is known to regulate *Tlr4* gene expression. In chapter 2, I describe the findings that MIF induces phosphorylation of ERK MAPK, and that this is associated with the regulation of proliferation in RA FLS. MAPK activation and cell proliferation have not been previously studied in MIF-/- cells.

Recent studies in other cell types have suggested that MAPK may be involved in the control of proliferation and apoptosis, but their contribution to the regulation of MIF mediated proliferation is not well characterised. In this chapter, I

demonstrate that endogenous MIF is required for IL-1β- and TNFα-induced proliferation of mouse dermal fibroblasts. Endogenous MIF is also required for maximal activation of MAPK by IL-1β and TNFα in mouse dermal fibroblasts. Together with the demonstration of the activation of MAPK by MIF in human synovial fibroblasts in chapter 2, these data support a crucial role for MIF in MAPK-dependent activation of cell proliferation.

3.3 Methods

3.3.1 Animals.

MIF-/- mice (Bozza et al. 1999a) (generously donated by Dr John R David, Harvard, Cambridge MA) were crossed for two generations with wt C57/Bl6 mice, generating MIF-/- and wt. These mice had 75% C57 background and 25% 129SV background. Experiments were performed on 8-10 week old female mice. Mice were housed in conventional animal housing facilities.

3.3.2 Isolation and culture of fibroblasts

Mouse dermal fibroblasts (MDF) were obtained from either MIF-/- or wt mice. Dermal fibroblasts were isolated by dissection of the dermal layer. A single cell suspension was obtained by digesting minced dermal tissue with 2.4mg/ml Dispase (grade II, 5 U/mg; Boehringer Mannheim, Melbourne, Australia), 1mg/ml collagenase (type II; Sigma, Melbourne, Australia) and DNase (type I; Boehringer Mannheim). MDF were propagated at a cell density of $1\text{-}5\times10^6$ cells per 10cm culture plates in RPMI (ICN Biomedicals, Cincinatti OH)/10 % FCS (ICN Pty Ltd, Melbourne, Australia) at 37°C in a 5% CO_2 humidified incubator. Cells were

used between passages 4 and 15. Comparisons between wt and MIF-/- cells were made using cells of the same passage.

3.3.3 Measurement of dermal fibroblast proliferation

To determine the effect of endogenous MIF production on proliferation, dermal fibroblast proliferation was measured. Dermal fibroblasts were seeded overnight at $1x10^5$ cells per well in 24 well tissue culture plates in RPMI/10% FCS. Cells were treated in RPMI/0.1 % bovine serum albumin (BSA, Sigma) and stimulated with 0.1ng/ml IL-1β or 1ng/ml TNFα for 48hr. Cells were pulsed for the last 18hr with 1μCi/ml [^{3}H]thymidine (Amersham International, Sydney, Australia). Quadruplicate cultures were used for each determination. Fibroblasts were detached using Trypsin-EDTA, harvested using a cell harvester, and the radioactivity incorporated into DNA determined using a Wallac 1409 liquid scintillation counter (Pharmacia, Turku, Finland).

3.3.4 Analysis of MAPK activation

The phosphorylation of ERK, p38 and JNK was assessed using Western blotting with mAbs and polyclonal Abs specific for the phosphorylated (activated) and total ERK, as described in section 2.3.3. Cells were seeded at 5×10^5 per well in a 6 well plate and starved for 24hr serum free (RPMI alone). Cells were treated with either 0.1ng/ml IL-1β, or 1ng/ml TNFα for 30min. Cells were disrupted by repeated aspiration through a 21-gauge needle. After incubation on ice for 10 min and micro centrifugation at 12000 rpm for 15 min (4 °C), the lysates were stored at -80 °C. Protein concentration was determined using a BCA protein assay

reagent kit (Pierce, Rockford, IL). Equal amounts (50μg) of cellular proteins were fractionated on 10% SDS-polyacrylamide electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Western blotting was performed using antibodies directed against phospho-p44/42 (ERK), phospho-p38, phospho-JNK, total p44/42 (ERK), total p38 and total JNK according to the manufacturers instructions (Cell Signaling Laboratories, Beverly, MA).

3.3.5 Statistical Analysis

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

3.4 Results

3.4.1 Effect of endogenous MIF on proliferation

The role of endogenous MIF in the regulation of proliferation was measured by measuring the level of proliferation in MIF-/- and wt dermal fibroblasts cultured in serum. MIF-/ dermal fibroblasts show a significant reduction in constitutive proliferation when compared to wt dermal fibroblasts, as measured by [^{3}H]thymidine incorporation (Figure 3.6.1) ($p < 0.05$). IL-1β and TNFα induced an increase in proliferation in wt dermal fibroblasts however neither cytokines had any effect on MIF-/- cells (Figure 3.6.3) ($p < 0.05$).

3.4.2 Effect of endogenous MIF on MAPK activation

Weak constitutive activation of ERK1/2, p38 and JNK1/2 MAPK was observed in wt cells. The level of constitutively activated ERK, p38 and JNK MAPK was reduced in MIF-/- dermal fibroblasts in comparison to wt dermal fibroblasts.

Stimulation with IL-1β induced strong and sustained activated of ERK1/2 in a time dependent manner in wt dermal fibroblasts. In contrast, reduced and transient activation of ERK1/2 was observed in MIF-/- dermal fibroblasts. Strong and transient activation of p38 and JNK1/2 was observed in wt dermal fibroblasts in response to IL-1β. Reduced p38 activation was observed in MIF-/- dermal fibroblasts, while JNK activation appeared to be delayed and reduced in response to IL-1β. (Figure 3.6.4)

Stimulation with TNFα activated ERK and JNK in wt dermal fibroblast in a time dependent manner. In contrast, very weak activation of ERK1/2 and JNK1/2 was observed in MIF-/- dermal fibroblasts. TNFα activated p38 in a time dependent manner in wt dermal fibroblasts, however delayed p38 activation was observed in MIF-/- fibroblasts (Figure 3.6.5).

3.5 Discussion

The importance of cytokines in the pathogenesis of RA has been highlighted by the dramatic increase in research into their role in RA. The development of efficacious treatments which target cytokines such TNF and IL-1 provide further evidence of their importance. Even with these new therapies, some patients remain unresponsive or incompletely responsive, which suggests that other cytokines are important in this disease (Firestein et al. 2002). These findings also indicate that the pathogenic mechanisms in RA are complex. Although there are numerous cytokines which influence pathogenesis in RA, the focus of my work has been MIF.

MIF, as previously discussed in chapter 1, is believed to be the first cytokine activity discovered. MIF potentially contributes to the pathogenesis of RA on a number of levels. On a cellular level, MIF has been localized to macrophages, endothelial cells, FLS, and to a lesser extent T cells in human RA synovial tissue (Calandra et al. 1994; Bacher et al. 1996; Bernhagen et al. 1996; Leech et al. 1999). The finding that MIF is present in synovial microvascular endothelial cells is consistent with its role in endothelial cell activation and angiogenesis (Chesney et al. 1999; Ogawa et al. 2000).

MIF is overexpressed in serum, synovial fluid, and cultured FLS from RA patients. The pro-inflammatory function of MIF has been directly evaluated in RA synoviocyte-conditioned medium (Leech et al. 1999). RA synoviocyte-conditioned medium stimulated monocytes to release TNF and this was abrogated with anti-MIF Ab treatment. It has also been shown that MIF stimulates macrophages to release TNF, IL-1, IL-6 and IL-8 (Calandra et al. 1995; Donnelly et al. 1997). Additionally, MIF has been reported to upregulate IL-1β mRNA in

FLS (Onodera et al. 2000). These results suggest that MIF can act as an upstream regulator of synovial cytokine expression in RA, particularly with regards to IL-1 and TNF. In addition to inflammation, RA is characterized by hypercellularity, potentially contributed to by dysregulated proliferation and apoptosis.

The result presented in this chapter demonstrates a role for endogenous MIF in the regulation of proliferation. MIF-/- mice develop normally in relation to size, behaviour and fertility, suggesting ontologic adaptation to the targeted disruption of this gene. The recently reported dramatic reduction in outgrowth of explant synovial cells in MIF-/- mice compared with wild-type explants does indicate however that endogenous MIF is a crucial facilitator of cell growth in the potentially challenging circumstance of ex vivo culture (Leech et al. 2003). As demonstrated in chapter 2, rMIF increases human RA FLS proliferation, this identifies its potential contribution to excessive synovial growth in human arthritis. Moreover, MIF expression has been described in a number of human cancers and animal models of neoplasia (Meyersiegler et al. 1996; Nishihira et al. 1996; Tampanaru-Sarmesiu et al. 1997). Transfection of murine colon carcinoma cells with an antisense MIF plasmid was associated with significant decreases in cell proliferation (Takahashi et al. 1998).

Endogenous MIF plays an important role in fibroblast proliferation. MIF-/- mouse dermal fibroblasts and synovial fibroblasts exhibited dramatically and statistically significantly reduced levels of proliferation. Other factors considered important in the induction of FLS proliferation include pro-inflammatory cytokines such as interleukin (IL)-1) (Aupperle et al. 1999; Schett et al. 2000; Inoue et al. 2001; Morita et al. 2001). As shown in chapter 2, the pro-proliferative effects of IL-1 and TNF on RA FLS can be abrogated by immuno-neutralization of MIF with

anti-MIF mAb. In this chapter, I show that endogenous MIF plays an important role in fibroblast activation in response to cytokine stimulation. MIF-/- mouse dermal fibroblasts were hyporesponsive to IL-1 and TNF stimulation, indicating that MIF amplifies cellular activation by these two important cytokines.

MIF co-localizes with Jab1 in the cytosol, and both endogenous and exogenously added MIF bind Jab1 following endocytosis (Kleemann et al. 2000). The intracellular binding of MIF to Jab1 is possibly a key molecular basis in cytokine signaling. Moreover, MIF-/- mice have been shown to have reduced cell surface expression of TLR-4, which was due to impaired PU.1 transcription activity, known to regulate *Tlr4* gene expression (Roger et al. 2001). The reduction in TLR-4 expression explains why MIF-/- macrophages have a reduction in the activity of NF-κB and the production of TNFα in response to LPS (Roger et al. 2001). The current finding, that MIF deficient cells display an altered MAPK activation pattern in response to IL-1 and TNF compared to wild type cells, indicates that MIF is necessary for signal transduction in response to cytokines which do not signal via TLR4. This is consistent with a broader regulatory role of MIF.

Experiments in MIF deficient mice have previously demonstrated a reduced capacity to proliferate in response to specific stimuli. Specifically, in a mouse model of wound healing, the contact hypersensitivity response was significantly reduced in MIF-/- mice compared to wt controls (Shimizu et al. 2003). Additionally, in a mixed lymphocyte reaction, the T cell proliferative response to alloantigen was significantly decreased in the MIF-/- mice compared with wt mice (Shimizu et al. 2003). This observation may relate to changes in proliferation, or in other aspects of the adaptive immune response influenced by MIF. A separate

study investigating MIF-/- fibroblasts found minor deviations in growth characteristic between MIF-/- and wt cells (Petrenko et al. 2003). Interestingly, the same study found growth retardation in the immortalized MIF-/- cultures, correlated with their reduced susceptibility to Ras-mediated transformation. Additionally, the resistance of MIF-/- cells to oncogenic transformation could be reversed by concomitant interference with p53- and E2F-response transcriptional control. These results indicate that MIF plays an important role in maintaining normal cellular proliferation and interacts with, or regulates p53. MIF involvement with p53 will be discussed in chapter 4.

In conclusion, MIF-/- fibroblasts exhibit reduced cytokine-induced MAPK activation and reduced constitutive and cytokine-induced proliferation. These data support a significant and essential amplifying role for MIF in cell activation by inflammatory stimuli. Further studies of differences in signal transduction events in MIF-/- cells are being carried by other scientists in the candidate's laboratory.

3.6 Figures

Figure 3.6.1

MIF-/- and wt dermal fibroblast proliferation

Basal proliferation of MIF -/- and wt dermal fibroblasts cultured in 0.1% BSA/RPMI for 48hr was measured. Proliferation was determined from quadruplicate cultures and expressed as [3H] thymidine incorporation (cpm). Values are the mean SEM of 4 experiments (* $p<0.01$).

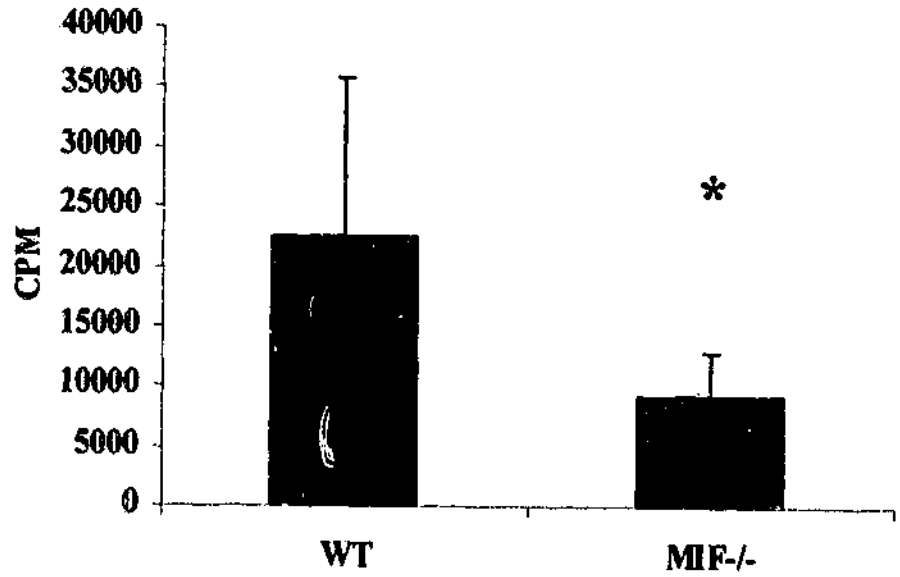

40000
35000
30000
25000
20000
15000
10000
5000
0
CPM
*
WT
MIF-/-

Figure 3.6.2

Proliferation of MIF-/- and wt dermal fibroblasts

Dermal fibroblasts were cultured in 0.1% BSA/RPMI, treated with 0.1ng/ml IL-β or 1ng/ml TNFα for 48hr, and proliferation determined from quadruplicate cultures labelled with 1μCi/ml [^{3}H]thymidine for the last 18hr. Results are expressed as mean ± SEM [^{3}H]thymidine incorporation (cpm). MIF-/- dermal fibroblasts showed a hyporesponsiveness to both IL-1β and TNFα (* $p<0.05$).

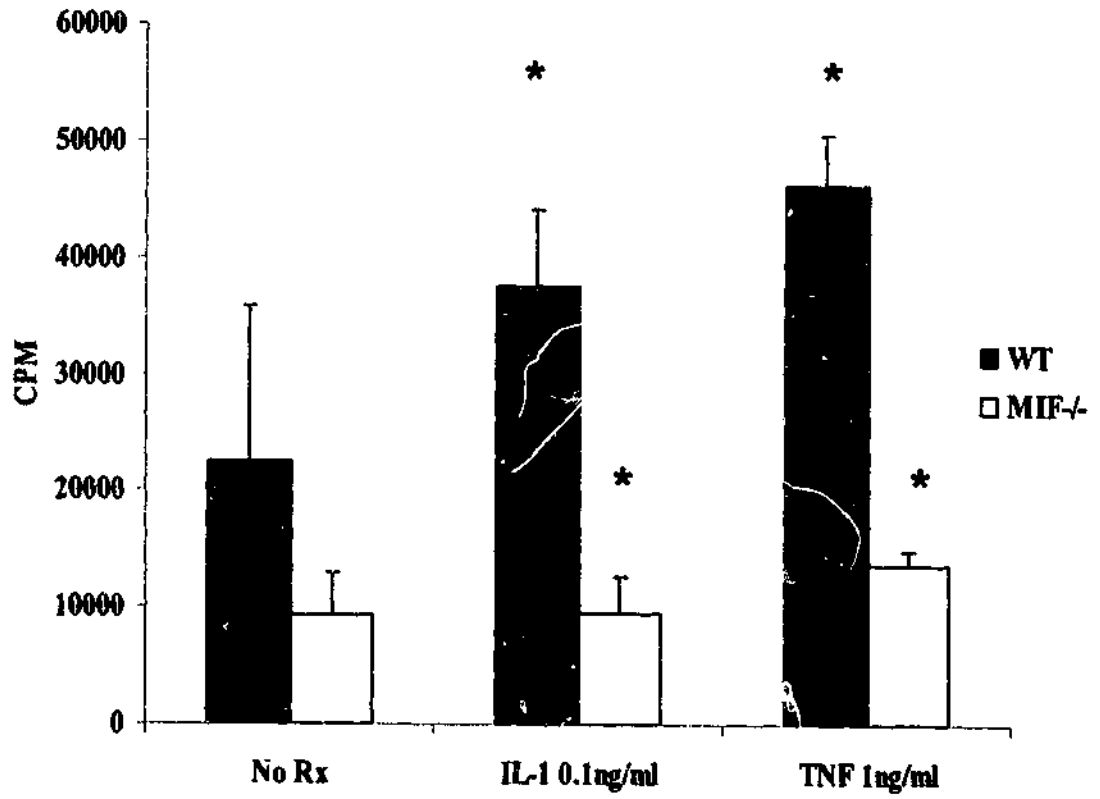

60000
50000
40000
30000
20000
10000
0
CPM
*
*
*
*
WT
MIF-/-
No Rx
IL-1 0.1ng/ml
TNF 1ng/ml

Figure 3.6.3

IL-1β activation of MIF-/- and wt dermal fibroblasts MAPK

Mouse dermal fibroblasts were stimulated with 0.1ng/ml IL-1β and the level of phosphorylated JNK1/2, ERK1/2, p38 and total ERK, were measured by Western blot analysis. Results are representative of 4 independent experiments.

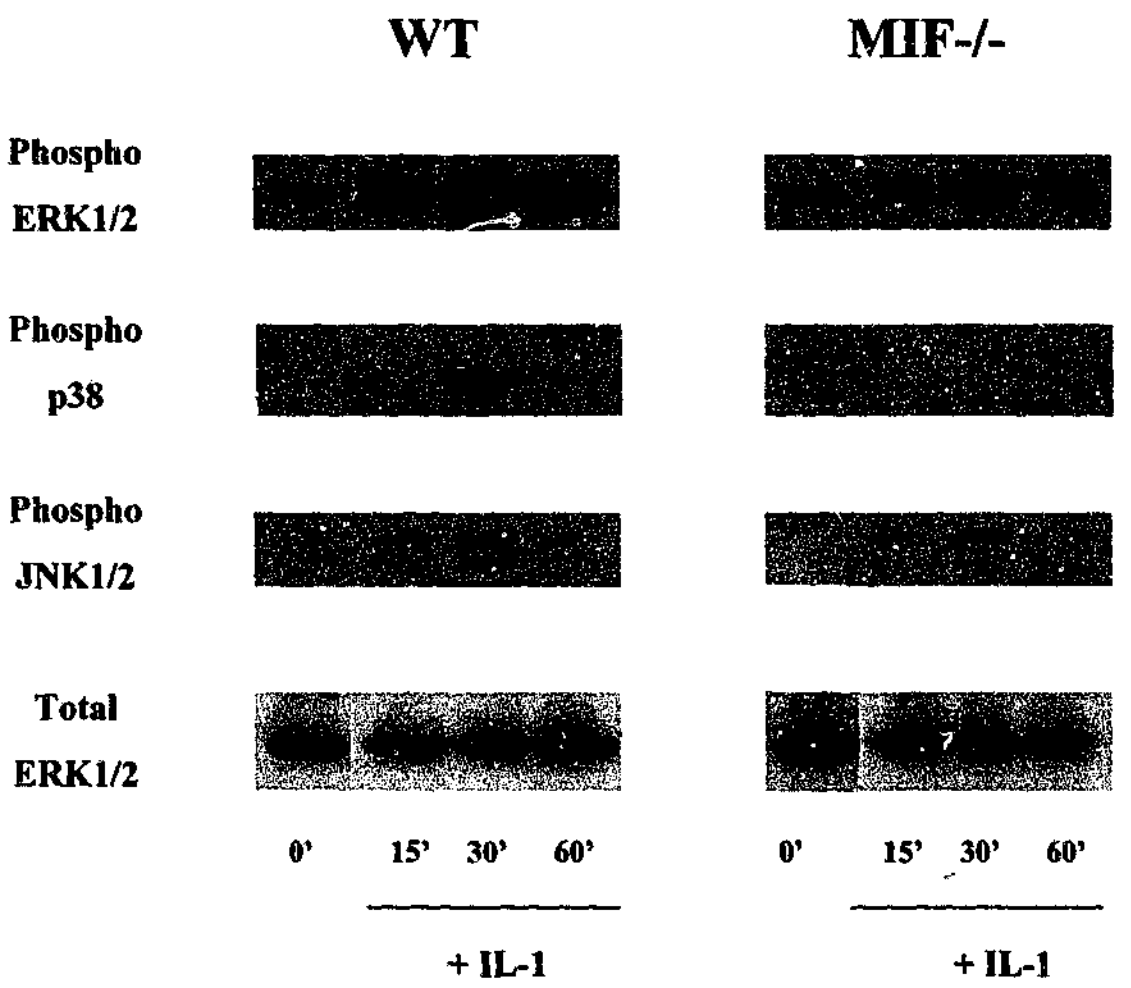
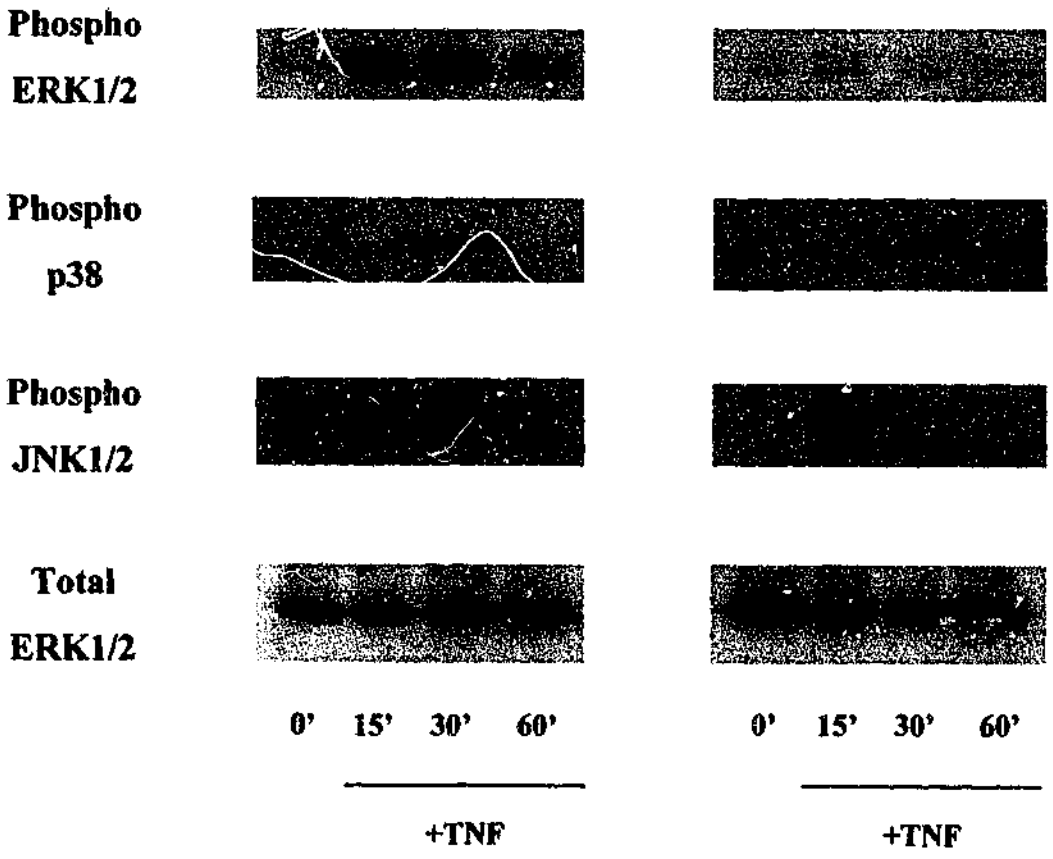

WT
MIF-/-
Phospho ERK1/2
Phospho p38
Phospho JNK1/2
Total ERK1/2
0'
15'
30'
60'
0'
15'
30'
60'
+ IL-1
+ IL-1

Figure 3.6.4

TNFα activation of MIF-/- and wt dermal fibroblast MAPK

Mouse dermal fibroblasts were stimulated with 1ng/ml TNFα and the level of phosphorylated JNK1/2, ERK1/2, p38 and total ERK, were measured by Western blot analysis. Results are representative of 4 independent experiments.


WT
MIF-/-
Phospho ERK1/2
Phospho p38
Phospho JNK1/2
Total ERK1/2
0' 15' 30' 60'
0' 15' 30' 60'
+TNF
+TNF

Chapter Four:

Macrophage migration inhibitory factor (MIF) regulates apoptosis and p53 in inflammatory arthritis.

4.1 Chapter Summary

This chapter examines the capacity of MIF to regulate apoptosis and p53 in human RA cells and mouse dermal fibroblasts cells.

Western blotting was used to measure p53 expression in cells. Apoptosis was detected in cells by flow cytometry using TUNEL and annexinV/ propidium iodide labelling.

In human RA fibroblast-like synoviocytes (FLS), MIF inhibited p53 expression. The levels of p53 were correspondingly increased in MIF-/-. Spontaneous and sodium nitroprusside-induced apoptosis were significantly increased in MIF-/- cells, and exposure of FLS to MIF reduced apoptosis *in vitro*.

Factors regulating p53 expression in inflammatory disease have not been previously identified. These results indicate a role for MIF in the regulation of p53 expression and p53-mediated events in the inflamed synovium, and support the hypothesis that MIF is of importance in the pathogenesis of rheumatoid arthritis.

4.2 Introduction

Rheumatoid arthritis (RA) is characterised by inflammation and hyperplasia of joint lining tissue. In addition to cell migration, imbalance between proliferation and apoptosis of resident cells including fibroblast-like synoviocytes (FLS) underpins this tissue expansion. Invasive synovial tissue, or pannus, and its products, mediate progressive destruction of cartilage and bone in RA. The presence of FLS in this most inflamed and destructive region of RA synovium, and increasing knowledge of the capacity of FLS to modify immune responses, has led to a reappraisal of their role in RA pathogenesis. FLS derived from RA synovial tissue display an autonomous invasive and proliferative phenotype (Zvaifler et al. 1994). In culture, RA FLS grow at a faster rate than normal synoviocytes, can escape contact inhibition, and grow in an anchorage-independent manner (Lafyatis et al. 1989a). In contrast to osteoarthritis FLS, rheumatoid FLS are capable of autonomous invasion of human cartilage in an engrafted co-culture system in the severe combined immunodeficiency mouse (Muller-Ladner et al. 1991). The dysregulated proliferation of RA FLS coupled with low rates of apoptosis observed in RA synovial tissue indicate that escape from p53 mediated cell cycle control may be a factor in this phenotype.

Programmed cell death or apoptosis is a cellular process for maintaining homeostasis by removing damaged cells from tissue, without causing adverse effects. Apoptosis can be induced internally by DNA damage, or externally by activation of death receptor pathways. DNA damage can be caused by numerous stimuli including, ultraviolet light, ionizing radiation, chemotherapeutic drugs or oxidative stress. The increased oxidative stress in the joints of RA patients is believed to cause much of the observed DNA damage. Fas ligand binding to Fas

on the cell surface activates Fas associated death domain and TNF binding to the TNF receptor activates the associated protein with death domain, are both external signals that can initiate apoptosis. There is elevated TNF and Fas expression in RA synovium, however neither appear to induce apoptosis within the joint (Firestein et al. 1995; Cantwell et al. 1997; Hasunuma et al. 1997; Beutler et al. 1998)

As I reviewed in chapter 1, p53 is a tumour-suppressor gene which has a key role in the regulation of normal cell growth, senescence and apoptosis. Once activated, p53 plays a crucial role in the cellular response to DNA damage by inducing cell cycle arrest. Mediation of growth arrest by p53 involves transcriptional activation of genes including p21, cyclin G and growth arrest and DNA damage-induced protein 45 (GADD45) (Chen et al. 1995; Okamoto et al. 1999), p53-mediated apoptosis is described to transcriptional and non-transcriptional activation of pro-apoptotic Bcl2 members including bax, PUMA and NOXA (Schuler et al. 2001). Functional inactivation of p53 is known to result from mutations within the p53 gene (Kern et al. 1991).

With regard to RA, experimental inactivation of endogenous p53 protein has been reported to lead to enhanced RA FLS proliferation, anchorage-independent growth, and invasiveness into cartilage extracts as well as impaired apoptosis (Pap et al. 2001). Overexpression of p53 and functional p53 mutations have been detected in cells and tissue derived from RA (Firestein et al. 1997). Difficulty clarifying the contribution of p53 mutations to synovial hyperplasia has resulted largely from discrepancies in the detection rate of p53 mutations within tissues and fibroblast-like synoviocytes (FLS) derived from RA patients (Yamanishi et al.

2002c). It has been suggested that p53 mutations occur late in RA, as a result of the genotoxic environment within the inflamed joint (Yamanishi et al. 2002c).

Fluctuations in the levels or function of wt p53 unrelated to mutation could also have an impact on synovial proliferative capacity. The levels of wt p53 have not been the focus of intense investigation in the past and this may largely relate to the difficulty of detection of wt p53 in synovial tissue. In normal cells, wt p53 protein is barely detectable because of its short half-life. The role of functional wt p53 has recently been examined in collagen-induced arthritis however, where more severe arthritis and earlier disease onset was observed in p53-/- mice (Yamanishi et al. 2002b). Moreover, the characteristic phenotype of RA FLS is reported to be induced in normal FLS by inhibition of p53 (Pap et al. 2001). Here I identify MIF as the first intrinsic product of FLS capable of inhibiting p53 and p53-mediated events in the setting of inflammation.

Evidence from *in vivo* studies using neutralizing mAbs suggest the pro-inflammatory cytokine MIF contributes to synovial hypercellularity in experimental models of RA (Leech et al. 1998a; Santos, L. et al. 2001). MIF is abundantly expressed in cells and tissue derived from RA synovium (Leech et al. 1999). Moreover, MIF was isolated in functional screening to be the only pro-inflammatory cytokine capable of functionally inactivating p53 (Hudson et al. 1999). Direct effects of MIF on p53 transcriptional targets including p21 have also been described (Liao 2002). This observation has not, however, been examined *in vivo* or extrapolated to specific inflammatory diseases, and no direct effect of MIF on p53 has been described. In this chapter I examine the hypothesis that MIF contributes to synovial hyperplasia through effects on synoviocyte apoptosis and p53.

Dysfunctional or incomplete apoptosis of synovial cells has been suggested as a possible mechanism for promoting synovial hyperplasia. Although high levels of DNA strand breaks and p53 protein have been detected in RA synovium, there is an absence of cells with an apoptotic morphology, possibly indicating defective apoptosis (Firestein et al. 1995; Firestein et al. 1996). More recently, studies have demonstrated that RA FLS rarely undergo apoptosis, compared with the high levels of apoptosis observed in sarcoid granulomas (Highton et al. 2003). A possible molecular mechanism for FLS resistance to apoptosis is their high expression of anti-apoptotic molecules such as bcl-2 (Matsumoto et al. 1996; Perlman et al. 2000), sentrin-1/SUMO-1 (Franz et al. 2000), and FLIP (Perlman et al. 2001). In this chapter, I show that the cytokine MIF can protect RA FLS from p53 mediated apoptosis and regulate p53 expression.

4.3 Methods

4.3.1 Isolation and culture of fibroblasts

Fibroblast-like synoviocytes (FLS) were obtained from synovium of RA patients undergoing joint replacement surgery. All patients satisfied the American College of Rheumatology criteria for the classification of RA (Arnett et al. 1988). Mouse dermal fibroblasts (MDF) were obtained by dissection as previously described in chapter 3. Fibroblasts were isolated using enzyme digestion and cultured in RPMI/10% fetal calf serum (FCS, ICN, Melbourne Australia) as previously described in chapter 2 and 3. A single cell suspension was obtained by digesting minced synovial tissue with 2.4mg/ml Dispase (grade II, 5U/mg; Boehringer Mannheim, Melbourne, Australia), 1mg/ml collagenase (type II; Sigma,

Melbourne, Australia) and DNase (type I; Boehringer Mannheim). FLS and MDF were propagated in 10cm culture plates in RPMI (ICN Biomedicals, Cincinatti OH)/10 % FCS at 37°C in a 5% CO_2 humidified incubator. Cells were used between passages 4 and 9. In each group of experiments, n refers to the number of individual human RA or mouse donor fibroblasts used.

4.3.2 Analysis of p53 expression

The expression of p53 was assessed using Western blotting with a mAb specific for wild type human p53 (Santa Cruz, Santa Cruz, CA). Cells were washed with cold PBS and then lysed with 2×SDS sample buffer. The protein samples were boiled for 10min and stored at -20°C. Samples were subjected to 10% Tris Glycine iGel SDS/PAGE (Gradipore, Sydney Australia), transferred to a Hybond C membrane and detected using ECL detection system (Amersham). Loading was confirmed using an antibody against β-tubulin (Santa Cruz, CA). Western blots were scanned and densitometry ratios obtained using Image Gauge software (version 3.46). Human RA FLS were cultured at 2×10^6 cells per well in a 6 well tissue culture plates in RPMI/10% FCS and stimulated with or without 100 ng/ml of MIF for 2-8hr.

4.3.3 Animals

MIF-/- mice (Bozza et al. 1999a) (generously donated by Dr John R David, Harvard, Cambridge MA) were crossed for two generations with wild type C57/Bl6 mice, generating MIF-/- and wt animals. These mice had 75% C57 background and 25% 129SV background. Experiments were performed on 8-10

week old female mice. Mice were housed in conventional animal housing facilities.

4.3.4 Apoptosis

Apoptosis was measured at baseline and after cells (RA FLS and mouse dermal fibroblasts) were treated with sodium nitroprusside (SNP). 2×10^5 cells per well in a 6 well plate were cultured overnight in 10%FCS/RPMI at 37°C in a 5% CO_2 humidified incubator. Medium was removed and replaced with 0.1%BSA/RPMI, and cells were treated with 0, 0.1 or 0.5μM/ml SNP for 6, 24 or 48hr. Apoptosis was measured using flow cytometric detection of annexin V binding and propidium iodide staining and confirmed using TUNEL as described (Bladier et al. 1997). All the cells from the wells were removed using trypsin/EDTA and labeled with annexin V and propidium iodide, or fixed and labeled using a fluorescein *in situ* death detection kit (Roche, Mannheim, Germany) according the manufacturer's instructions. Cells were analysed on a Dako-cytomation Mo-Flo (Fort Collins, CO, USA) flow cytometer. Laser alumination wave length 488nm, FITC (annexin V) was measured through 530nm ±20nm bandpass filter and propidium iodide was measured through 670nm ±20nm bandpass filter. Scatter in forward angle and 90° angle were collected. A minimum of 20,000 counts were collected on cells gated by their light scatter characteristics.

4.3.5 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.

4.4 Results

4.4.1 Effect of MIF on RA FLS p53 expression

To assess the relevance of MIF to human RA p53 expression, we performed experiments using recombinant MIF and cultured human RA FLS derived from 5 different RA donors. Using Western blotting, we detected constitutive expression of p53 in unstimulated RA FLS (Figure 4.6.1). Recombinant human MIF (100ng/ml) induced a substantial reduction in p53 protein content in RA FLS. The reduction in p53 content was evident within 2hr, and persisted for at least 8hr (Figure 4.6.1). This effect was consistently observed with rMIF treatment of 5 different RA donors. The maximal effect of MIF was observed at 6 hours of treatment. There was a significant reduction in p53 protein levels in rMIF treated cells compared to control cells as measured by densitometry ratios. The p53 protein densitometry ratio for rMIF treated at 6 hours was 0.48 ± 0.28 vs 1.03 ± 0.69 for control cells ($p< 0.02$).

4.4.2 Effect of endogenous MIF on p53

I next analysed p53 protein content in tissue and cells from wt and MIF-/- mice by Western blotting. Spleen tissue and mouse dermal fibroblasts (MDF) from wt

mice demonstrated detectable p53 under basal conditions. MIF-/- mice exhibited substantially increased levels of p53 in spleen tissue and MDF (Figure 4.6.2). These results were obtained in experiments undertaken in collaboration with Dr Jin Xue and Dr Michelle Leech, Monash University.

4.4.4 Effect of MIF on RA FLS Apoptosis

I next investigated the effect of recombinant MIF on human RA FLS apoptosis. As the basal rate of RA FLS apoptosis are very low, I used SNP to induce apoptosis via a p53 dependent pathway. FLS apoptosis induced by SNP was detected using annexin V/propidium iodide staining (Figure 4.6.4). Exposure of FLS to SNP significantly increased apoptosis (Figure 4.6.4). Treatment of RA FLS with MIF markedly and significantly reduced the induction of apoptosis by SNP ($p< 0.05$) (Figure 4.6.4).

This effect of MIF was confirmed using flow cytometric analysis of TUNEL staining (Figure 4.6.5). Exposure of FLS to SNP was associated with a significant increase in TUNEL staining, which was significantly inhibited by MIF (Figure 4.6.5).

4.4.3 Effect of endogenous MIF on dermal fibroblast apoptosis

The role of endogenous MIF in the regulation of fibroblast apoptosis was investigated using dermal fibroblasts cultured from MIF-/- and wt mice. Increased spontaneous apoptosis, as measured by annexin V/propidium iodide labelling, was observed in MIF-/- fibroblasts as compared to wt cells ($p<0.01$) (Figure 4.6.6). SNP exposure, which induces p53-dependent apoptosis in fibroblasts (Aupperle et

al. 1998; Tak et al. 2000), was associated with a significant increase in fibroblast apoptosis in MIF-/- cells compared to wt cells ($p<0.001$) (Figure 4.6.6 a).

Increased spontaneous apoptosis was confirmed in MIF-/- fibroblasts as compared to wt ($p<0.01$) as measured by flow cytometric analysis of TUNEL staining (Figure 4.6.6 b), consistent with the finding that the absence of MIF is associated with increased fibroblast apoptosis.

4.5 Discussion

In RA, the progressive destruction of articular cartilage is reliant at least in part on invasion of cartilage by hyperplastic synovial cells. Hyperplasia of synovium, and dysregulated proliferation and apoptosis of FLS, are well described in RA (Qu et al. 1994a; Zvaifler et al. 1994; Firestein 1996). Hitherto, endogenously produced reactive oxygen species and other genotoxic stresses of inflammation have been postulated to lead to the p53 mutations described in subsets of RA patients. Previous studies aimed at elucidating the impact of mutated or malfunctioning p53 have shown that *in vivo* invasiveness and proliferative capacity of RA FLS can be modulated by inhibition of endogenous wt p53. Furthermore, a "transformed", or aggressive RA-like phenotype can be induced normal FLS following p53 inhibition (Pap et al. 2001). In this study, I focused on down-regulation of wt p53. rMIF treatment of human RA FLS down regulated p53, and increased levels of p53 were detected in cells derived from MIF-/- mice. I also observed increased rates of apoptosis in cells obtained from MIF-/- mice. The combination of effects on apoptosis and proliferation (as shown in chapters 2 and 3) strongly suggest MIF as a critical mediator of synovial hypercellularity in arthritis.

MIF immunoneutralisation in animal models of arthritis is associated with substantial reductions in synovial cellularity (Leech et al. 1998a; Santos, L. et al. 2001). More recently, a study of experimental arthritis in MIF-/- mice showed decreased arthritis expression associated with increased apoptosis and p53 detection in the synovial lesion (Leech et al. 2003). In that study, very few proliferating cells were detected in AIA synovitis with no significant further reduction detectable in MIF-/- animals. This suggests that in addition to potential effects on leukocyte recruitment, the reductions in synovial hypercellularity in

MIF-/- mice with AIA are influenced by increased apoptosis rather than decreased proliferation.

MIF is a cytokine increasingly recognised as a participant in pathogenic mechanisms contributing to inflammatory, autoimmune and neoplastic processes. Its potential role in the development of RA is highlighted by studies in which immunoneutralisation of MIF resulted in profound inhibition of adjuvant arthritis in the rat, as well as delayed onset and lower frequency of collagen-induced arthritis and reduced severity of antigen-induced arthritis in the mouse (Mikulowska et al. 1997b; Leech et al. 1998a; Santos, L. et al. 2001). Previous studies have identified abundant expression of MIF in human RA synovial FLS, macrophages, and to a lesser extent T cells, and demonstrated its overexpression in RA compared to control tissues and cells (Leech et al. 1999). Recently, MIF detection in human cholesteatomas was found to correlate strongly with levels of the anti-apoptotic protein galectin and to reflect the biologic aggressiveness of these lesions (Choufani et al. 2001). The current study provides evidence that MIF can prevent apoptosis in human synoviocytes and that endogenous MIF has an anti-apoptotic-effect.

Cytokines including IL-4 and IL-13 have been shown to have anti-apoptotic effects in human synoviocytes, possibly via effects on caspases (Relic et al. 2001). To date these cytokines have not, however, been shown to influence p53 expression. Whilst IL-6 has previously been shown to override p53 in leukemic cells, this study was performed using transfected clones (Yonish-Rouach et al. 1991) and no direct effect on p53 expression was detected. The current study clearly demonstrates that MIF decreases the expression of p53 in cells derived from human RA synovium.

In a recent study aimed at isolating genes which can bypass p53-mediated growth arrest or apoptosis, MIF was found to influence p53 transcriptional activity without changing p53 protein levels or subcellular localisation (Hudson et al. 1999). Specifically, MIF was shown to inhibit p53 transcriptional activity, as measured by a luciferase reporter gene assay. MIF was also found to decrease p53-induced genes such as p21, cyclin G and MDM2. The functional significance of these findings was confirmed using biological assays in which rMIF suppressed apoptosis (Hudson et al. 1999). These studies were performed in murine embryonic cells and cell lines, not primary cells derived from an inflammatory site. Our findings demonstrate in contrast a MIF-induced reduction in p53 protein in human FLS, but similarly we were able to demonstrate that MIF provided protection from p53 dependent apoptosis. The difference in our finding to those of Hudson *et al* could be due to the fact that in my studies I modulated p53 in primary cells and avoided the need to artificially overexpress p53 by transfection. In addition, evidence that cells were synchronized prior to study is lacking in the paper by Hudson *et al.*

The effects of MIF on p53 activation and phosphorylation status, via effects on signal transduction pathways, are as yet unexplored. Extracellular receptor activated kinase (ERK)-mediated signal transduction has been implicated in MIF-mediated effects on fibroblast phospholipase A_2 activation (Mitchell et al. 1999). As shown in chapter 2 in human synovial cells, I have confirmed the ability of MIF to activate ERK MAPK. Nuclear factor κB (NF-κB) has been shown to influence synovial hypertrophy via the protection of cells from TNF-mediated apoptosis (Miagkov et al. 1998). As discussed in chapter 2, however, direct evidence of NF-κB activation by MIF is consistently lacking (Kleemann et al.

2000). NF-κB and p53 are thought to have mutually antagonistic effects on tissue growth via competition for co-activator molecules including CREB-binding protein (Wadgaonkar et al. 1999). This highlights a mechanism whereby MIF could indirectly influence NF-κB mediated events via down-regulation of p53, without any direct effect on NF-κB activation or translocation.

Abnormalities in the regulation of p53 may underlie the progression from initial synovial inflammation to the autonomous expansion and invasion displayed by the synovial pannus in established RA. Loss of p53 function in RA FLS resulted in increased anchorage-independent growth and increased cell proliferation (Aupperle et al. 1998). In a rat antigen-induced arthritis (AA) model, p53 expression increased gradually over the duration of the disease as measured by Western blotting and immunohistochemistry (Tak et al. 2000). Interestingly, p53 expression at the later stages of AA disease was significantly higher than that observed in tissue from RA patients with long standing disease. Apoptosis in rat AA joints, as measured by TUNEL staining, was found to be preceded by increased p53 expression (Tak et al. 2000). In a collagen-induced arthritis model, it was found that p53-/- mice developed disease earlier and had increased disease severity compared to wt mice, as measured by clinical and histological scoring (Yamanishi et al. 2002b). In a SCID mouse *in vivo* model of cartilage invasion, human RA FLS cells with functionally inactivated p53 had significantly increased invasion characteristics compared with parental FLS, as measured by histological evaluation of invasion and cartilage degradation (Pap et al. 2001).

Soluble factors influencing p53 in RA tissues have not previously been identified. Data presented in this chapter indicates that the pro-inflammatory cytokine MIF, produced abundantly by rheumatoid macrophages and synoviocytes, is capable of

decreasing p53 expression in FLS. This observation has important implications in terms of potential modulation of the invasive and hyperplastic phenotype displayed by RA FLS.

Normal expression of p53 is vital to allow cell cycle progression, and to induce growth arrest for DNA repair or apoptosis. It is conceivable however, that situations can emerge, such as at sites of acute inflammation, when it is necessary to temporarily inactivate p53 function and allow cell proliferation and tissue repair. Such a mechanism could permit resistance to or limit the damage from genotoxic conditions that could otherwise cause deleterious consequences to surrounding cells and tissue. The observed down regulation of p53 and protection from p53-mediated apoptosis by MIF provides a possible mechanism for this phenomenon. In chronic inflammatory diseases like RA, the ability of MIF to down regulate p53 protein could have deleterious consequences, providing opportunities for more lasting loss of p53 function. A number of chronic inflammatory conditions, such as ulcerative colitis, are associated with tumour development. Similarly, chronic *Helicobacter pylori* infection is associated with gastric cancer formation (Wotherspoon 1998). Escape from p53 mediated cell-cycle restraint is likely to contribute to enhanced proliferation and cellularity in circumstances where tissue hyperplasia has significant impact on tissue destruction and disease outcome. Future targeting of MIF with specific inhibitors may permit the therapeutic limitation of synovial hyperplasia and invasion, an aim that eludes contemporary therapies.

4.6 Figures

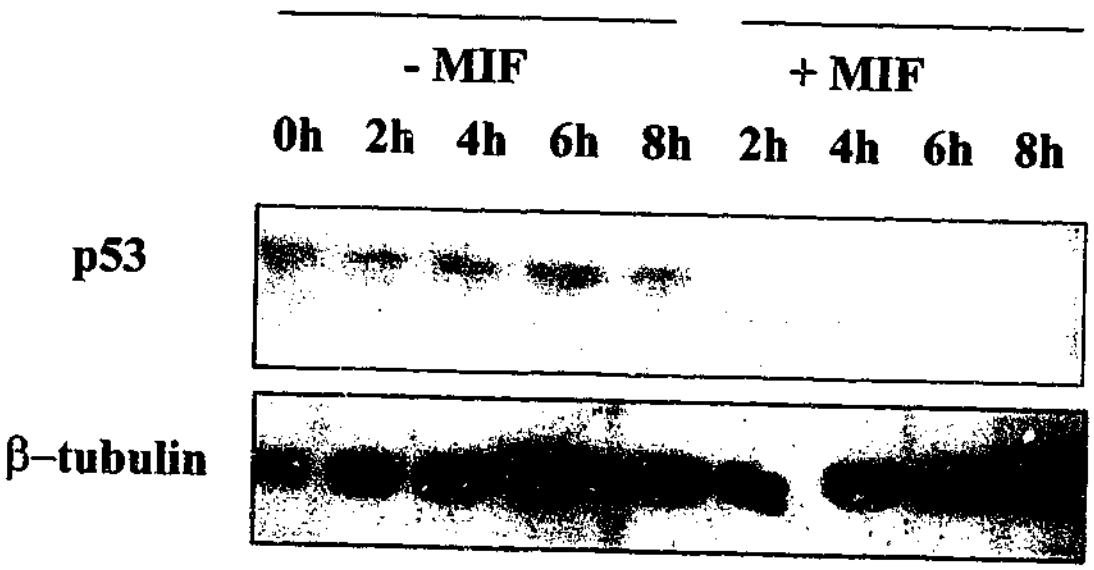
Figure 4.6.1

Effects of recombinant human MIF on human RA FLS p53

(a) p53 was examined using Western blotting with a monoclonal anti-human p53 antibody. The equivalence of protein loading was measured using detection of β-tubulin. Compared to untreated cells, MIF induced a reduction in FLS p53, evident within 2hr and persisting throughout the 8hr of the experiment. Representative of 5 separate experiments using cells from 5 individual RA patients.

(b) Densitometry was measured for each time point and expressed in a bar graph using an arbitrary scale.

a


- MIF
+ MIF
0h 2h 4h 6h 8h 2h 4h 6h 8h
p53
β–tubulin

b

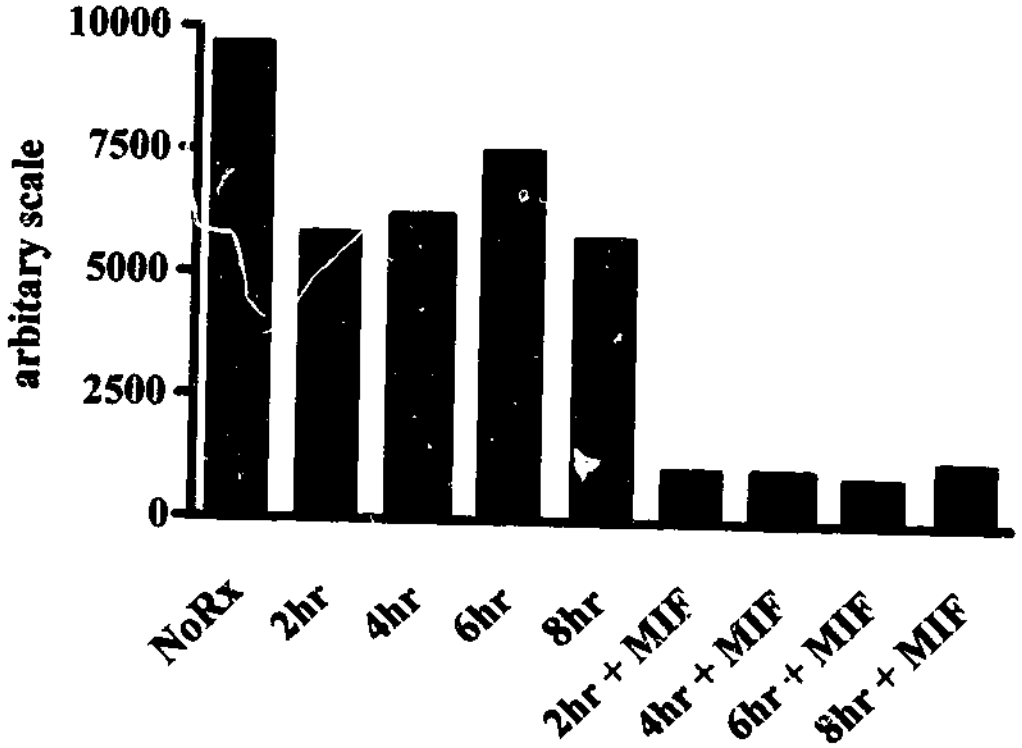

10000
7500
5000
2500
0
arbitary scale
NoRx
2hr
4hr
6hr
8hr
2hr + MIF
4hr + MIF
6hr + MIF
8hr + MIF

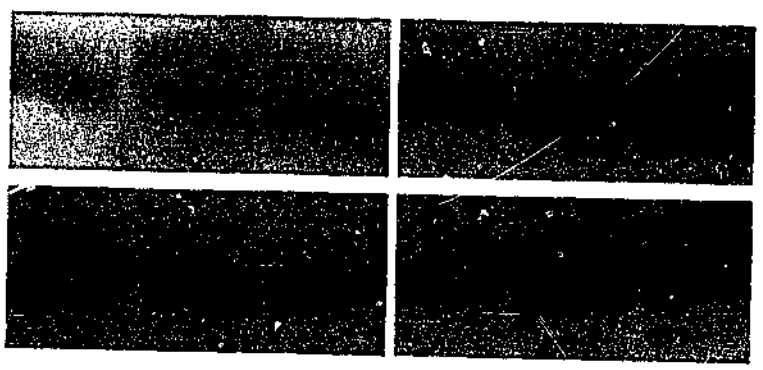
Figure 4.6.2

Effect of endogenous MIF on p53

(a) Spleens were obtained from MIF-/- and wt mice and p53 protein content detected by Western blotting with a monoclonal anti-mouse p53 antibody. The equivalence of protein loading was measured using detection of β-tubulin. Each lane represents a single MIF-/- or wt mouse. Compared to wt mice, MIF-/- spleens exhibited higher p53 content.

(b) Mouse dermal fibroblasts (MDF) were obtained from MIF-/- mice and wt mice and p53 detected using Western blotting as above. Compared to wt mice, MDF from MIF-/- mice exhibited higher p53 content. In figures (a) and (b) each lane represents a single MIF-/- or wt mouse. Lanes 1–3 in each case represent wt and lanes 4 – 6 represent MIF-/-cells or tissue.

a



b

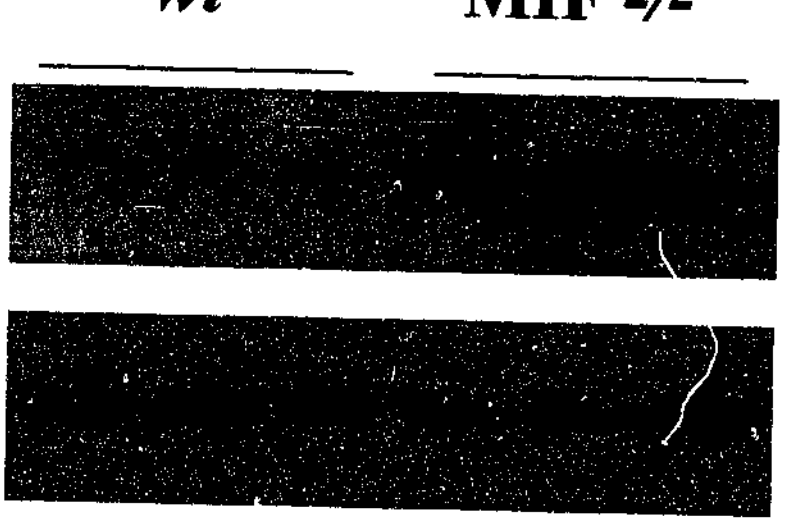


Figure 4.6.3

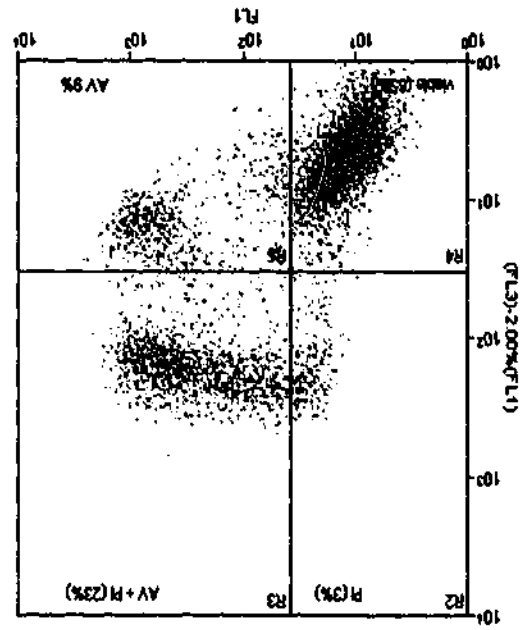
Effects of recombinant human MIF on RA FLS apoptosis

Apoptosis was examined using flow cytometric analysis of annexin V (horizontal axis) and propidium iodide (vertical axis).

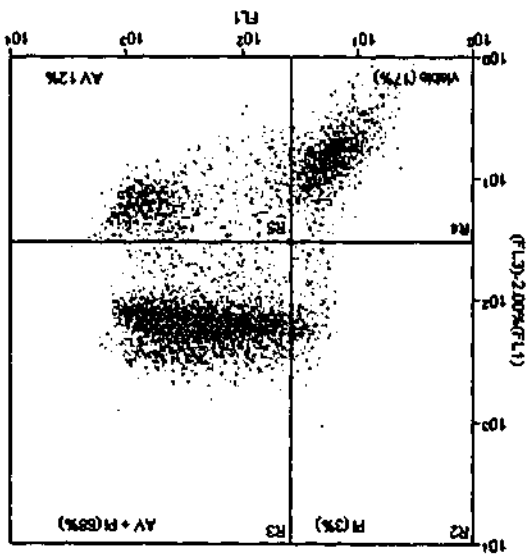
(a) RA FLS demonstrated low rates of spontaneous apoptosis.

(b) SNP induced a significant increase in annexin V/PI-positive cells ($p < 0.05$).

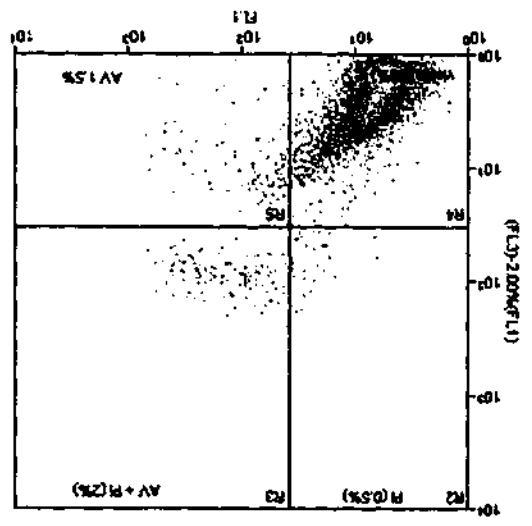
(c) Treatment of cells with recombinant human MIF significantly abrogated SNP-induced apoptosis ($p < 0.05$). Representative of 4 separate experiments.


FL1
(FL3)-2.00%(FL1)
R2
PI (3%)
R3
AV + PI (23%)
R4
R5
AV 9%

c


FL1
(FL3)-2.00%(FL1)
R2
PI (3%)
R3
AV + PI (58%)
R4
R5
AV 12%

b


FL1
(FL3)-2.00%(FL1)
R2
PI (0.5%)
R3
AV + PI (2%)
R4
R5
AV 1.5%

a

Figure 4.6.4

Effect of recombinant human MIF on RA FLS apoptosis

RA FLS were treated with media alone, 0.5mM of SNP, with or without 100ng/ml of MIF and apoptosis was measured by annexin V and propidium iodide. Treatment of RA FLS with MIF significantly reduced SNP induced apoptosis, and correspondingly increased viable cell numbers ($p<0.05$). Representative of 4 separate experiments.

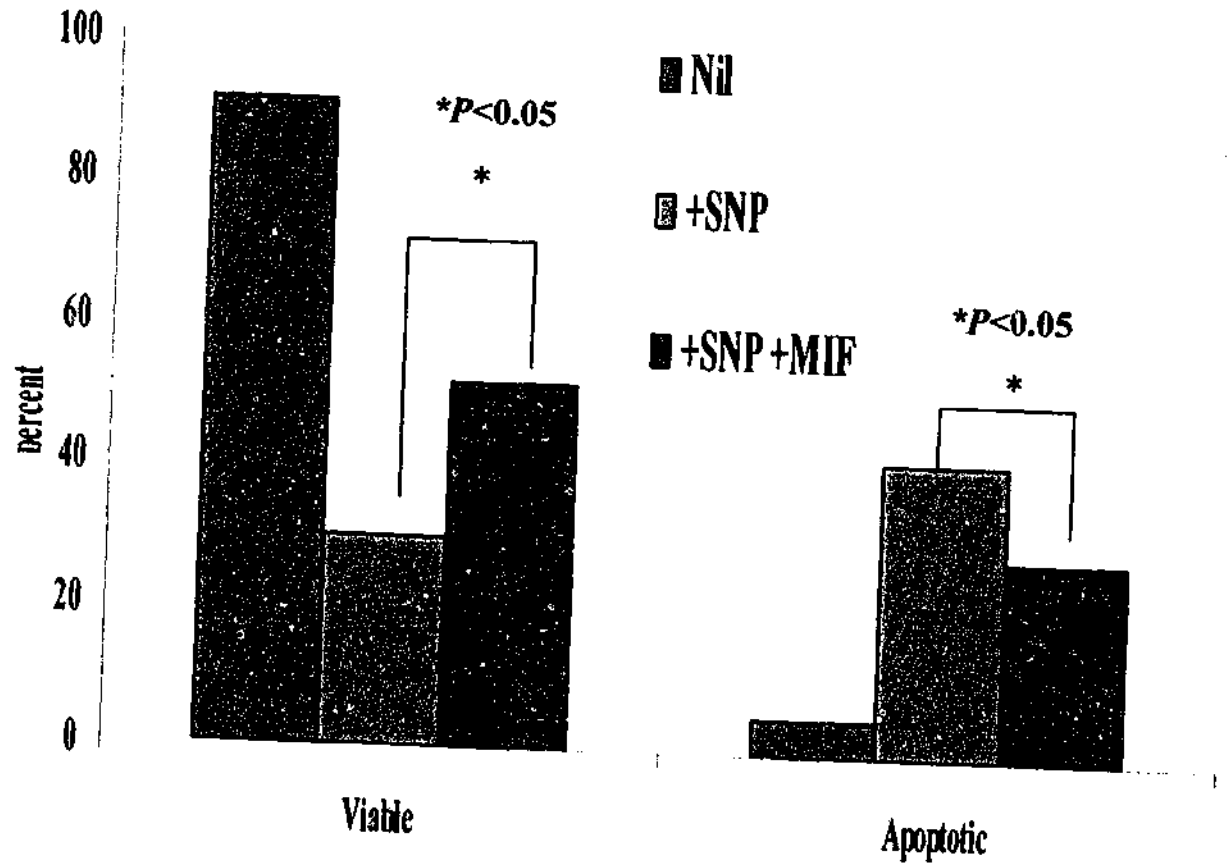
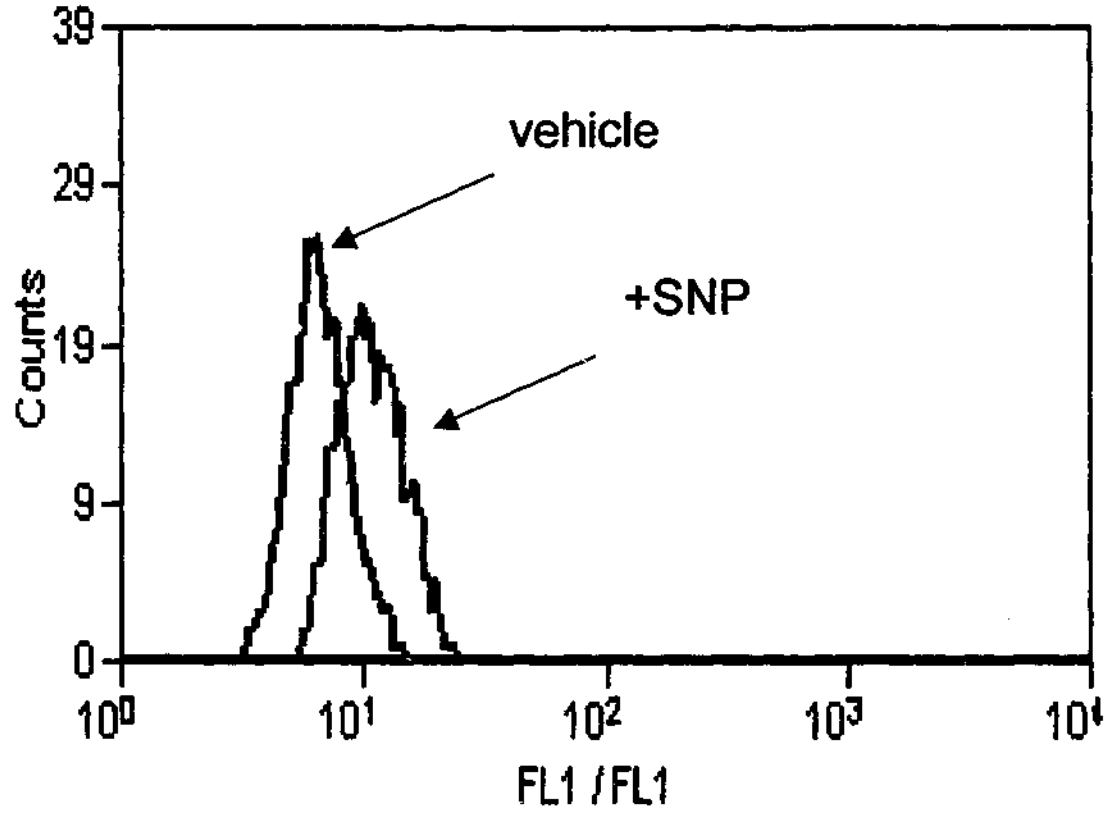

100
80
60
40
20
0
percent
*P<0.05
*
Nil
+SNP
+SNP +MIF
*P<0.05
*
Viable
Apoptotic

Figure 4.6.5

Effect of recombinant human MIF on RA FLS apoptosis

RA FLS were treated with medium alone, 0.5mM of SNP, and/or 100ng/ml MIF, and apoptosis was examined using TUNEL staining. Treatment of RA FLS with MIF significantly reduced SNP-induced apoptosis as measured by TUNEL staining ($p<0.05$). Representative of 4 separate experiments.


39
29
19
9
0
Counts
vehicle
+SNP
10^0
10^1
10^2
10^3
10^4
FL1 / FL1

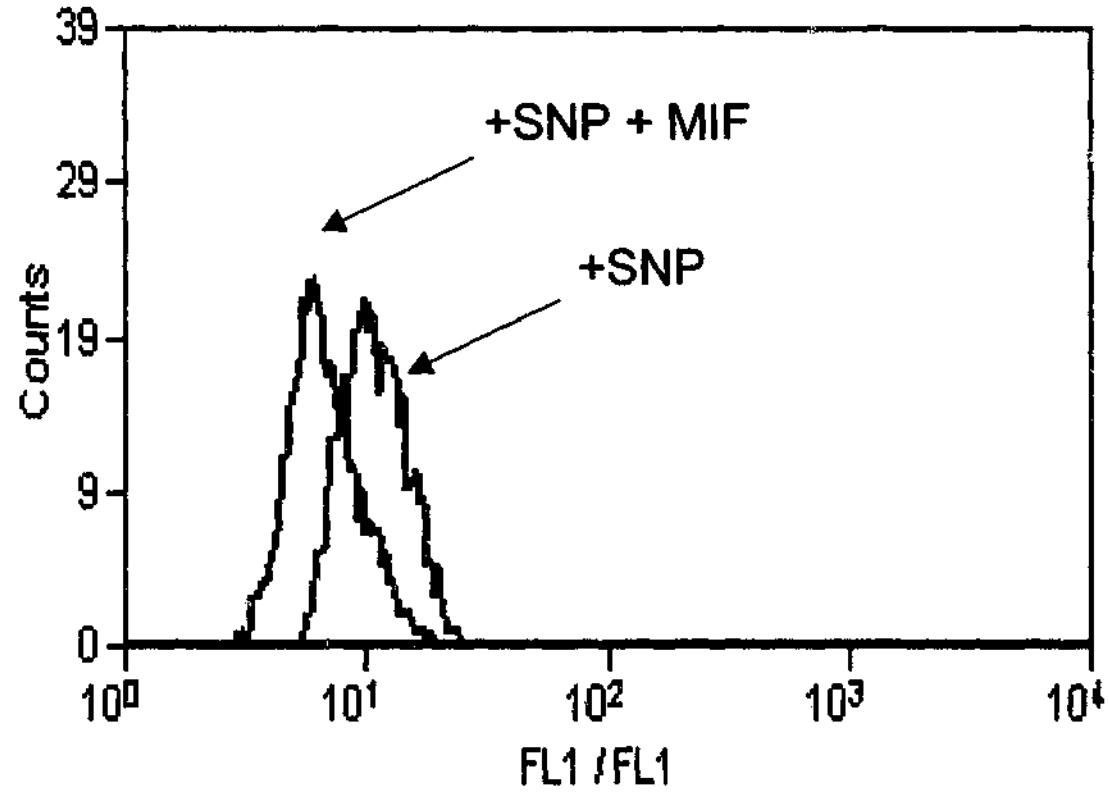
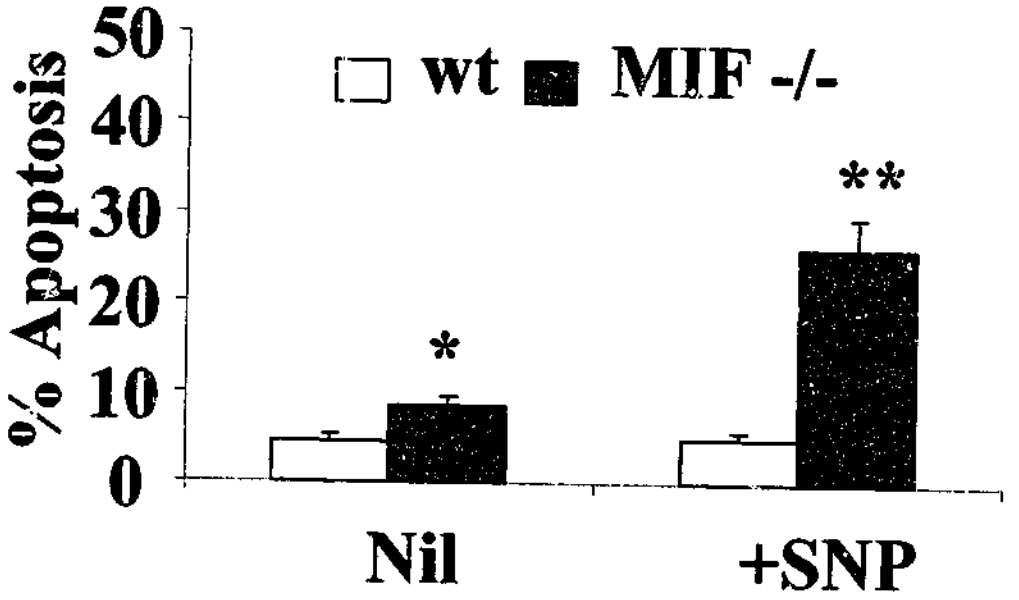

39
29
19
9
0
Counts
+SNP + MIF
+SNP
10^0
10^1
10^2
10^3
10^4
FL1 / FL1

Figure 4.6.6

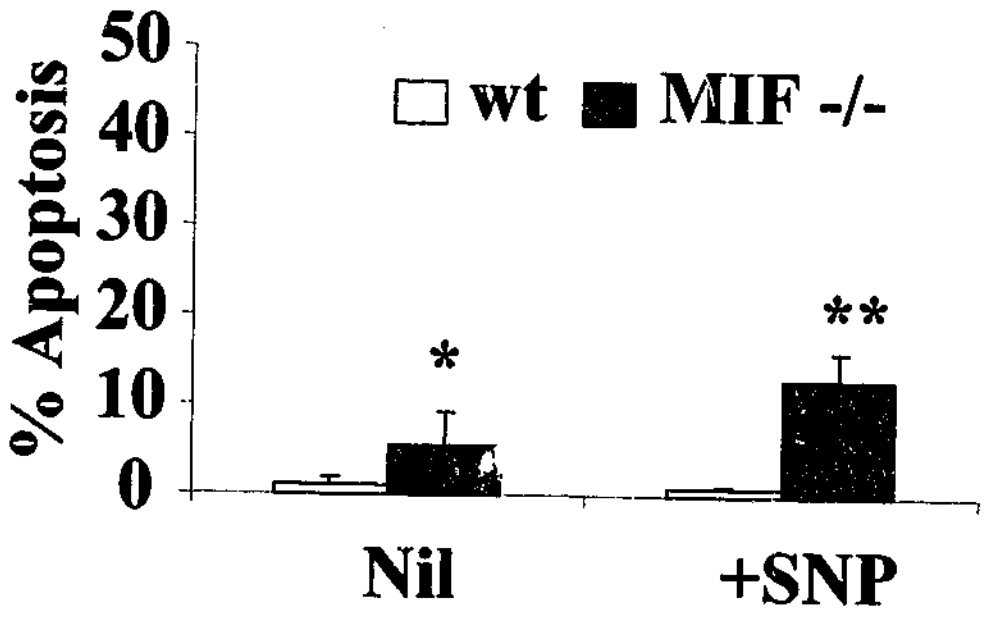
Effect of endogenous MIF on Dermal fibroblast apoptosis

Apoptosis was examined in cultured dermal fibroblasts from wt and MIF-/- mice (a) using annexin V and propidium iodide staining, and confirmed (b) using flow cytometric detection of TUNEL staining. Mean ± SEM of 4 separate experiments. MIF-/- cells showed increased spontaneous apoptosis (*$p < 0.01$), and increased apoptosis in response to SNP (**$p < 0.001$).

a


% Apoptosis
50
40
30
20
10
0
wt
MIF -/-
*
**
Nil
+SNP

b


% Apoptosis
50
40
30
20
10
0
wt
MIF -/-
*
**
Nil
+SNP

Chapter Five:

Regulation of human rheumatoid arthritis synoviocyte proliferation and apoptosis by mitogen activated protein kinases

5.1 Chapter Summary

Mitogen activated protein kinases (MAPK) have been shown to be important in rheumatoid arthritis (RA), particularly in relation to the regulation of pro-inflammatory gene expression. Less is known about the contribution of MAPK to the regulation of synovial hypercellularity. Studies presented in chapter 2 suggested that in addition to a role in cytokine induced proliferation, MAPK could be involved in the control of constitutive proliferation. This study was undertaken to ascertain whether MAPK influence the proliferation and/or apoptosis of RA fibroblast-like synoviocytes (FLS).

Activation of MAPK was measured using Western blotting with phospho-specific antibodies. Cell proliferation was measured by [^{3}H] thymidine incorporation. Apoptosis was induced with sodium nitroprusside (SNP), and measured using flow cytometric detection of annexin V binding and TUNEL staining. Activation of MAPK was antagonised with specific inhibitors.

Serum-induced proliferation of RA FLS was associated with phosphorylation of ERK1/2, p38 and JNK MAPK. Antagonism of the JNK and ERK pathways significantly inhibited the proliferation of RA FLS, where p38 inhibition had no effect. IL-1β and TNFα also significantly increased FLS proliferation. In addition, the effects of IL-1β and TNFα were also prevented by JNK and ERK pathway inhibition.

SNP significantly induced FLS apoptosis, associated with phosphorylation of ERK1/2 and JNK. JNK pathway antagonism increased RA FLS sensitivity to SNP induced apoptosis, whereas neither ERK nor p38 inhibition had any effect.

The activation of JNK and ERK are important in the regulation of RA FLS proliferation. JNK activation is also an important survival factor in SNP-induced

apoptosis. This study highlights the potential importance of individual MAPK in the regulation of FLS expansion in RA.

5.2 Introduction

Rheumatoid arthritis (RA) is characterised by chronic inflammation of the joints and synovial hyperplasia. The development of pannus, and subsequent invasion and destruction of cartilage and bone is mediated by resident synovial cells, such as fibroblast-like synoviocytes (FLS) (Firestein 2003). The hyperplastic phenotype of FLS is well described. FLS from RA patients show increased expression of proto-oncogenes such as c-myc and ras (Muller-Ladner et al. 1995; Muller-Ladner et al. 1998), while cells in the superficial lining layer express proliferation cell nuclear antigen (PCNA) (Qu et al. 1994b). FLS derived from RA synovial tissue display an autonomous invasive and proliferative phenotype (Zvaifler et al. 1994; Firestein 1996). In culture, RA FLS grow at a faster rate than control cells, can escape contact inhibition, and grow in an anchorage-independent manner (Lafyatis et al. 1989a). In contrast to osteoarthritis FLS, rheumatoid FLS are capable of autonomous invasion of human cartilage in an engrafted co-culture system in the SCID mouse (Muller-Ladner et al. 1991). A number of studies have shown evidence of defects in apoptosis in RA synovium (Firestein et al. 1995; Sugiyama et al. 1996; Catrina et al. 2002), suggesting dysregulated apoptosis may contribute to synovial hyperplasia.

Factors which regulate the activation of FLS do so via specific intracellular signal transduction pathways. Signal transduction pathways leading to activation of NFκB have been previously studied in relation to the regulation of FLS proliferation and apoptosis using various techniques (Makarov 2001; Yamasaki et al. 2001). Most studies conclude that cytokine-induced NFκB is important for the apoptosis resistance of FLS in the presence of potentially pro-apoptotic stimuli such as TNF (Van Antwerp et al. 1996). MIF, a cytokine with importance in RA,

and which regulates FLS proliferation and apoptosis (see chapter 4), has been shown to act independently of NFκB (Lacey, D.C. et al. 2003; Leech et al. 2003), suggesting an important independent role of other pathways in the regulation of cell growth in RA.

Mitogen-activated protein kinase (MAPK) pathways have also been implicated in RA pathogenesis. The MAPK pathways which have been shown to be relevant to RA include extracellular signal-regulated kinases (ERK1/2), the c-jun amino-terminal kinases (JNK1/2) and p38 MAPK as reviewed in chapter 1. It has previously been reported that activated MAPK expression is significantly increased in RA tissue compared to osteoarthritis (OA) tissue, and that important pro-inflammatory cytokines such as IL-1 and TNF activate all three MAPK in RA FLS (Schett et al. 2000).

Recent studies in other cell types have suggested that MAPK may be involved in the control of proliferation and apoptosis. However, their contribution to the regulation of FLS proliferation and apoptosis has not been studied. Moreover, MAPK have been observed to mediate diverse and in some cases opposite effects in regards to apoptosis depending on the cell or tissue under examination. This highlights the need to study their effects directly in cells relevant to a given disease such as RA.

In this chapter I report that ERK1/2 and JNK activation can each support synoviocyte proliferation *in vitro*. In addition, JNK activation is a survival factor providing partial resistance to SNP induced apoptosis.

5.3 Methods

5.3.1 Isolation and culture of fibroblast-like synoviocytes

Fibroblast-like synoviocytes (FLS) were obtained from synovium of rheumatoid (RA) patients undergoing joint replacement surgery. All patients satisfied the American College of Rheumatology criteria for the classification of RA (Arnett et al. 1988). FLS were isolated using enzyme digestion and cultured in RPMI/10% foetal calf serum (FCS, ICN, Melbourne Australia) as described in previous chapters. A single cell suspension was obtained by digesting minced synovial tissue, with 2.4mg/ml Dispase (grade II, 5 U/mg; Boehringer Mannheim, Melbourne, Australia), 1mg/ml collagenase (type II; Sigma, Melbourne, Australia) and DNase (type I; Boehringer Mannheim). FLS were propagated in 10cm culture plates in RPMI (ICN Biomedicals, Cincinatti OH)/10 % FCS (Trace Biosciences Pty Ltd, Melbourne, Australia) at 37°C in a 5% CO_2 humidified incubator. Cells beyond third passage were more than 99% CD45-. Cells were used between passages 4 and 9. In each group of experiments, n refers to the number of individual human RA donor FLS used.

5.3.2 Measurement of fibroblast-like synoviocyte proliferation

RA FLS proliferation was measured by [^{3}H] thymidine incorporation as described in chapter 2. RA FLS were seeded overnight at $1x10^5$ cells per well in 24 well tissue culture plates in RPMI/10% FCS. Cell growth was synchronised by culturing FLS in RPMI/0.1 % bovine serum albumin (BSA) (Sigma, Melbourne, Australia) for 24hr. To determine the effect of MAPK on FLS proliferation, FLS were stimulated with FCS (CSL, Melbourne, Australia), IL-1β 0.1ng/ml (Sigma,

Melbourne, Australia) or TNFα 1ng/ml (Biosource, Carmarillo, CA) with or without specific MAPK inhibitors PD98059 (50μM) (Alexis Biochemicals, San Diego, CA) (Alessi et al. 1995), SB203580 (5μM) (kindly provided by Dr Alison Badger, SmithKline Beecham Pharmaceuticals, King of Prussia, PA) (Lee et al. 1994), SP600125 (50μM) (kindly provided by Dr Brydon Bennett, Celgene, San Diego, CA) (Bennett et al. 2001) or vehicle (DMSO). MAPK inhibitors were added to cultures 30min prior to treatment. To determine proliferation, FLS were cultured for 72hr, pulsed for the final 18hr with 1μCi/ml [^{3}H]thymidine (Amersham International, Sydney, Australia). Triplicate cultures were used for each determination as noted. FLS were detached using Trypsin-EDTA, harvested using a cell harvester (Packard, CT, USA), and the radioactivity incorporated into DNA determined using a Wallac 1409 liquid scintillation counter (Pharmacia, Turku, Finland).

5.3.3 Analysis of MAPK activation.

The phosphorylation of ERK, p38 and JNK was assessed using Western blotting, as described in chapter 3. Cells were disrupted by repeated aspiration through a 21-gauge needle, incubated on ice for 10 min and micro centrifuged at 3000 rpm for 15 min (4 °C), and lysates were stored at -80 °C. Protein concentration was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL). Equal amounts (50μg) of cellular proteins were fractionated on 10% SDS-polyacrylamide electrophoresis gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Western blotting was performed using antibodies directed against phospho-ERK, phospho-p38, phospho-JNK, total ERK, total p38 and total JNK according to the manufacturer's instructions (Cell

Signaling Laboratories, Beverly, MA). Western blots were scanned and densitometry ratios obtained using Image Gauge software (version 3.46).

5.3.4 Apoptosis

FLS ($0.2x10^5$ per well) were seeded onto a 6 well plate for 24hr in 10%FCS/RPMI. Cells were then treated in 0.1%BSA with 0, 0.1, or 0.5mM of sodium nitroprusside (SNP) with or without MAPK inhibitors PD98059 (50μM), SB203580 (5μM), SP600125 (50μM) or vehicle (DMSO) for 24hr. Cells were removed and apoptosis was measured using flow cytometric detection of annexin V binding and propidium iodide (both from Roche, Mannheim, Germany) staining as described in chapter 4, and confirmed using TUNEL staining performed using a fluorescein *in situ* death detection kit (Roche, Mannheim, Germany) as described in chapter 4.

5.3.5 Statistical Analysis

Results are expressed as the mean ± SEM. Statistical analysis was performed using the one-way ANOVA, with values of $p < 0.05$ regarded as statistically significant.

5.4 Results

5.4.1 Effect of serum on RA FLS proliferation

This experiment was carried out by my colleague Dr Annnalise Sampey and was submitted as part of her B.Sc Honors thesis, but not published elsewhere. I include

these data as a prelude to my own work and to demonstrate that fetal calf serum is required for FLS proliferation.

To determine whether fetal calf serum (FCS) is essential for RA FLS proliferation, proliferation was measured at various concentrations of FCS. As shown in Appendix 1, Table 1, the lowest rate of [^{3}H] thymidine incorporation was observed in synoviocytes cultured in RPMI alone (844±110 cpm), indicating low proliferative rates for synoviocytes when maintained in the absence of serum. Culturing FLS in 10% and 20% (v/v) FCS resulted in an increase in proliferation by at least a factor of ten. No significant difference in proliferation was observed between cells cultured in 10% and 20% FCS. All remaining studies in this thesis were preformed by the candidate. See Appendix 1 Table 1.

5.4.2 Effect of serum on MAPK activation

To determine the role of MAPK in serum induced FLS proliferation, I cultured FLS with serum and measured the level of phosphorylated and total JNK1/2, ERK1/2, and p38 by Western blot analysis.

Weak basal activation of ERK1 and strong basal ERK2 activation was detected in serum-starved FLS. Rapid and sustained activation of ERK1/2 was observed after stimulation of FLS with serum. There was no detectable increase in total ERK1/2 protein levels over the course of the experiment (Figure 5.6.1a).

Very weak basal activation of JNK1/2 was detected in serum starved FLS. Rapid transient activation of JNK1/2 was observed after stimulation of FLS with serum, which returned to baseline by 60 mins. There was no detectable increase in total JNK1/2 protein levels over the course of the experiment (Figure 5.6.1c).

Weak basal activation of p38 was detected in serum starved FLS. Rapid activation of p38 was observed after stimulation with serum, which returned to baseline by 60mins. There was no detectable increase in total p38 protein levels over the course of the experiment (Figure 5.6.1e).

5.4.3 Effect of MAPK antagonism on RA FLS proliferation

The role of MAPK has not been well defined with respect to the control of proliferation in RA FLS. To examine the role of MAPK activation in RA FLS proliferation, specific MAPK inhibitors were employed. Treatment with the MEK inhibitor PD98059, which blocks ERK activation, resulted in a significant inhibition of serum induced proliferation ($p<0.01$) (Figure 5.6.2). Treatment with the JNK inhibitor SP600125, resulted in a dramatic decrease in serum induced proliferation, ($p<0.01$), (Figure 5.6.2). In contrast, treatment with the p38α/β inhibitor SB203580 had no effect on serum induced proliferation (Figure 5.6.2).

5.4.4 Effect of MAPK antagonism on cytokine induced RA FLS proliferation

To determine any additive effect of IL-1β and TNFα on serum induced proliferation, RA FLS were stimulated with 0.1ng/ml of IL-1β or 1ng/ml of TNFα for 72hrs in RPMI/10%FCS. Figure 5.6.3a shows that IL-1 ($p<0.01$) and TNF ($p<0.05$) both induced significant increases in proliferation, between 50-100% greater than that observed with serum alone.

Given the importance of IL-1β and TNFα in RA and their well-described ability to activate MAPK, we measured the effect of MAPK antagonism on IL-1β (Figure 5.6.3b) and TNFα (Figure 5.6.3c) induced proliferation. ERK pathway antagonism

resulted in significant inhibition of IL-1 and TNF induced proliferation, similar to the effects observed for serum induced proliferation. JNK pathway antagonism resulted in significant inhibition of IL-1 and TNF induced proliferation, similar to the effects observed for serum induced proliferation. Antagonism of p38 had no statistically significant effect. However, p38 antagonism was associated with a trend of inhibition towards baseline of the IL-1 and TNF induced increases in proliferation.

5.4.5 Effect of sodium nitroprusside on MAPK activation

Dysregulation of apoptosis and low observed levels of spontaneous apoptosis in RA synovium are both thought to be important in the pathogenesis of RA. Having determined a role of MAPK activation in FLS proliferation, I examined the participation of MAPK in the regulation of FLS apoptosis. As shown in chapter 4, nitric oxide donor sodium nitroprusside (SNP) induces significant apoptosis in RA FLS *in vitro*. To determine whether MAPK are activated by SNP, I measured the level of JNK1/2, ERK1/2, and p38 phosphorylation by Western blot analysis.

Rapid and sustained activation of ERK1/2 was observed after stimulation of FLS with serum. There was no detectable increase in total ERK1/2 protein levels over the course of the experiment (Figure 5.6.4a).

Rapid transient activation of JNK1/2 was observed after stimulation of FLS with serum, which returned to baseline by 60 mins. There was no detectable increase in total JNK1/2 protein levels over the course of the experiment (Figure 5.6.4c).

SNP did not detectably activate p38 in RA FLS. There was no detectable increase in total p38 protein levels over the course of the experiment (Figure 5.6.1e).

5.4.6 Effect of MAPK antagonism on sodium nitroprusside induced apoptosis

I next examined the role of MAPK in SNP induced apoptosis using specific MAPK inhibitors. As was observed in chapter 4, SNP induced apoptosis in RA FLS in a dose-dependent manner (Figure 5.6.5a).

Treatment with the MEK inhibitor PD98059, which blocks ERK activation, had no effect on baseline apoptosis or on SNP induced apoptosis (Figure 5.6.5b). Treatment with the JNK inhibitor SP600125 had no effect on baseline apoptosis (Figure 5.6.5c). Treatment with the JNK inhibitor SP600125 did significantly increase SNP induced apoptosis ($p<0.02$) (Figure 5.6.5c). This effect of JNK inhibition on apoptosis was confirmed by TUNEL staining ($p<0.001$) (Figure 5.6.6). Treatment with the p38α/β inhibitor SB203580 had no effect on baseline apoptosis or on SNP induced apoptosis (Figure 5.6.5d).

5.5 Discussion

In recent years, there has been increasing interest in the role of MAPK in the regulation of gene expression in RA synovium. This has led to an increased understanding and importance of the role of MAPK in regulating cytokine expression and cell activation in the context of RA pathogenesis. Previous studies have focused on the role of MAPK in inflammation and joint destruction in RA (Han et al. 1999; Han, Z. et al. 2001). RA is characterised by massive synovial hyperplasia, thought to be due to dysregulation of resident cell proliferation and apoptosis as well as leukocyte migration. This hyperplasia is required for the evolution of pannus, which is responsible for cartilage and bone erosion. Given that both proliferation and apoptosis may be important in synovial hyperplasia, we sought to investigate the role of MAPK in both phenomena.

MAPK are grouped into at least three main families. The JNK and p38 pathways were initially believed to be responsible for mediating responses to inflammatory cytokines, osmotic shock and agents that induce apoptosis (Karin 1995), while the ERK pathway was thought to be responsible for transducing growth factor-dependent proliferation signals (Gille et al. 1995). This assertion was made because of the ability of p38 and JNK to transduce signals resulting in cytokine gene transcription, whereas ERK phosphorylates and activates Elk-1, which trans-activates the serum response element and thereby promotes growth and differentiation (Gille et al. 1995). It is now clear that the roles of MAPK are not as discrete as was previously hypothesised. Cytokines such as IL-1 and TNF can activate all three MAPK pathways (Barchowsky et al. 2000), and all three can activate Elk-1, which mediates growth factor responses by activating the c-fos promoter (Whitmarsh et al. 1995; Whitmarsh et al. 1997). It has previously been

reported that activated MAPK expression is significantly higher in RA tissue compared to osteoarthritis (OA) tissue, and that IL-1 and TNF activate all three MAPK in RA FLS (Schett et al. 2000). The expression of c-fos and c-jun correlates with synovial hyperplasia in RA synovial tissue (Roivainen et al. 1996). As pharmacological inhibitors of MAPK are in preclinical or clinical development in RA, an understanding of their role in the control of synovial hypercellularity is required.

My results show that serum, which is essential for RA FLS proliferation *in vitro*, can activate all three MAPK. When the ERK specific inhibitor (PD98059) is used, JNK activity should be unaffected and yet proliferation was prevented. Similarly, when the JNK specific inhibitor (SP600125) was used, ERK activity should be unaffected and yet proliferation was prevented. This indicates that activation of both JNK and ERK MAPK pathways are operative in RA FLS serum-induced proliferation *in vitro*. Although p38 MAPK was activated by serum in RA FLS, antagonism of the p38 pathway with the specific p38 inhibitor (SB203580) had no statistically significant effect on proliferation.

ERK1/2 nuclear translocation and persistent activation in the G1 phase of cell cycle has been shown to be critical for fibroblast proliferation (Pages et al. 1993; Brunet et al. 1999). *In vitro,* ERK1 deficient mouse embryonic fibroblasts proliferated similarly to wild type, suggesting that ERK2 expression was sufficient for fibroblast proliferation (Pages et al. 1999). T cells from ERK1-/- mice demonstrated significantly reduced proliferation in response to stimuli (Pages et al. 1999). In addition, MEK1-/- mice exhibit an embryonic lethal phenotype at day 10.5, while MEK1-/- embryonal fibroblasts fail to migrate in

response to fibronectin (Giroux et al. 1999). My results confirm that ERK1/2 activation is required for RA FLS proliferation.

The role of JNK in the control of proliferation has been studied in gene knockout mice. It has been shown that JNK1-/- and JNK1/2-/- mouse embryonic fibroblasts (MEF) proliferate slower than wild type MEF (Tournier et al. 2000). MEF lacking c-jun or MEF with a mutation in the JNK bind site of c-jun also proliferate slower than wild type MEF (Johnson, R.S. et al. 1993; Behrens et al. 1999). These data demonstrate that JNK activation is required for normal proliferation in these cells.

Given the importance of IL-1β and TNFα in RA, I investigated the effect of both cytokines on RA FLS proliferation, and the involvement of MAPK in this phenomenon. IL-1β and TNFα treatment increased serum-induced FLS proliferation by between 50%-100%. The ability of IL-1β and TNFα to activate all three MAPK pathways in RA FLS has been demonstrated previously (Lu et al. 2000; Schett et al. 2000). It has also been shown that both IL-1 and TNF can induce an increase in RA FLS proliferation (Arend et al. 1995). However, no link between MAPK activation and IL-1β or TNFα induced RA FLS proliferation has been made. JNK and ERK pathway antagonism significantly inhibited IL-1β and TNFα induced proliferation. These results indicate that JNK and ERK activation are essential for proliferation of RA FLS, whether stimulated by growth factors alone or by these cytokines.

It has been established that IL-1 and TNF utilize the p38 pathway as one of a number of pathways to elicit other pro-inflammatory effects. The antagonism of p38 with SB 203580 in an animal model of arthritis, for example, was associated with inhibition of inflammatory cytokine production and reduced inflammation (Badger et al. 1996). In the studies presented in this chapter, antagonism of p38

was associated with a trend towards partially inhibition of IL-1 and TNF induced proliferation. These results suggest that IL-1β and TNFα may utilize the p38 pathway to enhance RA FLS proliferation, but appear to require JNK and ERK pathway activation for maximal effects. Clearly, p38 pathway activation is not a prerequisite for FLS proliferation.

The importance of apoptosis in maintaining homeostasis in the synovium has gained acceptance in recent years. It is now believed that dysregulation of apoptosis plays an important role in RA synovial hyperplasia. (Okamoto et al. 1997) (Franz et al. 2000). Nitric oxide (NO) has been shown to mediate synovial cell and chondrocyte apoptosis in RA joints (van't Hof et al. 2000). However, understanding of the role of MAPK in RA FLS apoptosis is limited. Antagonism of JNK, ERK and p38 pathways had no effect on basal apoptosis. My results show that exposure to SNP, at concentrations which induce apoptosis, is associated with activation of JNK and ERK in RA FLS. Given the lack of p38 phosphorylation in response to SNP, it was not unexpected that p38 antagonism had no effect on FLS apoptosis. Interestingly, antagonism of ERK also had no effect on SNP induced apoptosis. In other studies both ERK and p38 have been implicated in rabbit chondrocyte NO-induced apoptosis (Kim et al. 2002). ERK activation was reported to be anti-apoptotic, while p38 activation was reported to be pro-apoptotic in rabbit chondrocytes. p38 antagonism blocks IL-1 induced inducible nitric oxide synthase (iNOS) production in bovine cartilage derived chondrocytes (Badger et al. 1998), however apoptosis was not measured in that study.

Antagonism of JNK significantly increased SNP induced apoptosis of RA FLS. My results indicate that activation of JNK by SNP has a pro-survival role during SNP induced apoptosis. This supports findings by Hong Zhang *et al*, whom

demonstrated that antagonism of JNK by SP600125 in a human colorectal carcinoma cell line (HCT116) increased nocodazole induced apoptosis (Zhang et al. 2002). Embryonic stem (ES) cells lacking MEKK1 (an upstream activator of the JNK pathway) have reduced JNK activity in response to nocodazole, cold stress, mild hyperosmolarity, and microtubule disruption. MEKK1-/- cells also show an increased sensitivity to nocodazole induced apoptosis (Yujiri et al. 1998). In activated T cells, the JNK/c-jun pathway promotes the pro-survival signal of insulin-like growth factor-1 (IGF-1) receptor mediated protection from Fas-induced death (Walsh et al. 2002). These findings highlight the pro-survival function of JNK in different cell types and in response to diverse stimuli.

In contrast, it has also been shown that JNK has pro-apoptotic effects in other cell types. JNK1/2-/- mouse embryonic fibroblasts (MEF), for example, show resistance to UV induced apoptosis (Tournier et al. 2000). Suppression of JNK and p38 activity by PKC transfection protected RAW264.7 cells from SNP mediated apoptosis, by inhibiting caspase 3 activity (Jun et al. 1999). The demonstration of opposite effects of JNK activation in the regulation of apoptosis in different cell types mandate the study of its role directly in each cell of interest, in this case RA FLS.

My finding that JNK is involved in the regulation of RA FLS proliferation and apoptosis supports data obtained in animal models of arthritis, which suggest that JNK plays an important role in joint destruction. It was reported that administration of SP600125 in rat adjuvant-induced arthritis reduced joint damage (Han, Z. et al. 2001). The study of JNK2 knockout mice in a passive murine collagen-induced model of arthritis has also demonstrated a role for JNK in matrix degradation (Han et al. 2002). Neither study investigated the role of JNK in

synovial proliferation or apoptosis. In the light of findings, it is possible that the reduced joint destruction associated with JNK pathway antagonism or disruption in animal models of arthritis could in part be due to decreased proliferation or increased apoptosis of resident cells.

The opposing roles MAPK have in different cell types under different stimuli supports investigation to elucidate the roles of MAPK in all the cells in the RA pannus. Potential clinical application of MAPK inhibitors has focused on the need for specific antagonism of individual kinases. The current data suggest JNK antagonism is most likely to result in a favourable result in terms of synoviocyte proliferation and apoptosis.

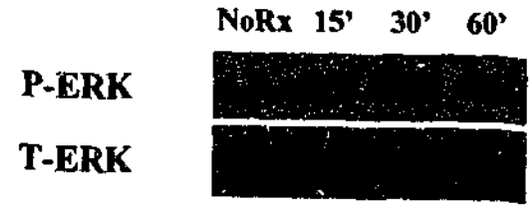
5.6 Figures

Figure 5.6.1

Serum activation of RA FLS MAPK

RA FLS were stimulated with 10% serum and the level of phosphorylated and total JNK1/2, ERK1/2, and p38 were measured by Western blot analysis (Figure 2a, c, and e respectively). Densitometry of phosphorylated JNK1/2, ERK1/2 and p38 was also measured (Figure 2b, d and f respectively). Results are representative of 4 independent experiments.

a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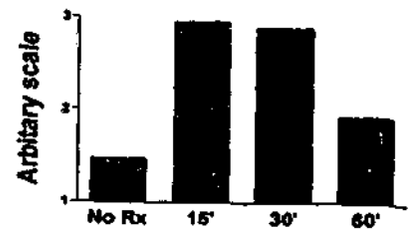
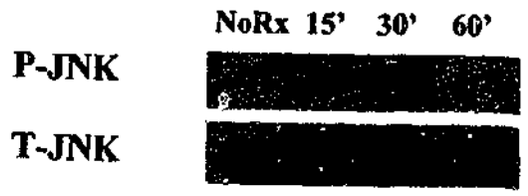
P-ERK
T-ERK

b


Arbitary scale
No Rx
15'
30'
60'

c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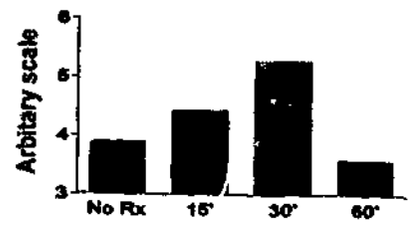
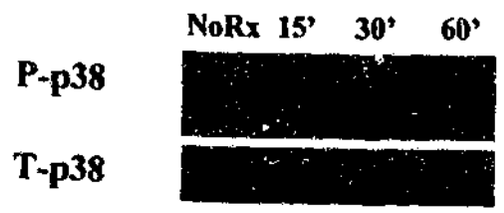
P-JNK
T-JNK

d


Arbitary scale
No Rx
15'
30'
60'

e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P-p38
T-p38

f

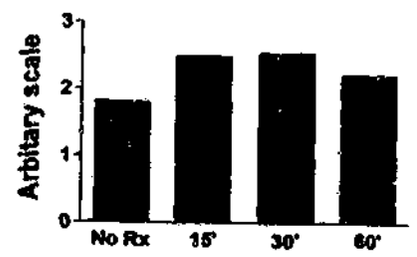
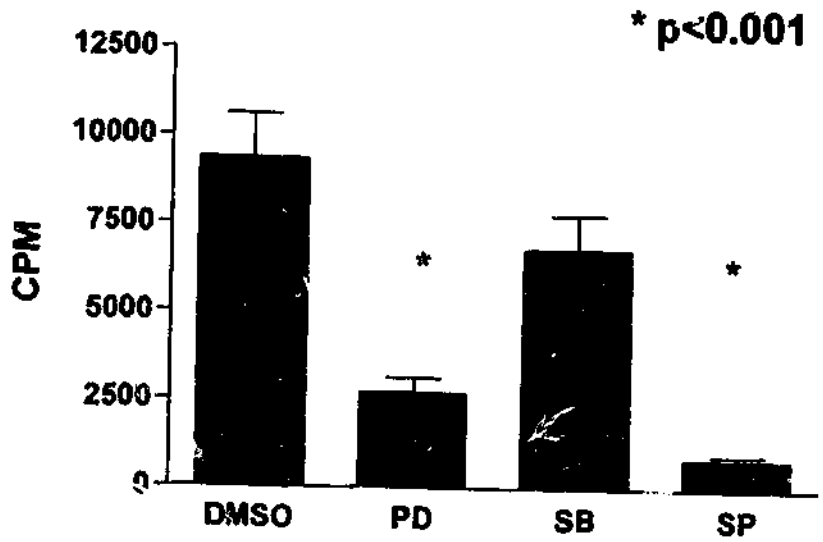

Arbitary scale
No Rx
15'
30'
60'

Figure 5.6.2

MAPK Antagonism of RA FLS Proliferation

RA FLS were incubated with specific MAPK inhibitors for ERK (PD98059:PD), p38 (SB203580:SB), JNK (SP600125:SP), or vehicle (DMSO) during serum stimulated proliferation. Synoviocyte proliferation was estimated by measuring [3H]thymidine incorporation. Results are expressed as mean cpm ± SEM of 4 independent experiments performed in triplicate (*p<0.001).


* p<0.001
12500
10000
7500
5000
2500
0
CPM
*
*
DMSO
PD
SB
SP

system in the SCID mouse. These characteristics have been described as indicating that RA FLS have a "transformed" phenotype.

FLS proliferate *in vitro*, however by passage 10-12 growth gradually diminishes and cells become somewhat senescent. During long term culture with addition of no other stimuli, RA FLS can constitutively secrete a number of cytokines and growth factors, such as IL-6, transforming growth factor (TGF) β, macrophage migration inhibitory factor (MIF) and fibroblast growth factor (FGF).

As reviewed in chapter 1, MIF is the only known pro-inflammatory cytokine that is induced by physiologic concentration of glucocorticoids (GC) and which antagonizes the actions of GC. MIF has a broad range of actions including upregulation of macrophage and T cell function, and induction of macrophage cytokine and mediator release. MIF has been reported in RA synovium and FLS, and a critical role has been identified for MIF in animal models of arthritis. Furthermore, MIF induces PGE_2 production, and PLA_2 and COX2 expression in RA FLS. More recently, MIF has also been shown to suppress p53-mediated growth arrest *in vitro,* which is in keeping with its suggested role in tumorigenesis. The mechanisms by which MIF exerts its effects have not been fully elucidated. It has been demonstrated that MIF does not utilizes the NF-κB signal transduction pathway; however MIF induction of proliferation in NIH3T3 cells is associated with ERK1/2 pathway activation.

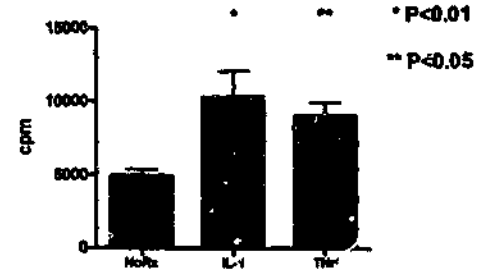
This thesis examines the signal transduction pathways utilized by MIF to exert its effects on RA FLS. In chapter 2, data is presented which indicates that MIF activates cells via MAPK pathways. MIF induced phosphorylation of ERK1/2 and p38 MAPK in a time dependent manner in RA FLS as measured by Western blotting. Antagonism of the ERK pathway with the specific MEK1/2 inhibitor

Figure 5.6.3

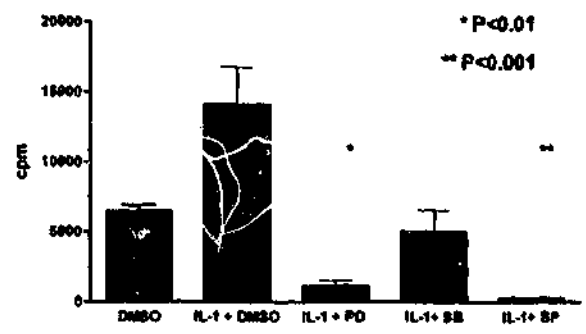
MAPK Antagonism of cytokine induced FLS proliferation

FLS were stimulated with 0.1ng/ml IL-1β or 1ng/ml TNFα for 72hr in RPMI containing 10% FCS with or without specific MAPK inhibitors for ERK (PD98059:PD), p38 (SB203580:SB), JNK (SP600125:SP), or vehicle (DMSO) (Figures 3a-c respectively). Synoviocyte proliferation was estimated by measuring [3H]thymidine incorporation. Results are expressed as mean cpm ± SEM of 4 independent experiments performed in triplicate.

a


* P<0.01
** P<0.05
cpm
15000
10000
5000
0
IL-1

b


* P<0.01
** P<0.001
cpm
20000
15000
10000
5000
0
DMSO
IL-1 + DMSO
IL-1 + PD
IL-1+ SB
IL-1+ SP

c

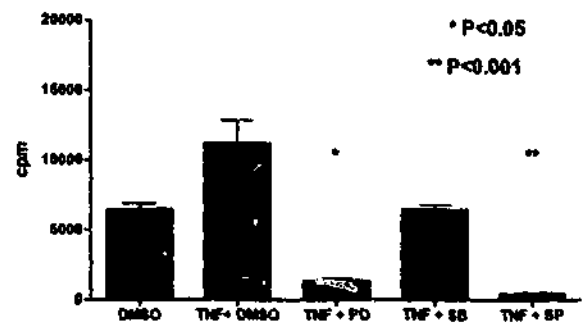
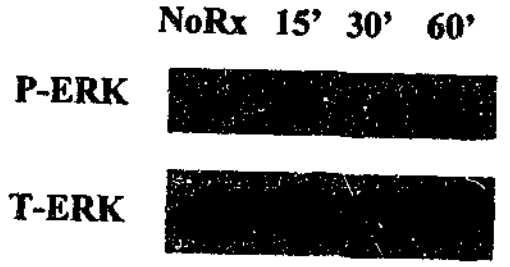

* P<0.05
** P<0.001
cpm
20000
15000
10000
5000
0
DMSO
TNF+ DMSO
TNF + PD
TNF + SB
TNF + SP

Figure 5.6.4

SNP activation of RA FLS MAPK

RA FLS were cultured with 0.1mM SNP and the level of phosphorylated and total JNK1/2, ERK1/2, and p38 was measured by Western blot analysis (Figure 4a, c, and e respectively). Densitometry of phosphorylated JNK1/2, ERK1/2 and p38 was also measured (Figure 4b, d and f respectively). Results are representative of 4 independent experiments.

a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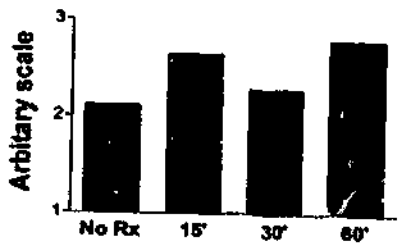
P-ERK
T-ERK

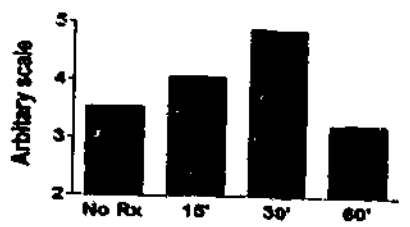
b


Arbitary scale
3
2
1
No Rx
15'
30'
60'

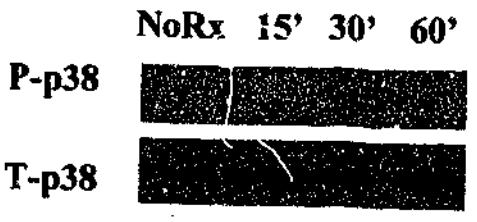
c


NoRx 15' 30' 60'
P-JNK
T-JNK

d


Arbitary scale
5
4
3
2
No Rx
15'
30'
60'

e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P-p38
T-p38

f

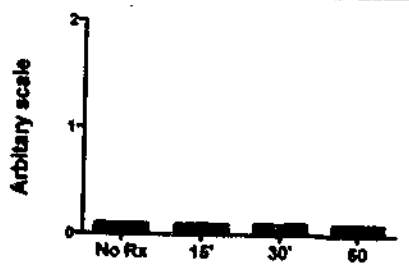
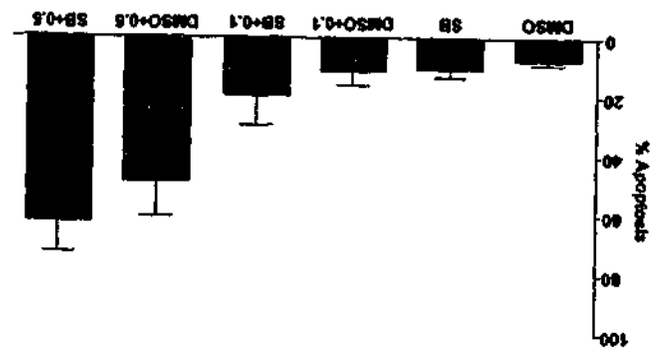

Arbitary scale
2
1
0
No Rx
15'
30'
60

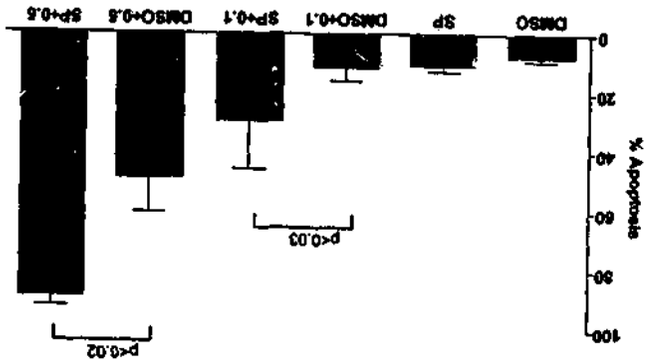
Figure 5.6.5

Effect of MAPK inhibition on SNP induced apoptosis of RA FLS.

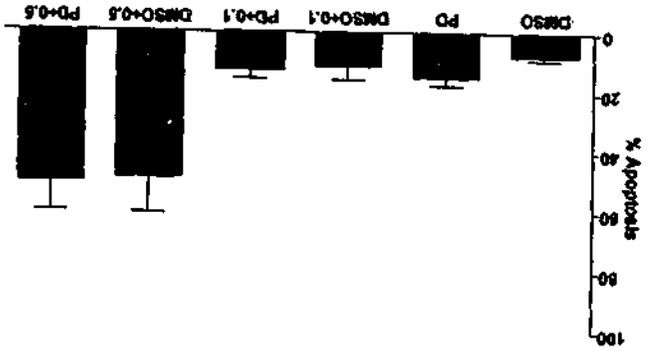
RA FLS were incubated with vehicle (DMSO) or specific MAPK inhibitors for JNK (SP600125:SP), ERK (PD98059:PD), or p38 (SB203580:SB), during SNP induced apoptosis (Figure 5a-d respectively). Results are expressed as mean % ± SEM of apoptotic cells as measured by Annexin V and PI staining. Results are mean ± SEM of 6 independent experiments performed in duplicate.


DMSO
SB
DMSO+0.1
SB+0.1
DMSO+0.5
SB+0.5
% Apoptosis
0
20
40
60
80
100

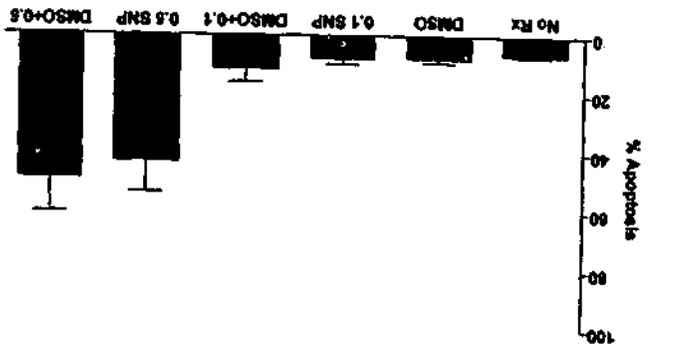
d


DMSO
SP
DMSO+0.1
SP+0.1
DMSO+0.5
SP+0.5
p<0.02
p<0.03
% Apoptosis
0
20
40
60
80
100

c


DMSO
PD
DMSO+0.1
PD+0.1
DMSO+0.5
PD+0.5
% Apoptosis
0
20
40
60
80
100

b

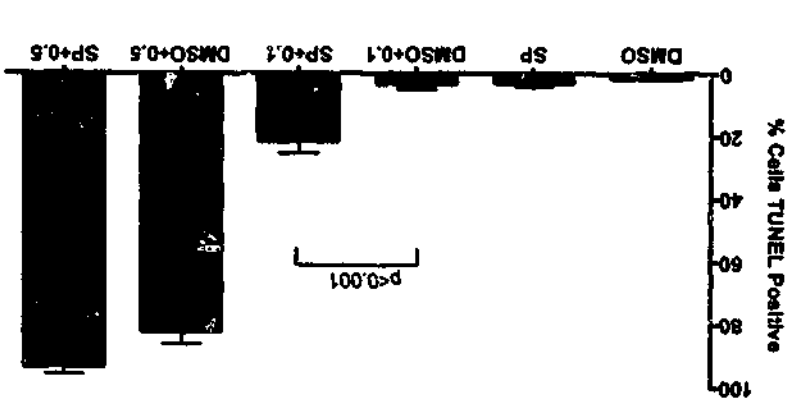

No RX
DMSO
0.1 SNP
DMSO+0.1
0.5 SNP
DMSO+0.5
% Apoptosis
0
20
40
60
80
100

a

Figure 5.6.6

Effect of JNK inhibition on TUNEL Staining in RA FLS

RA FLS were incubated with vehicle (DMSO) or a specific MAPK inhibitor for JNK (SP600125:SP), during SNP induced apoptosis. Results are expressed as mean ±SEM of TUNEL positive cells. Results are of 6 independent experiments performed in duplicate.


DMSO
SP
DMSO+0.1
SP+0.1
DMSO+0.5
SP+0.5
0
20
40
60
80
100
% Cells TUNEL Positive
p<0.001

Chapter Six:

Summary and conclusions

6.1 Summary and conclusions

Rheumatoid arthritis (RA) is a chronic systemic disease that affects approximately 1% of the population. RA results in inflammation and deformity preferentially involving the metacarpal and interphalangeal joints of the hands and feet. The pathology of RA is increasingly well understood, characterised by synovial hyperplasia, inflammation and altered systemic immune responses. Chronic synovial inflammation involves dysregulated proliferation and apoptosis of resident synovial cells, and infiltration by activated leukocytes. Destruction of articular cartilage and subchondral bone, and subsequent erosion of joints, is mediated in large part by invasive synovial tissue.

As outlined in chapter 1, highly vascularised inflamed synovium that resembles granulation tissue and is located at the synovial interface with cartilage and bone is known as pannus. The pannus consists mainly of macrophages and fibroblasts, which are thought to be invasive synovial lining cells. The cells within the pannus are responsible for the active invasion of cartilage and bone, and ultimately, joint destruction. These cells also release cytokines and prostaglandins, which perpetuate the inflammatory process, as well as matrix metalloproteinases (MMPs), that perpetrate tissue damage.

The expansion of FLS in synovial hyperplasia is important in the generation of pannus and hence joint destruction; the mechanisms behind this synovial lining expansion remain controversial. FLS derived from RA synovial tissue display an autonomous invasive and proliferative phenotype. In culture, RA FLS grow at a faster rate than control cells, can escape contact inhibition, and grow in an anchorage-independent manner. In contrast to osteoarthritis FLS, RA FLS are capable of autonomous invasion of human cartilage in an engrafted co-culture

PD98059 suppressed MIF-induced proliferation. Antagonism of the p38 pathway with the specific p38 inhibitor SB203580 had no effect on MIF-induced proliferation. This result indicates that although MIF can activate the ERK and p38 pathways in RA FLS, only the ERK pathway is involved in MIF-induced proliferation. Additionally, MIF is involved in IL-1β- and TNFα-induced proliferation of RA FLS, as inhibition of MIF with anti-MIF Abs abrogates the effects of IL-1β and TNFα on FLS proliferation.

The ability of endogenous MIF to modulate the amplitude of IL-1β and TNFα stimulation on fibroblasts was explored in chapter 3. MIF-/- dermal fibroblasts demonstrated a significant reduction in proliferation in response to IL-1β and TNFα compared to wild type fibroblasts. This phenomenon in MIF-/- fibroblasts was associated with hypo-activation of MAPK in response to IL-1β and TNFα. MIF-/- fibroblasts demonstrated both delayed and reduced activation of ERK1/2, p38 and JNK1/2 MAPK. These results suggest that MIF may act to prime cells or to amplify cytokine signaling via MAPK, and thereby modulate the degree of stimulation induced by other pro-inflammatory cytokines. The mechanisms MIF utilizes to accomplish this remain to be elucidated. These results also support a role for MIF as an upstream regulator of synovial hyperplasia in RA.

IL-1β and TNFα are two important cytokines in RA pathogenesis as demonstrated by clinical improvement in a subset of RA patients who respond to anti-IL-1β and anti-TNFα therapies. My results indicate a possible mechanism by which MIF suppresses the effects of glucocorticoids, through modulating the degree of MAPK activation by IL-1β and TNFα. My results also suggest that antagonism of MIF could potentially regulate the inflammatory action of IL-1β, TNFα and possibily other cytokines in a single therapy. Through inhibiting the inflammatory

effects of proinflammatory cytokines, MIF targeted therapeutics could increase the potency of glucocorticoids and allow for lower doses of glucocorticoids to be administered. This would reduce side effects associated with high dose glucocorticoids. This may to be accomplished by MIF effects on MAPK pathways via an as yet unkown mechanism. Some possible mechanisms by which MIF acts could include the regulation of the IL-1 and TNF receptors by a mechanism similar to MIF's known effects on *TLR4*. Alternatively, MIF may regulate the levels or activation state of intracellular signaling proteins such as protein kinases, scaffolding and/or accessory proteins and thereby allow signals by pro-inflamnatory cytokines to flow through the MAPK pathways.

In addition, a new class of drug is currently in development which targets individual signal transduction pathways, such as Vertex 745, which targets p38 MAPK. In blocking all signaling through a particular signaling module, such drugs could have adverse side effects, as not all signals through a given signaling module are deleterious. My results demonstrate that MIF is required for complete activation of MAPK pathways by IL-1β and TNFα. Given this result, MIF targeted therapies could antagonise multiple MAPK pathways in reponse to proinflammatory stimuli, without blocking potentially beneficial signals through the same MAPK pathways.

A number of studies have shown evidence of defects in apoptosis in RA synovium, suggesting that dysregulated apoptosis may contribute to synovial hyperplasia. Recently it has been demonstrated that there is little apoptosis in RA synovium. Although there is a relatively high degree of DNA strand breaks in RA FLS, there may be an inability of RA FLS to complete apoptosis. The description of somatic mutations of p53 in RA FLS could explain the aggressive and invasive

phenotype of RA FLS. Experiments where p53 was functionally inactivated in normal FLS resulted in a RA FLS-like phenotype, thereby providing support for p53 inactivation as a contributing factor in RA. In contrast, a separate study in patients in another part of the world reported that no mutations in p53 were detected. MIF has recently been identified as being the only pro-inflammatory cytokine capable of functionally inactivating p53. Given the overexpression of MIF in RA, and its ability to functionally inactivate p53, as well as the importance of p53 in RA, I investigated the influence of MIF on p53 and apoptosis in RA FLS.

In chapter 4, I presented data demonstrating the ability of exogenous MIF to down regulate p53 protein levels in RA FLS. Examination of p53 protein levels in MIF-/- dermal fibroblasts confirmed endogenous MIF down regulates p53 protein levels. The functional relevance of this phenomenon was tested by measuring MIF effects on p53 mediated apoptosis. I show that MIF protects RA FLS from SNP mediated apoptosis, a process which utilizes p53 dependent pathways. Similarly, MIF-/- fibroblasts displayed increase basal apoptosis and increased sensitivity to p53 mediated apoptosis compared to wt fibroblasts. A transient decrease of p53 protein at sites of inflammation can conceivably be beneficial. This would enable immune cells to execute their functions in a potentially genotoxic environment without undergoing apoptosis. Alternatively, at sites of chronic inflammation, prolonged overexpression of MIF could have deleterious consequences, impairing apoptosis in cells that would otherwise undergo apoptosis due to DNA damage. This scenario could allow DNA mutations to occur at site of chronic inflammation such as the joints of RA patients.

MIF targeted therapies could restore balance between apoptosis and proliferation of RA FLS. This could lead to a reduction in FLS numbers and pannus size in patients with RA and a subsequent reduction in joint inflammation. In the broader context of human disease, MIF blockadge in cancers, for example, could increase the effectiveness of anti-cancer therapies by increasing apoptosis. My results demonstrate that the presence of MIF protects cells from apoptosis.

MAPK have multiple roles in different cells and it is necessary to be aware of their functions in individual cell types. MAPK are important in a number of cellular processes and have been shown to be highly expressed in RA synovium compared to OA and normal tissue. In chapter 5, I present data showing in RA FLS that activation of the ERK and JNK MAPK pathways but not the p38 MAPK pathway are required for RA FLS proliferation. Secondly, activation of the JNK MAPK pathway has a protective effect against SNP induced RA FLS apoptosis, as antagonism of the JNK MAPK pathway but not the ERK or p38 MAPK pathways increased RA FLS sensitivity to SNP induced apoptosis. MAPK targeted therapeutics, which blocks all signaling through a give MAPK module are being developed, and have progressed to clinical trials. It is therefore important to establish the range of functions of individual MAPK modules induced by various stimuli. Although traditionally it has been thought that the JNK pathway transduced apoptotic signals, my results demonstrate that the JNK pathway can transduce pro-survival signals in FLS. This highlights the importance of investigating the function of MAPK pathways in individual cell types and by different signals. In addition, comparison of my results in FLS with results reported in other cell types indicate that blocking one MAPK pathway may have

the desired effect in one cell type, but may have the opposite effect in another cell type.

Further experiments which would be desirable as a result of the data presented in this thesis include an assessment of direct activation of JNK in FLS by MIF. A more detailed assessment of the signal transduction events that are impaired in the absence of MIF would be achieved by adding MIF back to MIF-/- cells. A more detailed investigation of the relationship between MIF and p53, including effects on p53-induced genes is of great interest, however, such experiments have been limited by the limitied availability of active recombinant MIF protein.

Studies presented in this thesis extend knowledge of MIF, MAPK and p53 in relation to RA. These data identify MIF as a therapeutic target in the treatment of RA with novel effects on synoviocyte MAPK activation, p53 expression, proliferation, and apoptosis. The development of novel therapeutic drugs targeting signal transduction components and in particular components of the MAPK pathways, herald a new class of drugs for regulating inflammation. It is necessary to understand the multiple and complex functions of each component of each MAPK module in order to target and design drugs without serious side effects. Therapeutic suppression of MIF could conceivably inhibit MAPK activation and synoviocyte proliferation, while simultaneously increasing p53 and apoptosis.

Appendix

Appendix 1

Serum concentration	Fetal calf serum
% (v/v)	av cpm±SEM
0%	844.1±110.0
10%	10703.6±570.0
20%	8906.4±728.5

Table 1

Serum induced proliferation of FLS

RA FLS were plated at a density of 1 x 10^5 cells/plate. Cells were incubated for 72hr in RPMI containing 0%, 10% or 20% (v/v) of FCS and pulsed with [3H]thymidine in the last 18hr. FLS proliferation was estimated by measuring [3H]thymidine incorporation. Results are expressed as mean ± SEM cpm of 4 independent experiments performed in triplicate.

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