Regulation of Matrix Metalloproteinases, their Inhibitors and IL-8 in Inflammatory Rheumatic Diseases: Effects of Cytokines and Anti-rheumatic Agents

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Erratum

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22.	Duche et al is replaced by Williamson et al, 1993
32.	Dinarello et al, 1989
33.	Ruscetti et al, 1992
36.	Mukaida et al, 1994 (Mokaida is incorrect spelling)
47.	Koehler is correct spelling
48.	Furst & Kremer, 1988
48.	Segal et al, 1990 b
48.	Songsiridej and Furst 1990
49.	Bannwarth et al, 1994
49.	Brauer et al, 1994
50.	Brinckerhoff et al, 1987
63.	Lyons et al, 1989
79.	McNaul et al, 1990
90.	Chomczynski et al, 1987
97.	Legend for Figure 3.2. a) Collagenase and b) TIMP-1
102.	The asterisks apply to both GSTM as well as GSTG
108.	It is possible that GSTM and TM have independent effects on the mRNA expression for collagenase, VCAM-1 and E-selectin.
111.	Blackburn et al, 1991
124.	Figure 4.6. The double asterisk which pertains to the collagenase result at dexamethasone 100 mM is misplaced below the X axis.
125.	Legend for Figure 4.7. The concentration of dexamethasone should read nM.
134.	Brinckerhoff et al, 1987
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138.	Lennarz and Strittmatter 1991
139.	Overall et al, 1989
139.	Whal 1987
157.	Spalla et al, 1985
178.	Harant et al, 1996
178.	Mukaida et al, 1992b
178.	Calandre et al, 1997
181.	Whether gold compounds act by inhibiting transcription or by promoting transcript degradation remaind to be determined.

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Declaration

This thesis contains no material which had been accepted for the award of any other degree or diploma in any University or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Publications and Presentations

F.Shabani, J.D.McNeil, L.Tippett. The oxidative inactivation of Tissue inhibitor of Metalloproteinases (TIMP-1) by hypochlorous acid (HOCl) is suppressed by anti-rheumatic drugs. (Accepted for publication in Free Radical Research September, 1997).

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F.Shabani, J.D.McNeil, S. Usher, R.Shicchitano and M.Holmes. Inhibition of IL-8 mRNA synthesis and secretion in rheumatoid synoviocytes as a mechanism of action for glucocorticoids. (Presentation at the Annual Scientific Meeting of the Australian and New Zealand Rheumatology Association, May, 1997).

F.Shabani, J.D.McNeil, L.Tippett. Inactivation of Tissue inhibitor of Metalloproteinases (TIMP-1) by oxidants. (Australian and New Zealand Journal of Medicine 1996, 26, No. 1:135).

F.Shabani, J.D.McNeil, L.Tippett. Anti rheumatic drugs inhibit the oxidative inactivation of the Tissue inhibitor of Metalloproteinases (TIMP-1) by hypochlorous acid (HOCl). (Australian and New Zealand Journal of Medicine 1996, 26, No. 1:134).

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F.Shabani, J.D.McNeil, R.Shicchitano and M.Holmes. Transforming Growth Factor-β (TGF-β) regulates the expression of collagenase and tissue inhibitor of metalloproteinases (TIMP-1) genes in human synovial fibroblasts. (Presentation at the 39th Annual Meeting of the Canadian Federation of Biological Societies, 1996).

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Thesis summary

Joint inflammation is a major cause of morbidity in modern society. Rheumatoid arthritis is the most common form of chronic joint inflammation in Australia and other western countries. The morbidity associated with this condition is mediated by the damaging effects that products of inflammation have on the extracellular components of the joint. The most potent damage is mediated by enzymes capable of breaking down the structural proteins that are present within cartilaginous and ligamentous structures. The family of enzymes most responsible for this are the matrix metalloproteinases (MMPs) particularly collagenase and stromelysin. Their activity is antagonized by their inhibitor, tissue inhibitor of metalloproteinases (TIMP-1). TIMPs are produced by synovial lining cells and chondrocytes as well as the neutrophils present in inflamed area.

In this study, I examined the influence of various factors including anti-rheumatic agents on the expression and activity of MMPs and TIMP-1. Furthermore, the effects of these anti-rheumatic agents have been examined on the expression of specific genes related to regulatory cytokine network, such as, interleukin-8 (IL-8) in stimulated human synoviocytes. The specific areas examined were:

A. The complex structure of TIMP-1 maintained by disulfide bonds is such that one would expect it to be inactivated by oxidants. The presence of neutrophils in the acute and chronically inflamed joints provides a potent source of the oxidants HOCl and N-chlorotaurine (NCT). Human recombinant TIMP-1 (hrTIMP-1) was inactivated with HOCl, but not with NCT, as measured by its ability to inhibit the BC1 collagenase activity in vitro. Anti-rheumatic agents differentially altered the oxidative inactivation of the hrTIMP-1.

B. Gold sodium thiomalate (GSTM) is a well established disease modifying anti-rheumatic agent used in the treatment of rheumatoid arthritis (RA). Human synovial fibroblasts in monolayer culture expressed the specific genes for collagenase and TIMP-1 as determined by northern analysis. This expression was significantly increased in response to the pro-inflammatory cytokine IL-1β. GSTM but not its thiomalate (TM) salt, down regulated the level of collagenase gene expression in cytokine stimulated synoviocytes. The ineffectiveness of TM suggests that the relative inhibition of collagenase gene expression appears to be mediated by gold rather than its thiomalate component.

C. The effect of a range of anti-rheumatic agents on cytokine stimulated synoviocytes was examined. enhanced the level of MMP-1 but not TIMP-1 gene expression. Tenidap effectively reduced the gene level of MMP-1, MMP-3 and TIMP-1 in cultured OA synoviocytes. Dexamethasone significantly reduced MMP-1 and MMP-3 gene expression, but not TIMP-1. Cyclosporin-A also partially reduced the expression of MMP-1, whereas methotrexate was inactive.

D. Transforming growth factor-β (TGF-β) is an anti-inflammatory cytokine which is known to regulate the level of MMP-1 and TIMP-1 secretion. In pro-inflammatory cytokine stimulated synoviocytes, TGF-β inhibited the catabolic effects of cytokines on collagenase gene secretion. TGF-β in combination with IL-1β or TNF-α synergistically increased the expression of TIMP-1 mRNA. Similarly, TGF-β also inhibited the stimulatory effects of IL-1β and TNF-α on PGE_2 production. Taken together, TGF-β appears to be capable of protecting extracellular matrix by modulating the expression of collagenase and TIMP-1 mRNA as well as the secretion of inflammatory mediators, e.g. PGE_2.

E. The chemotactic cytokine, interleukin-8 (IL-8) is considered as a major mediator of the continuous accumulation and activation of neutrophils in RA conditions. Since neutrophils are known to be a significant source of MMPs and TIMP-1 in RA joints, the importance of IL-8 is underlined. Besides, IL-8 is likely to retain its activity and to

have a long lasting effect on neutrophil recruitment. Therefore antagonising or inhibiting the synthesis of this cytokine may have important pathophysiological and possibly therapeutical implications. RA synoviocytes secreted substantial amount of IL-8 mRNA and protein in response to the pro-inflammatory cytokines, IL-1β and TNF-β, but not constitutively. IL-8 mRNA and protein levels were determined using northern analysis and enzyme-liked immunosorbent assay (ELISA). Dexamethasone exerted a potent suppressive effect on the production of IL-8 from cultured synoviocytes in the presence of the pro-inflammatory cytokines.

In conclusion, in this thesis I have explored pathways by which therapeutic agents may affect the inflammatory reaction in rheumatoid arthritis. I have confirmed and expanded observation on anti-rheumatic agents that are capable of regulating the activity as well as the expression and production of a variety of inflammatory mediators related to tissue destruction in the inflamed joint.

Glossary

AF	Auranofin
αMs	α-Macroglubolins
AP-1	Activating protein-1
APMA	Aminophenylmercuricacetate
CSF	Colony stimulating factor
CyA	Cyclosporin-A
Cys	Cysteine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra acetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
FCS	Foetal calf serum
FIB-CL	Fibroblast collagenase
FMLP	N-formyl-methionyl-leucyl-phenylalanine
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde-3-phosphate dihydrogenase
GM-CSF	Granulocyte-macrophages colony stimulating factor
GR	Glucocorticoid receptor
GRh	Glucocorticoid receptor-hormone complex
GSTG	Gold sodium thioglucose
GSTM	Gold sodium thiomalate
GuHCl	Guanidine hydrochloride
H_2O_2	Hydrogen peroxide
HBSS	Hank's balanced salt solution
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
His	Histidine
HPLC	High performance liquid chromatography
hrTIMP-1	Human recombinant tissue inhibitor of metalloproteinase-1
IC_{50}	Inhibition constant (50%)
ICAM-1	Intracellular adhesion molecule-1
IFN-gamma	Interferon-gamma
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta

IL-1R	IL-1 receptor
IL-1ra	IL-1 receptor antagonist
IL-1RI	Type I IL-1 receptor
IL-1RII	Type II IL-1 receptor
IL-6	Interleukin-6
IL-8	Interleukin-8
Kb	Kilobases
KDa	Kilodaltons
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic peptide
MDNCF	Monocyte-derived neutrophil chemotactic factor
MIP	Macrophage inflammatory peptide
MME	Macrophage metalloelastase
MMP-1	Matrix metalloproteinase 1, collagenase
MMP-10	Matrix metalloproteinase 10, stromelysin 2
MMP-2	Matrix metalloproteinase 2, gelatinase A
MMP-3	Matrix metalloproteinase 3, stromelysin 1
MMP-11	Matrix metalloproteinase 11, stromelysin 3
MMP-9	Matrix metalloproteinase 9, gelatinase B
Mr	Molecular weight
MT-MMP	Membrane-type matrix metalloproteinase
NADPH	β-nicotinamide adenine dinucleotide phosphate
NAP-2	Neutrophil activating peptide-2
NCT	N-chlorotaurine
NEM	N-ethylmaleimide
NMR	Nuclear magnetic resonance
OA	Osteoarthritis
PAF	Platelet activating factor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E_2
PKC	Protein kinase-c
PLA_2	Phospholipase A_2
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocyte
PMN-CL	Polymorphonuclear collagenase
PMSF	Phenyl methyl sulfonyl fluoride
PUMP-1	Putative metalloproteinase-1, matrilysin

RA	Rheumatoid arthritis
rh	Recombinant human
RI	Relative intensity
RT	Room temperature
SBTI	Soy bean trypsin inhibitor
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
sTNFR	Soluble tumour necrosis factor receptor
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TGF-β	Transforming growth factor beta
TIMP-1	Tissue inhibitor of metalloproteinase-1
TIMP-2	Tissue inhibitor of metalloproteinase-2
TIMP-3	Tissue inhibitor of metalloproteinase-3
TIMP-4	Tissue inhibitor of metalloproteinase-4
TIMPs	Tissue inhibitor of metalloproteinases
TM	Thiomalate
TNF-α	Tumour necrosis factor alpha
TNFR	Tumour necrosis factor receptor
TPA	Tetra decanoyl phorbol acetate

Chapter 1

Introduction and review

1.1. General introduction

A hallmark of rheumatic disease is the irreparable degradation of the extracellular matrix (ECM). Enzymatic action of proteinases on the various components of the ECM, causes much of this degradation (Birkedal-Hansen et al, 1993). All four classes of proteinases i.e., serine, cysteine, aspartic, and metallo- contribute to matrix destruction. The matrix metalloproteinases (MMPs) are proteinases that contain tightly bound zinc. MMPs have a major role in physiological resorption of collagen and other macromolecules in developement and postnatal remodeling as well as in pathological resorption associated, for example the destruction of joints in rheumatoid arthritis (RA). MMPs are members of a large subfamily of proteinases and comprise a multi-gene family of at least 16 members. MMP genes are among the most abundant of those expressed by cells in inflammatory and malignant lesions (Matrisian 1992). The MMPs have several structural features in common that include a propeptide domain that contains the "cysteine switch", the catalytic zinc-binding domain, and a hemopexin-like domain. Membership in this family requires that four criteria be met: the enzyme should display proteolytic activity and function outside the cell, and its cDNA should code for a protein sequence for the cysteine switch mechanism (PRCGxPD) and for the binding of the catalytic zinc (HExGHxxGxxHS/T). The members of this family, can be grouped into three main classes based on substrate specificity. All are synthesized and secreted in a latent proenzyme form and require proteolytic cleavage for activation.

The expression of MMPs is regulated at the transcriptional level in response to a variety of stimuli. Cytokines and growth factors such as interleukin-1α (IL-1α) and IL-1β, epidermal growth factors (EGF), platelet-derived growth factor (PDGF), and tumour

necrosis factor-α (TNF-α) are all potent inducers of both collagenase and stromelysin. They are also regulated at the protein level by a number of inhibitors particularly their specific inhibitor, tissue inhibitor of metalloproteinases (TIMP). Connective tissue cells including fibroblasts, osteoblasts, and chondrocytes are the major source of these enzymes and their inhibitors. Studies with connective tissue cells have shown a positive correlation between metalloproteinase expression and tissue damage.

Because connective tissue destruction by collagenase and stromelysin is largely irreversible, an understanding of the way to inhibit their expression and activation is an important area to consider in designing effective therapeutic strategies. There are 2 principle ways to decrease levels of MMPs: inhibition of enzyme synthesis or inhibition of enzyme activity.

TIMPs are produced locally by chondrocytes and synovial fibroblasts. It is likely that much of the connective tissue destruction seen in RA and osteoarthritis (OA) is due to an imbalance between activated MMPs and TIMPs. The other means of decreasing MMP levels is to decrease the actual synthesis of these proteins. 3 classes of compounds comprising transforming growth factor-β (TGF-β), retinoids, and glucocorticoid hormones, appear to suppress MMP synthesis by suppressing gene transcription. Recent studies suggest that transcriptional mechanisms alone are insufficient to account for the regulation of metalloproteinase gene expression, and post-transcriptional effects also appear to be important (Vincenti et al, 1994).

1.2. The family of MMPs

1.2.1. Introduction

Matrix metalloproteinases (MMPs) are a family of metal ion dependent enzymes which play a pivotal role in the degradation and remodelling of the extracellular matrix (ECM) components. ECM is a complex network composed of protein constituents including collagens and elastin, glycoproteins such as laminin, fibronectin, as well as various proteoglycans and glycosaminoglycans. The integrity of the ECM is controlled by a balance between synthesis and degradation of its components. MMPs are believed to be the main physiologically relevant mediators of matrix degradation. This family of proteinases, can hydrolyse peptide bonds in proteins at neutral pH, and require a zinc ion at their active site (Lennarz et al., 1991). Due to the physiological importance of the substrates susceptible to cleavage by MMPs, their activity is tightly regulated at several levels. Thus controlling the activity of MMPs has direct and indirect effects on the destruction of matrix macromolecules (Wilde et al, 1994). The first level of control is at the transcriptional level, regulating the amount of enzyme produced. MMPs are synthesised as inactive zymogens and their activation forms the second level of the control. Finally these enzymes have natural inhibitors, TIMPs, which form the third level of control. These inhibitors inhibit MMPs by making inactive complexes with active and sometimes latent enzymes (Bodden et al, 1994 ; Birkedal-Hansen et al, 1993).

1.2.2. Definition and common properties

MMPs are a family of enzymes which require intrinsic Zn^{+2} and extrinsic Ca^{+2} for full activity. They are secreted from a variety of connective tissue cells and polymorphonuclear leucocytes in an inactive pro enzyme or zymogen form, which can be activated by losing 10-12 KDa in molecular weight at the active site (Emonard et al, 1990). Synthetic inhibitors interact with or remove the Zn^{2+} at the active site of the

enzyme. These include chelators, such as ethylene diamine tetra acetic acid (EDTA), and 1,10-phenanthroline. Biological inhibitors are α_2-Macroglobulin (α_2-M) and TIMPs.

1.2.3. Classification of MMPs

The MMP family have a number of common structural and functional features, however they differ in their substrate specificity (Matrisian et al, 1992) . These enzymes are divided into five subclasses based on their substrate specificity: collagenases, gelatinases, and stromelysins, membrane bound and others. (Table 1.1 is organised, using Nagase et al, 1992; Parsons et al, 1997; Mattei et al, 1997).

Table 1.1. Matrix Metalloproteinase family and substrates

Enzyme	Abbreviation	MMP#	Molecular weight(Da) Latent	Main substrates
Collagenases				
Fibroblast-type collagenase	FIB-CL	MMP-1	57,000/ 52,000	Collagen I. II, III, (III>>I), VII, VIII, X; gelatin; Proteoglycan (PG) core protein
Neutophil/ PMN-type collagenase	PMN-CL	MMP-8	75,000	Same as FIB-CL
Collagenase 3		MMP-13	65,000	
Gelatinases				
Gelatinase A type (IV) collagenase	GL-A	MMP-2	72,000	Gelatin; collagen IV, V, VII, X, XI, elastin; fibronectin; PG core protein
Gelatinase B type (IV) collagenase	GL-B	MMP-9	92,000	Gelatin; collagen IV, V; elastin; PG core protein
Stromelysins				
Stromelysin-1	SL-1	MMP-3	57,000/59,000	Proteoglycan, gelatins, fibronectin, laminin, collagen III, IV, IX, X
Stromelysin-2	SL-2	MMP-10	57,000	Same as SL-1
Others				
Stromelysin-3	SL-3	MMP-11	55,000	Fibronectin, laminin, collagen IV
Macrophage metalloelastase	MME	MMP-12	53,000	Elastin
Putative Metallo-proteinase (Matrilysin)	PUMP-1	MMP-7	28,000	Fibronectin, laminin, collagen IV, gelatin, proCL, PG core protein
Membrane bound Membrane-type (MT)				
MT1-MMP	MMP-14		66,000	Activate Pro-MMP-2
MT2-MMP	MMP-15		64,000	Activate Pro-MMP-2
MT3-MMP	MMP-16		?	?

1.2.3.1. Collagenases (EC 3.4.24.7)

There are three collagenases in the MMP family: fibroblast collagenase (FIB-CL) (MMP-1), polymorphonuclear collagenase (PMN-CL) (MMP-8) and most recently collagenase-3 (MMP-13). FIB-CL with molecular mass of 52-57 KDa is secreted by fibroblasts from skin, mucosa, synovium, cornea and uterus. PMN-CL which is made in PMN leucocytes and stored in granules, is highly glycosylated with the molecular mass of 75 KDa whereas only 70-80% of FIB-CL is glycosylated (57 KDa form). FIB-CL is readily activated by trypsin and plasmin but PMN-CL is not. FIB-CL preferentially cleaves type III collagen whereas PMN-CL has a preference to digest type I collagen. However both of these enzymes can cleave all three α chains of native types I, II and III collagen. This cleavage occurs at Gly-Leu or Gly-Ile bond (residue 775-776), located at a distance three quarters away from the amino-terminus. FIB-CL and PMN-CL can also cleave type VII collagen within helical sequences, generating two fragments of approximately 83 and 80 KDa (Emonard et al, 1990). Collagenase-3 (MMP-13) with molecular weight of 54 KDa is found in tumour cells (Freije et al, 1994). With only 50% identity to the MMP-1 amino acid sequences, the MMP-13 protein more closely resembles the interstitial collagenase isolated from rats and mice (Vincenti et al, 1996).

1.2.3.2. Gelatinases (EC 3.4.24.24)

72 KDa gelatinase A (type IV collagenase) (MMP-2) and 92 KDa gelatinase B (MMP-9) (EC 3.4.24.35) are the two members of this class. They have substrate specificity for denatured collagens (gelatins) and intact type IV, V, VII, basement membrane collagen, elastin and proteoglycan core protein.

1.2.3.3. Stromelysins (EC 3.4.24.17)

Stromelysin 1 (MMP-3), 2 (MMP-10), and 3 (MMP-11) are the three enzymes of this class, which cleave proteoglycan core protein, fibronectin, laminin, gelatins, elastin and types III, IV, V, IX collagens.

The smallest member of the MMP family is known by a number of names, putative metalloproteinase-1, (PUMP-1), matrilysin, matrix metalloproteinase-7, MMP-7 (EC 3.4.24.23) and has a molecular weight of 28 KDa. Matrilysin seems to have the most potent proteoglycanase activity of the MMPs tested. The other member of this family of enzymes is macrophage metalloelastase (MME) (MW, 53 KDa) (MMP-12) (EC 3.4.24) (Birkedal-Hanson et al, 1993 ; Emonard et al, 1990; Bodden et al, 1994) . Membrane-type MMP (MT-MMP), 66 KDa has been identified as an integral plasma membrane protein capable of activating MMP-2. (Sato et al, 1994)

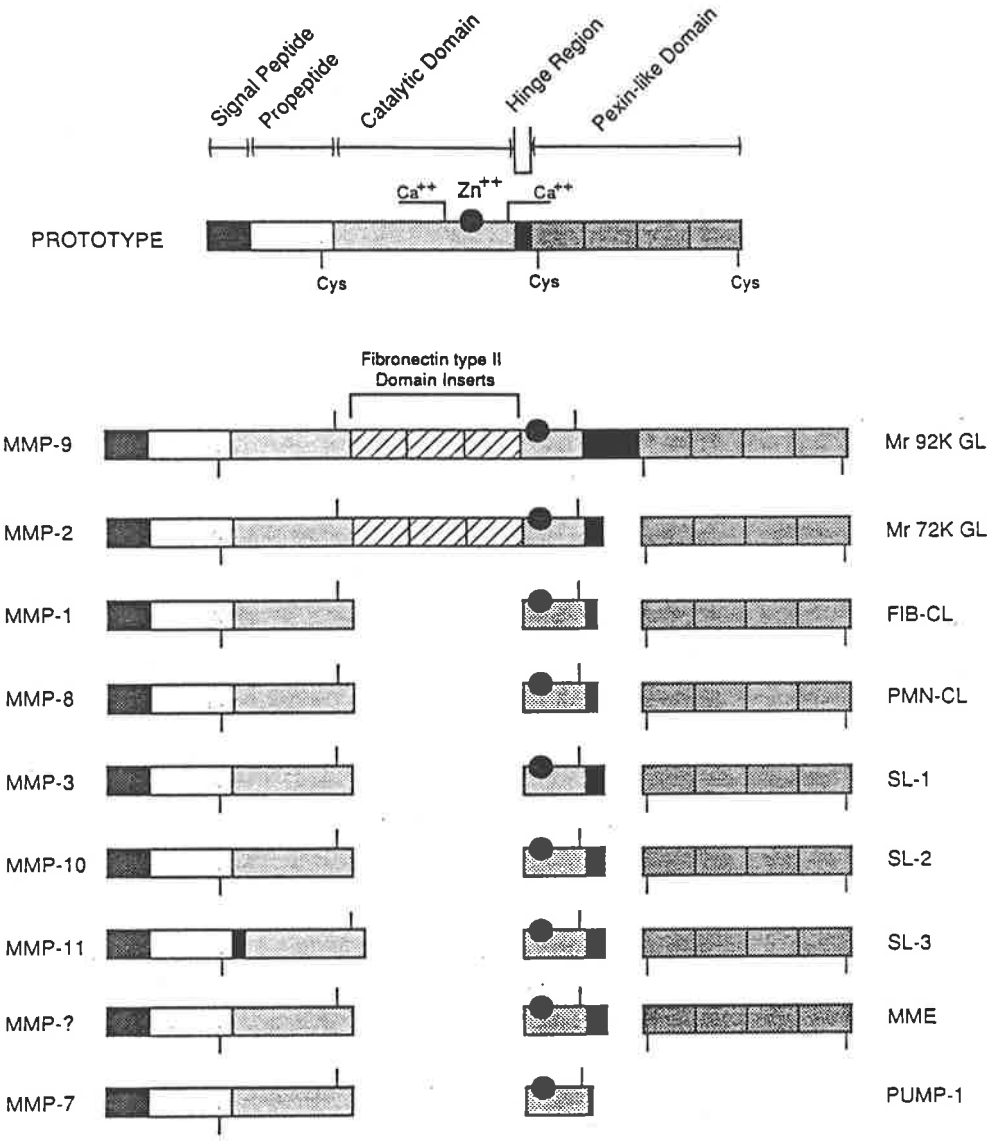
1.2.4. Domain structure of MMPs

The primary sequence of various members of the MMPs suggests that these enzymes are formed of several distinct domains, conserved among family members. These enzymes may be regarded as derivatives of the five-domain modular structure characteristic of collagenases and stromelysins formed either by addition or deletion of domains (Figure 1.1). The first 17-29 residues of the NH_2-terminal domain of the MMP precursor is a leader sequence called a signal peptide. This signal peptide consists of a hydrophobic signal sequence that targets the enzymes for secretion. The following 77-87 residue propeptide is responsible for the latency of the enzyme. The removal of this domain results in activation of the enzyme. A single unpaired cysteine residue (Cys^{73}) in a highly-conserved region of this pro domain (PRCGVPD) is shown to be responsible for the maintenance of enzyme latency (Windsor et al, 1991) . The catalytic domain contains the conserved sequence: HEXGHXXGXXH in human fibroblast collagenase. His-199, His-203, and His-209 are the three ligands that bind zinc which is present in the active

site (Windsor et al, 1994). The next domain is a 5-50 residue, proline-rich hinge region. The COOH terminal region is a hemopexin (a heme-binding protein) or vitronectin (the extracellular matrix component) -like domain consisting of approximately 200 residues, which are held together by a single disulfide bond composed of two Cys residues . This last domain appears to play a role in encoding substrate specificity. The replacement of the carboxy terminal domain from collagenases and stromelysin make these enzymes unable to degrade collagen. This suggests that this domain has an important role in substrate degradation (Matrisian 1992) . PUMP-1 which is the smallest member of this family lacks this domain. The gelatinases contain three repeats of fibronectin type II modules in their catalytic domain that allow the active and latent forms of these enzymes to bind gelatin (Birkedal-Hansen et al, 1993).

Figure 1.1

Domain structure of MMPs. Pro MMPs are consist of five conserved domains, including: signal peptide, propeptide, catalytic domain, hinge region and pexin like C-terminal domain. Figure adapted from Birkedal-Hansen et al, 1993.


Signal Peptide
Propeptide
Catalytic Domain
Hinge Region
Pexin-like Domain
PROTOTYPE
Ca++
Zn++
Ca++
Cys
Cys
Cys
Fibronectin type II
Domain Inserts
MMP-9
MMP-2
MMP-1
MMP-8
MMP-3
MMP-10
MMP-11
MMP-?
MMP-7
Mr 92K GL
Mr 72K GL
FIB-CL
PMN-CL
SL-1
SL-2
SL-3
MME
PUMP-1

1.2.5. Activation of pro MMPs

The activation process of the pro MMPs in the extracellular milieu is an additional key step in the regulation of collagenolysis (Suzuki et al, 1990) . The latent forms of MMPs can be activated by a variety of chemical and physical means. These include treatment with: proteases, conformational perturbants, such as the surfactant sodium dodecyl sulphate (SDS), chaotropic ions (SCN^-), organomercurials aminophenyl mercuricacetate (APMA), oxidants such as NaOCl, disulfide compounds such as oxidised glutathione, and sulfhydryl alkylating agents such as N-ethylmaleimide (NEM) (Van Wart et al, 1990) . Each of these activation mechanisms leads to modification, exposure, or proteolytic release of the Cys residue from the active site Zn^{2+} that is linked to this propeptide residue (Cys^{73} in human FIB-CL) (Springman et al, 1990) (Figure 1.2). In the latent form, the enzyme form is folded to form a covalent bond between the thiol group of the cysteine residue and the active site zinc atom. After cleavage, the protein opens. At this stage an H_2O molecule provides the fourth ligand for the Zinc ion (Figure 1.3). This water molecule appears to be necessary for hydrolysing the peptide bond of the substrate according to the mechanism proposed by Lennarz (Lennarz et al, 1991) . Once the Cys-Zn bond is cleaved, the enzyme itself generates the fully active form by further cleavages at its pro domain. SL-1, SL-2 and PUMP-1 can cause maximum activation in pro collagenases known as "superactivation". The biologic activation of MMPs is still incompletely understood (Figure 1.4).

Figure 1.2

Three different proMMP activation pathways. The latent MMPs may be activated through each of the three different pathways, leading to a open form of the enzyme. These include: cleavage of the pro enzyme by the proteinases (left column) or reaction of the pro domain site cysteine residue with metal ions, organomercurials, oxidizing agents (middle column) and thiol-reactive agents (right column) . Figure adapted from Birkedal-Hansen et al, 1993.

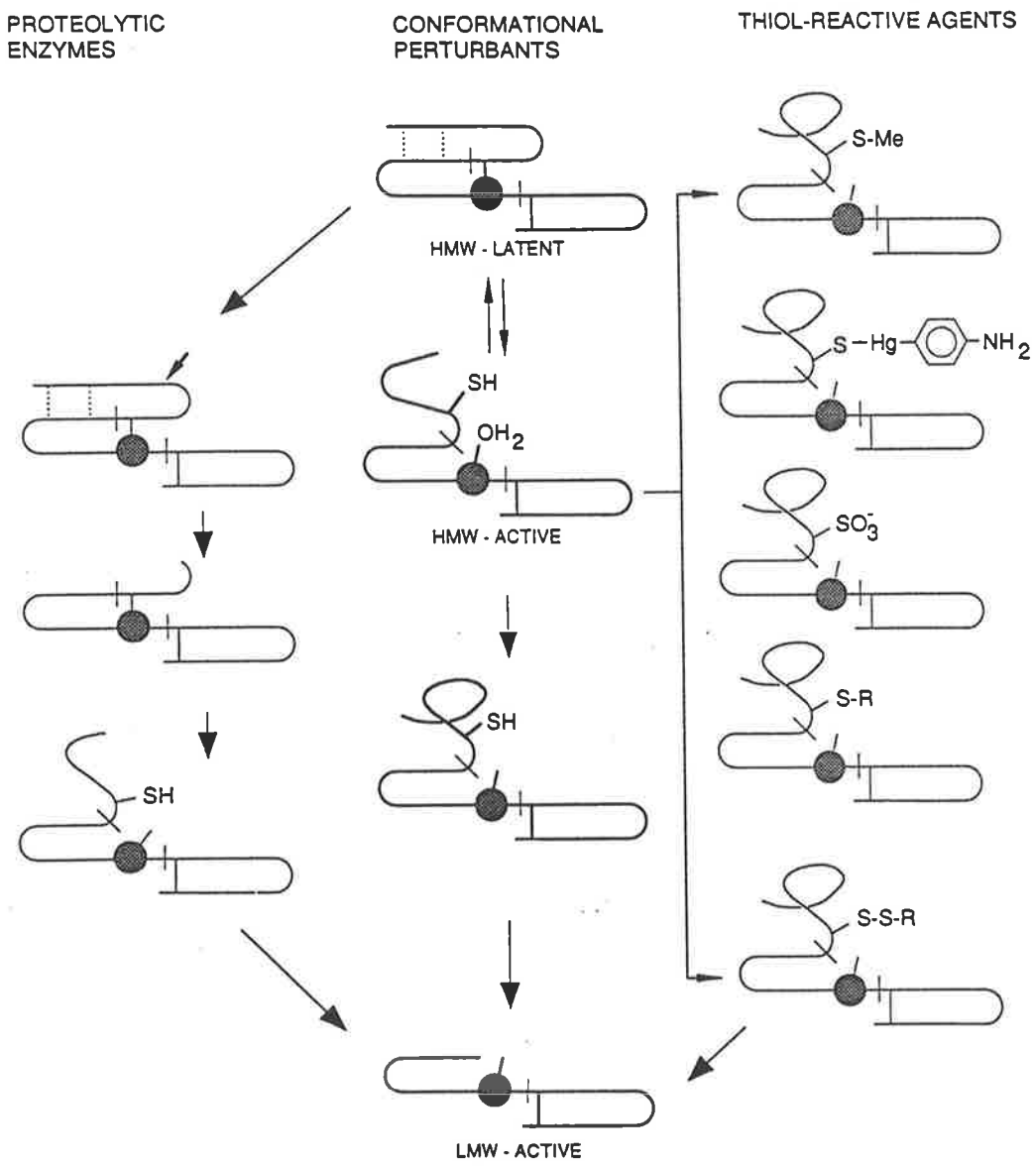
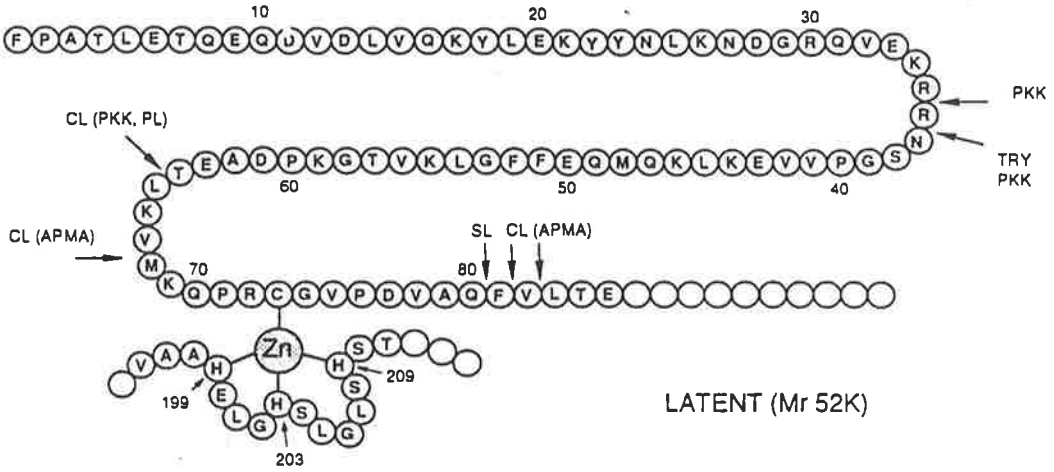
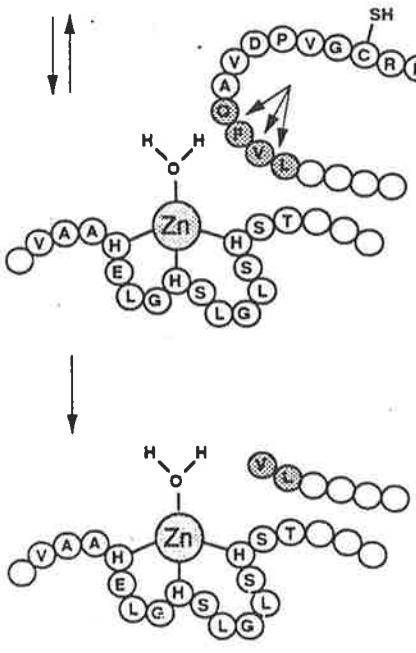

PROTEOLYTIC ENZYMES
CONFORMATIONAL PERTURBANTS
THIOL-REACTIVE AGENTS
HMW - LATENT
HMW - ACTIVE
LMW - ACTIVE
SH
OH2
S-Me
S—Hg—NH2
SO3-
S-R
S-S-R

Figure 1.3

Activation mechanism of latent human FIB-CL according to Cysteine switch model. TRY: trypsin cleavage site; PKK: plasma kallikrein cleavage site; CL (PKK, PL): autolytic cleavage site after the initial cleavage by PKK or PL; CL (APMA): autolytic cleavage site after reaction with 4- APMA; SL: stromelysin cleavage site. Figure adapted from Suzuki (1990) .



LATENT (Mr 52K)

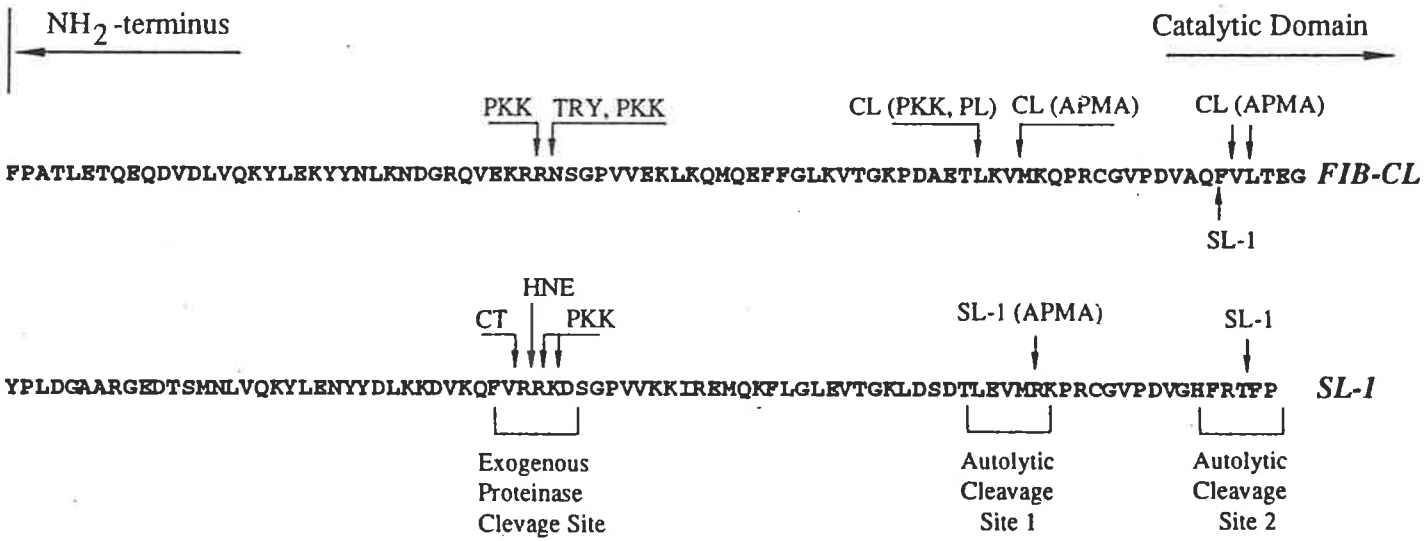


ACTIVE (Mr 52K, 46K, 43K)

ACTIVE (Mr 41K)

Figure 1.4

Proteolytic cleavage of the propeptide associated with activation of human FIB-CL and SL-1. Exogenous proteinases (plasma kalikrein [PKK], trypsin [TRY], chemotrypsin [CT], human neutrophil elastase [HNE], and human stromelysin-1 [SL-1], cleave the proenzyme at different peptide bonds identified by arrows. Autolytic cleavage sites are followed after activation by plasma kallikrein (CL [PKK]) or APMA (CL [APMA]; SL-1 [APMA]). Figure adapted from Suzuki 1990, and Nagase 1990.


NH2 -terminus
Catalytic Domain
PKK
TRY, PKK
CL (PKK, PL)
CL (APMA)
CL (APMA)
FPATLETQEQDVDLVQKYLEKYYNLKNDGRQVEKRRNSGPVVEKLKQMQEFFGLKVTGKPDAETLKVMKQPRCGVPDVAQFVLTEG
FIB-CL
SL-1
HNE
CT
PKK
SL-1 (APMA)
SL-1
YPLDGAARGEDTSMNLVQKYLENYYDLKKDVKQFVRRKDSGPVVKKIREMQKFLGLEVTGKLDSDTLEVMRKPRCGVPDVGHFRTFP
SL-1
Exogenous
Proteinase
Clevage Site
Autolytic
Cleavage
Site 1
Autolytic
Cleavage
Site 2

1.2.6. Gene structure of MMPs

Recent analysis of the exon and intron structure of the genes for MMP members suggests that, the modular structure of MMP genes is highly conserved. The CL, SL-1, and SL-2 genes each contain 10 exons (Figure 1.5). PUMP-1 which only contains the pre, pro, and catalytic domains, lacks exon 6 encoding the hinge region and also exons 7 to 10 encoding carboxy terminal domain. Gelatinase A and B genes each contain 13 exons and 12 introns of 26-27 Kb, and thus have larger genes compare to other MMPs. This is because the three additional fibronectin domains in their catalytic site are encoded with three additional exons.(Huhtala et al, 1991; Matrisian 1992) .

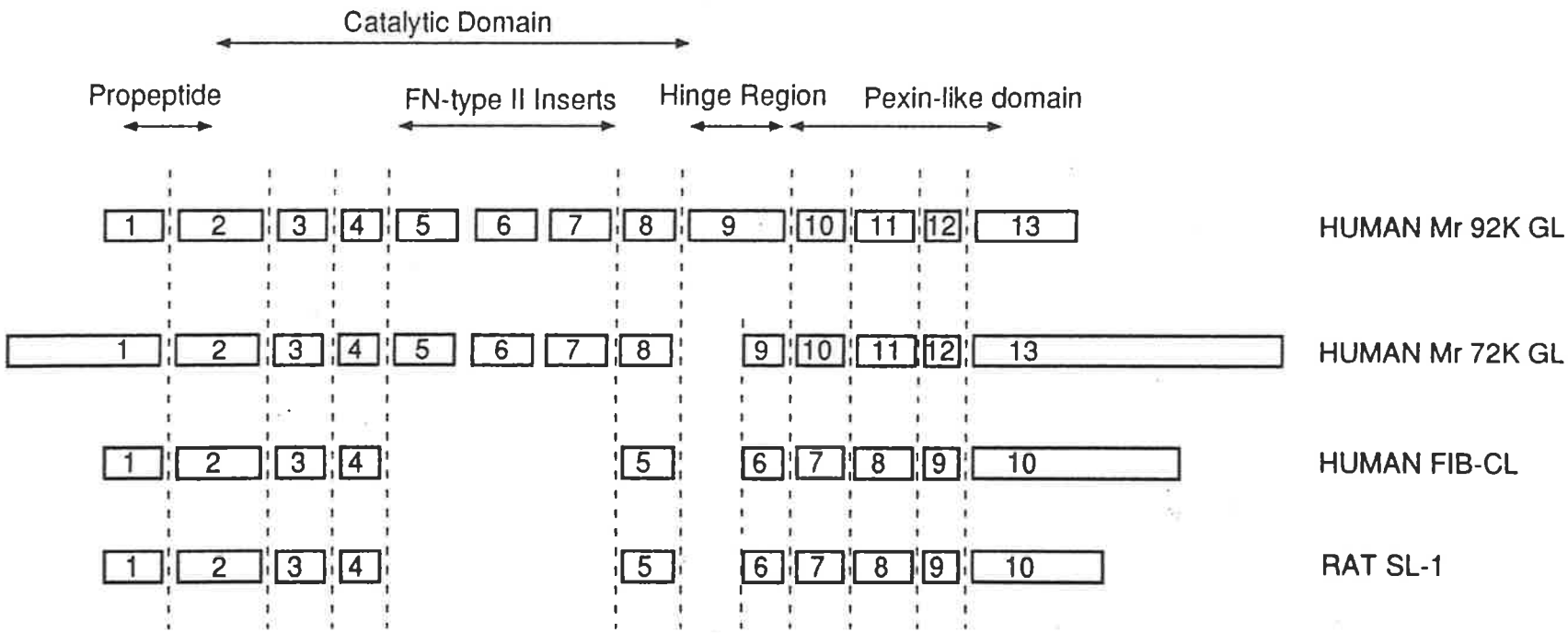
1.2.7. MMP gene expression

The transcriptional mechanisms that control MMP gene expression are still at an early state of definition. Generally transcription of the MMP genes requires the involvement of a number of cis- and trans-acting factors that mediate basal and activated transcription .

The 8-base pair sequence 5'-TGAGTCAC-3', is a phorbol responsive element (TRE), which is located at approximately position -11 in the promoter region of collagenase (-73) and stromelysin (-77). This sequence is called the activator protein-1 (AP-1) binding site (Krane et al, 1990). The AP-1 site binds members of the Fos and Jun families of transcription factors including, c-Fos, Fra-2 and Jun-D. There are currently three identified Jun proteins, c-Jun, JunB and Jun-D, and four Fos related proteins, c-Fos, FosB, Fra-2. There is a second AP-1 element at -186 in the rabbit promoter, 5'-TTAATCA-3', which bind members of Fos and Jun family. Larger fragments of the promoter also enhance transcription, suggesting a contribution from upstream elements, such as the PEA-3 site at 94, 5'-GAGGATGT-3', which is located just upstream of the AP-1 site (White and Brinckerhoff 1995). Recent studies indicate that basal transcription by PMA, cytokines and growth factors requires the specific interaction of AP-1 with other cis-acting elements, particularly PEA3 sites.

Figure 1.5

Exon structure of MMP genes. The exon structure of human FIB-CL, gelatinase B (72 KDa) and gelatinase A (92 KDa) and rat stromelysin is showed associated to the different domains of the proMMPs. Figure adapted from Birkedal-Hansen et al, 1993 and Matrisian 1992 .


Catalytic Domain
Propeptide
FN-type II Inserts
Hinge Region
Pexin-like domain
1 2 3 4 5 6 7 8 9 10 11 12 13
HUMAN Mr 92K GL
1 2 3 4 5 6 7 8 9 10 11 12 13
HUMAN Mr 72K GL
1 2 3 4 5 6 7 8 9 10
HUMAN FIB-CL
1 2 3 4 5 6 7 8 9 10
RAT SL-1

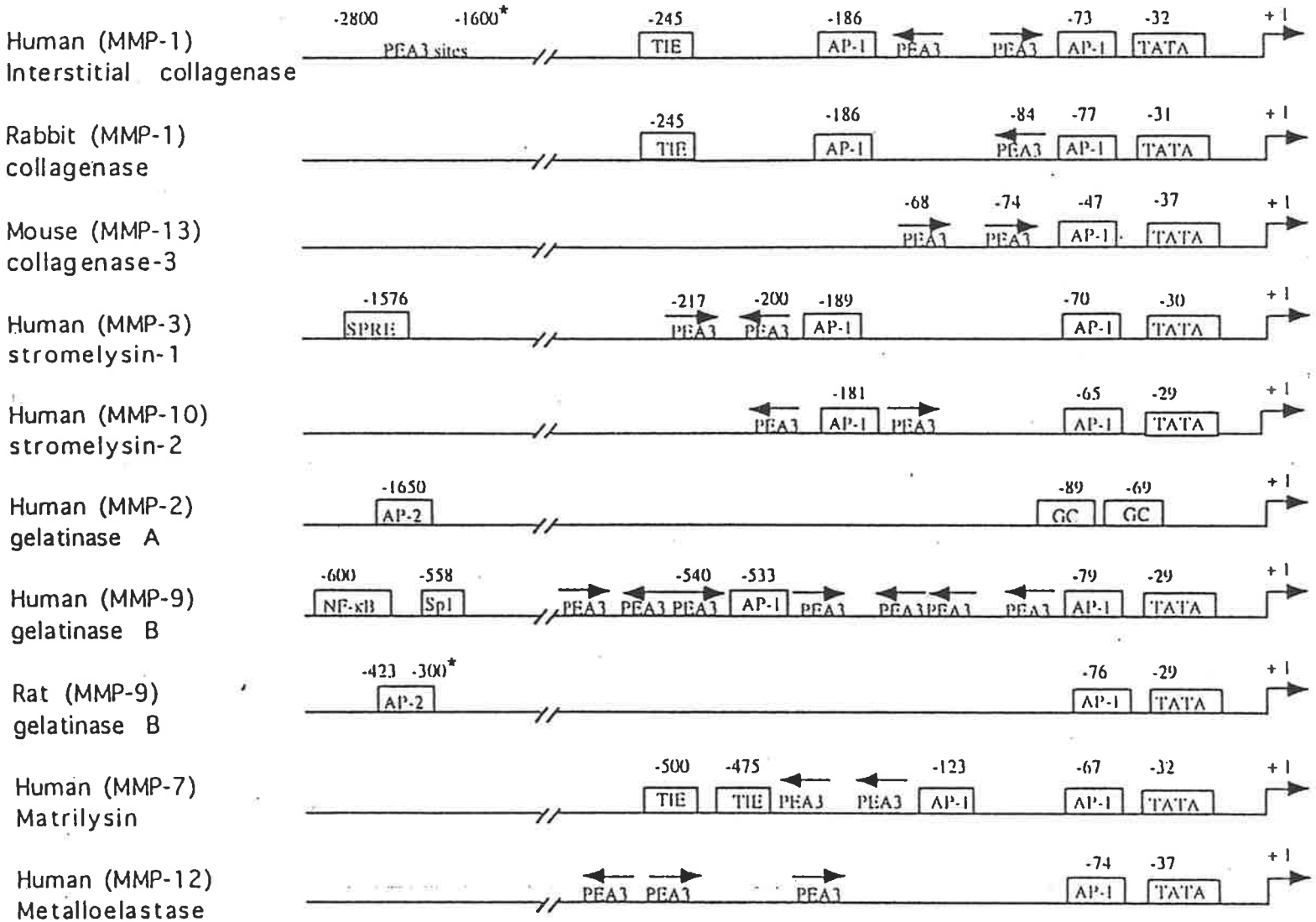
While the AP-1 site is involved in expression of MMPs, the presence of one or more AP-2 elements appears critical. Thus the AP-1 site alone does not regulate transcription of MMPs (Benbow and Brinckerhoff 1997). The promoters of the MMPs exhibit a high degree of similarity in their cis-acting regulatory elements (Figure 1.6), although there are also some differences. MMP-1 and MMP-3 are often but not always coordinately regulated. The similarity between their DNA sequence may explain their coordinate expression in some cells. In contrast, the differential spacing of their PEA-3 sites, and perhaps different binding affinities for transcription factors, may explain their different expression patterns in other cells. Together, complex interactions of the AP-1 site and other cis-acting sequences in the promoters with certain transcription factors that bind to these sequences, control the transcription of the MMPs in response to particular inducers.

In addition to the AP-1 site, other sequences located upstream in the promoter region, are also important, and may even be necessary. Some of these upstream sequences may function independently of the AP-1 site, while others may act in cooperation with it (Brinckerhoff et al, 1992). Recently a binding site for the transcription factor PEA3 has been shown 9 bases upstream from the AP-1 site in the collagenase gene, this protein acts synergistically with AP-1 after phorbol induction (Woessner 1991).

Thus interaction of multiple elements with the promoter appears to be essential for transcriptional control. A variety of stimuli under a variety of normal and pathologic conditions can influence those elements to regulate the metalloproteinase gene expression (Vincenti et al, 1994).

Figure 1.6

Model of MMP promoters showing the location of cis-acting elements known to participate in transcription. Where the exact location of the element is known , the position is indicated. (*) indicates the regions containing elements implicated in transcription. (AP-1, Activator Protein-1 site; PEA3, Polymavirus Enhancer A-binding Protein-3 site; TIE, TGF-β Inhibitory element; GC, Sp-1 binding sites). Figure adapted from Benbow and Brinkerhoff (1997).


Human (MMP-1)
Interstitial collagenase
-2800
-1600*
PEA3 sites
-245
TIE
-186
AP-1
PEA3
PEA3
-73
AP-1
-32
TATA
+1
Rabbit (MMP-1)
collagenase
-245
TIE
-186
AP-1
-84
PEA3
-77
AP-1
-31
TATA
+1
Mouse (MMP-13)
collagenase-3
-68
PEA3
-74
PEA3
-47
AP-1
-37
TATA
+1
Human (MMP-3)
stromelysin-1
-1576
SPRE
-217
PEA3
-200
PEA3
-189
AP-1
-70
AP-1
-30
TATA
+1
Human (MMP-10)
stromelysin-2
-181
PEA3
AP-1
PEA3
-65
AP-1
-29
TATA
+1
Human (MMP-2)
gelatinase A
-1650
AP-2
-89
GC
-69
GC
+1
Human (MMP-9)
gelatinase B
-600
NF-κB
-558
Sp1
-540
-533
PEA3
PEA3
PEA3
AP-1
PEA3
PEA3
PEA3
PEA3
-79
AP-1
-29
TATA
+1
Rat (MMP-9)
gelatinase B
-423
-300*
AP-2
-76
AP-1
-29
TATA
+1
Human (MMP-7)
Matrilysin
-500
TIE
-475
TIE
PEA3
PEA3
-123
AP-1
-67
AP-1
-32
TATA
+1
Human (MMP-12)
Metalloelastase
PEA3
PEA3
PEA3
-74
AP-1
-37
TATA
+1

1.2.8. Regulation of MMP gene transcription

The synthesis of the mRNAs that encode MMPs, is stimulated by exposure to agents such as growth factors and cytokines. FIB-CL, stromelysin-1, stromelysin-3 and gelatinase-B genes are stimulated with growth factors whereas gelatinase-A appears to be only moderately induced by the same factors. The second group of modulators of the MMPs are hormones, which can mediate transcriptional regulation of MMP expression. For example progesterone and medoxy-progesterone can inhibit the production of collagenase and inhibit matrix remodelling in uterine tissues. The third group of factors are physical signals or events that can stimulate MMP expression, such as heat-shock, phagocytosis of particulate matter, or treatment with cytochalasin B. Some studies indicated that cell-shape changes often can induce MMP expression. The presence of substrate alone is also suggested to be an MMP expression modulator as the cell-substrate adhesion can often dictate changes in the cell shape (Benbow and Brinkerhoff 1997; Vincenti et al, 1996).

1.2.9. Involvement of MMPs in various physiological processes

MMPs are involved in various physiological processes under normal and pathological conditions (Table 1.2). Connective tissue cells such as fibroblasts, osteoblasts, chondrocytes and endothelial cells, as well as neutrophils, macrophages or tumour cells are involved in a variety of processes leading to ECM degradation.

Table 1.2. MMPs involved in tissue remodelling

Normal processes	Pathological processes
Ovulation	Cancer invasion
Endometrical cycling	Tumour metastasis
Trophoblast/Blastocyst implantation	Leukemic dissemination
Embryogenesis	Rheumatoid arthritis
Salivary gland morphogenesis	Osteoarthritis
Mammary development/involution	Periodontal disease
Cervical dilatation	Fibrotic lung disease
Foetal membrane rupture	Liver cirrhosis
Uterine involution	Corneal ulceration
Bone growth plate	Gastric ulcer
Bone remodelling	Dilated cardiomyopathy
Tooth eruption	Aortic Aneurism
Hair follicle cycle	Arteriosclerosis
Wound/Fracture healing	Osteosclerosis
Angiogenesis	Epidermolysis bullosa
Macrophage function	Glomerulonephritis
Neutrophil function	Encephalomyelitis

Adapted from Ries et al, 1995.

1.2.10. Inhibition of MMPs

1.2.10.1. Synthetic inhibitors and antibodies

Chelating agents such as EDTA and 1,10-phenanthroline can inhibit the activity of the enzyme by removing or blocking the active site zinc ion. Although these agents are potent inhibitors of MMPs, they have limited analytical or therapeutic potential because of their lack of selectivity. The most potent synthetic inhibitors contain a thiol group which is believed to form a coordination bond with the active site Zn^{2+}.

HS-(CH_2-R-Leu)-Phe-Ala-NH_2 and HS-(CH_2-R-Leu)-Trp-Ala-NH_2 are two examples of thiol containing inhibitors with IC_{50} in the 40-70 nM range. Hydroxamate inhibitors with a terminal hydroxyl group and a vicinal carbonyl group together form a bi-dentate Zn^{2+} ligand. (HONH-CO-CH_2-CH(Bu)-CO-NH-CH(Bu)-CO-NH-CH(Me)-COOEt)

The mechanism of inhibition by tetracyclines and their synthetic analogues is suspected to be dependent on the chelating properties of these compounds. These inhibitors inhibit PMN-CL with a KI in the μM range whereas they are less effective inhibitors for FIB-CL, but the reason for this selectivity is not known.

A range of blocking antibodies with effective and specific inhibitory properties have been produced. Some of these inhibiting antibodies are highly specific and are as effective as good synthetic inhibitors. Monoclonal and polyclonal antibodies, purified by affinity columns can both attain an IC_{50} in the range of (20-200 nM). Inhibiting antibodies have also been shown to block matrix degradation by live cells in several model systems (Birkedal-Hansen et al, 1993).

1.2.10.2. Natural inhibitors

Naturally occurring inhibitors include, the α-Macroglobulins (αMs), which are non-specific, and the TIMPs which are specific. The proteinase-binding αMs are large glycoproteins found in the plasma of vertebrates and invertebrates, and in bird and reptile egg whites. αMs function as molecular traps for proteinases. Most αMs are tetramers assembled from two 360-KDa disulfide-bridged dimers. The αMs form complexes with a wide variety of proteinases. Once the complex is made the proteinase is largely protected from reaction with large substrates and inhibitors. Different mammalian αMs are known such as: human α_2M, rat α_1M, rat α_2M and rat α_1I_3. Human α2M and bovine α2M form complexes with several different collagenases including FIB-CL and PMN-CL. Only active collagenase reacts with the αMs. Different αMs can form complexes in which 95% of collagenase is covalently bound in a very high molecular weight complex. Complex formation is initiated by specific limited proteolysis in the so-called bait region located as residues 681-712 in human α2M. This event triggers a set of conformational changes resulting in the formation of a tight complex (Sottrup et al, 1989). α2-M is perhaps the best known of collagenase inhibitors. This 780 KDa general protease inhibitor shows >95% of the anti collagenolytic properties of serum. However the large size of this protein appears to limit its biological importance in the connective tissue matrix. For this role various tissue derived inhibitors identified in tissue extracts or media of cell or explant cultures are likely to be more important.(Stricklin et al, 1983)

1.3. The family of TIMPs

1.3.1. Introduction

Breakdown of connective tissue is an important event in many normal and pathological processes. The TIMP family of proteins are thought to play an important role in connective tissue catabolism by regulating the activity of matrix-degrading enzymes (MMPs) (Williamson et al, 1993) . TIMPs act as the final point for the limitation of tissue proteolysis. TIMPs are secreted by connective tissue cells in balance with secreted metalloproteinases so that an equilibrium between enzyme and inhibitor exists (Baragi et al, 1994) .

1.3.2. Members of the TIMP family

There are four members of the TIMP family: TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Greene et al, 1996). They have 40% similarity in their amino acid sequence with 25% identity that includes 12 cysteine residues forming six disulfide bonds (Willimson et al, 1994). Natural disulfide bonds stabilize the native conformation of proteins by lowering the entropy of the unfolded form (Duche et al, 1994). TIMP-1 with a M_r 28 KDa is a complex glycoprotein with heterogeneous glycan units attached at Asn 30 and Asn 78 with a M_r 20 KDa protein core. 30% of the molecular mass of TIMP-1 is glycoprotein, the removal of which does not change TIMP-1 inhibitory function. In addition to binding at the active site of the MMPs, TIMP-1 forms a 1:1 complex with the 92 KDa progelatinase B (Williamson et al, 1994; Birkedal-Hansen et al, 1993). TIMP-2 is a nonglycosylated protein with M_r 22 KDa which forms a complex with the 72 KDa progelatinase A. Although both human TIMPs inhibit a range of metalloproteinases, they show different specificities. TIMP-2 is the more effective inhibitor of both 72 KDa gelatinase (MMP-2) and 92 KDa gelatinase (MMP-9), while TIMP-1 is the better inhibitor of MMP-1. The third human TIMP (hTIMP-3) has been cloned and sequenced

from phorbol ester-differentiated THP-1 cells stimulated with bacterial lipopolysaccharide (LPS). This 21.6 KDa protein includes 211 amino acids and contains an N-linked glycosylation site near the carboxyl terminus. Human TIMP-3 shows 44.3% amino acid sequence similarity with TIMP-2 and 38.4% with TIMP-1, however it has 80.8% sequence similarity with the TIMP-3 from chicken embryo fibroblasts called chIMP-3 (Wilde et al, 1994). Human TIMP-4 shares 37% sequence identity with TIMP-1 and 51% identity with TIMP-2 and TIMP-3. The expression of TIMP-4 protein has been identified in MDA-MB-435 human breast cancer. Using Northern analysis, Green and his colleagues have determined a unique expression pattern for TIMP-4, where very low levels of its transcript were detected in the kidney, placenta, colon, and testes whereas it was found in abundance in the adult heart. (Greene et al, 1996)

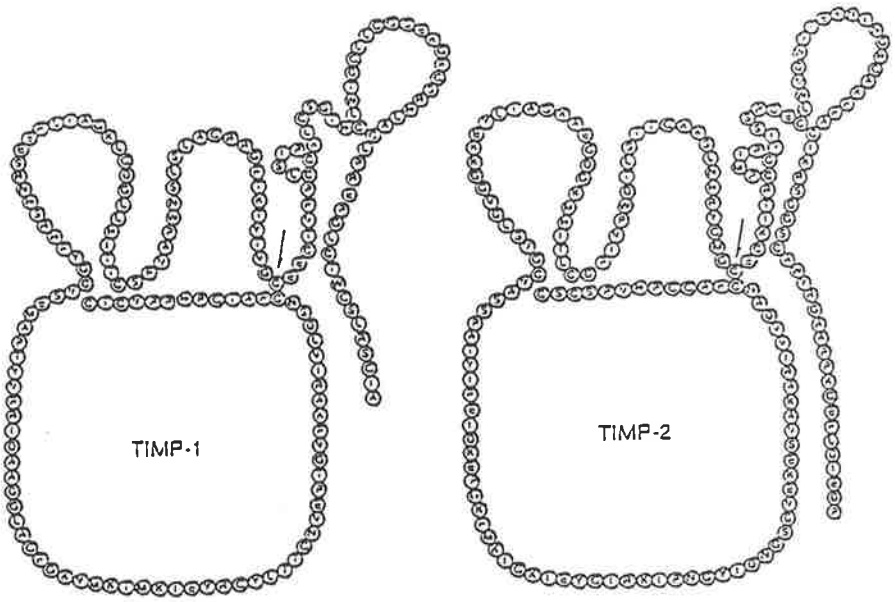
1.3.3. TIMP structure

The sequences of the eight TIMPs from different organisms show a highly conserved secondary structure including six conserved disulfide bonds. Reduction of the disulfide bonds inactivates the protein, whereas it is relatively stable to change in temperature and acidic conditions. The TIMP molecule consists of two main domains: a large 3-loop, N-terminal domain possessing the inhibition of the MMPs and a small 3-loop, C-terminal domain (Figure 1.7). The C-terminal domain is likely to be important in protein localization or complex formation with the progelatinases. The most strongly conserved region is the first 24 N-terminal amino acids, where 17 of the residues are identical in all 8 proteins. (Figure 1.6. Adapted from Williamson et al, 1993).

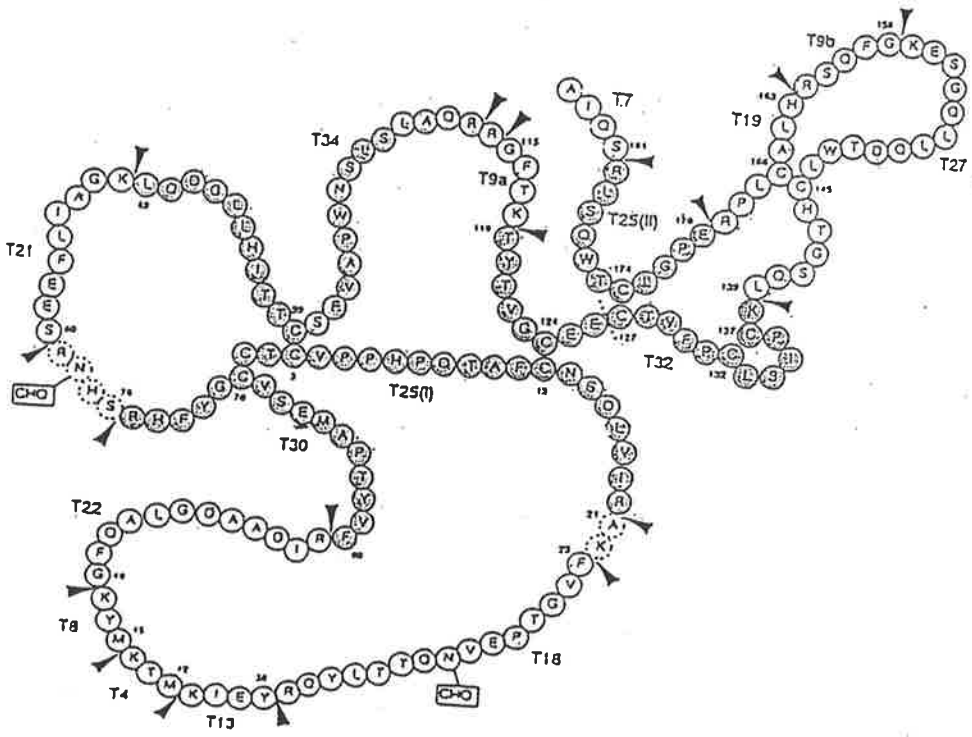
Figure 1.7

A, Two dimensional structure of human TIMP-1 and TIMP-2. B, Arrangement of the native disulfide bonds in a two-dimensional representation of the primary structure of TIMP-1. Carbohydrate attachment sites are denoted [CHO], predicted tryptic cleavage sites are denoted t and the insoluble tryptic core peptide is denoted by shaded circles. The extent of the truncated $D_{127\text{-}184}$ TIMP-1 molecule is shown by the dashed line (– – – –). Figure adapted from Birkedal-Hansen et al, 1993 and Williamsom et al, 1993 .

A.


TIMP-1
TIMP-2

B.


T9b
T19
T27
T7
T34
T9a
T25(II)
T21
T32
T25(I)
T30
T22
T8
T18
T4
T13
CHO
CHO

1.3.4. Complex formation

TIMP is known to bind to active enzyme with a 1:1 stoichiometry to form an inactive, non-covalent complex, with a dissociation constant ranging from 10^{-9} -10^{-10} M (Birkedal-Hansen et al, 1993). Binding can be reversed yielding fully active inhibitor at acidic conditions in the presence of a chelating agent. TIMP is not modified during complex formation and retains inhibitory activity after dissociation and appears to be structurally unchanged. TIMP can also bind to the pro-enzyme form of gelatinases A and B, against which the two forms of inhibitor show distinct specificity (Williamson et al, 1993). Precise details of the interaction between TIMP and the MMPs are unknown, but recent findings have shown that the N-terminal three loops alone are sufficient for both the binding and inhibition of active MMPs. However it is indicated that the C-terminal three loops of TIMP-1 play a part in the binding to progelatinase B. Chemical modification of TIMP-1 has indicated that histidine residues are required for inhibitory activity (O'Shea et al, 1992). It has been reported that the progelatinase A-TIMP-2 complex can autoactivate in the presence of organomercurials to produce enzyme that has low activity toward gelatin and can be inhibited by the further addition of free TIMP-2, suggesting the presence of two distinct TIMP-2 binding sites on gelatinase A. The complex between progelatinase and TIMP-2 can also inhibit collagenase activity indicating there are two distinct metalloproteinase binding sites on TIMP-2. By removal of the C-terminal domain, TIMP-2-gelatinase A affinity is decreased, which indicates an additional TIMP binding site on the C-terminal domain of active gelatinase A that is distinct from the TIMP-2 specific binding site on the proenzyme (ie. active site). Both TIMPs show similar affinities for gelatinase suggesting that the TIMP-binding sites on the active enzyme are common to both inhibitors (Murphy et al, 1992).

1.4. Tissue destruction by neutrophil oxygen metabolites

1.4.1. Introduction

A variety of the tissue destructive events that involve the pathological destruction of collagen are characterized by the presence of large numbers of neutrophils. Triggered human neutrophils simultaneously release and activate large amounts of their lysosomal enzymes including elastase and collagenase. These proteolytic enzymes must be activated in order to catalyse collagen degradation. The process of enzyme activation is controlled by an oxygen-dependent mechanism. Oxygen metabolites are among as the most destructive toxins released from the cell. This oxidation appears to cause partial unfolding of the native structure of the protein. After the proteins are modified by unfolding, they are more susceptible to degradation by proteolytic enzymes. Recent studies have shown that the oxidants and proteolytic enzymes act in a cooperative manner to allow neutrophils to release their full destructive potential (Weiss 1989).

1.4.2. Factors which activate neutrophils

A variety of soluble mediators are capable of stimulating neutrophils to release their granule constituents and/or to generate destructive oxygen metabolites. Cytokines released from activated macrophages or T-lymphocytes are effective stimulators of the respiratory burst of PMNs. Among different cytokines, tumour necrosis factors (TNFs) are reported to stimulate this release of oxygen metabolites by granulocytes. TNF is a factor in serum produced in vivo by mononuclear cells upon exposure to endotoxin. TNF-α and TNF-β are showed to be direct stimulants of the respiratory burst and degranulation of neutrophils. However their ability to stimulate this neutrophil oxidative burst is markedly enhanced in response to chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (FMLP) and the tumour promoter phorbol myristate acetate (PMA) (Berkow et al, 1987; Test 1991).

1.4.3. The NADPH oxidase system

The neutrophil's ability to mediate acute inflammatory tissue damage is attributed to a number of agents. These agents can be divided into two groups: those present in the intracellular granules and those produced at the plasma membrane. Granule-associated enzymes such as latent collagenase and latent gelatinase are capable of hydrolysing many polypeptides. These are released once the neutrophil has been exposed to the appropriate stimulus. Whereas the plasma membrane is the site of a membrane-associated enzyme called, NADPH oxidase, which is activated in triggered neutrophils. NADPH oxidase participates in the generation of three reactive oxidants, superoxide anion ($O_2^{\circ -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH°). Superoxide anion is an oxygen molecule that has accepted an extra, unpaired electron. The extra electron gives $O_2^{\circ -}$ a net negative charge (12 protons, 13 electrons) and the unpaired extra electron gives accounts for the molecule being a free radical.

$$2\,O_2 + NADPH \xrightarrow{\text{oxidase}} 2\,O_2^{\circ -} + H^+ + NADP^+$$

This reaction appears to be catalysed in human neutrophils by a pyridine-nucleotide dependent oxidase, which is able to shuttle electrons from the appropriate electron donor to oxygen. Superoxide anion can act as a reducing agent, an oxidising agent, a ligand, a base or a nucleophile. It is also cytotoxic. However its ability to participate in these reactions depends on the solvent. Two molecules of superoxide anion can react to form the hydroxyl radical (OH°) and hydrogen peroxide by a spontaneous dismutation reaction.

$$O_2^{\circ -} + O_2^{\circ -} + 2\,H^+ \longrightarrow H_2O_2 + O_2$$

The OH° is a powerful oxidant which is produced by the further reaction between H_2O_2 and $O_2^{\circ -}$.

$$O_2^{\cdot -} + H_2O_2 \longrightarrow OH^{\cdot} + OH^- + O_2$$

There are two strong lines of evidence that oxygen-derived free radicals produced by stimulated leukocytes are important mediators of inflammatory tissue damage. First, free radicals injure tissues and irreversibly alter macromolecules (i.e. collagen). Second, the scavengers of oxygen-derived free radicals such as superoxide dismutase (SOD) can prevent injuries caused by these free radicals. One mechanism by which oxygen-derived free radicals can provoke inflammation is by the inactivation of natural anti-proteinases. For example oxidation of the methionine thioether residue at the active site of the α1-proteinase inhibitor can inactivate this proteinase inhibitor. This finding suggests that naturally occurring proteinase inhibitors can be oxidized in vivo.

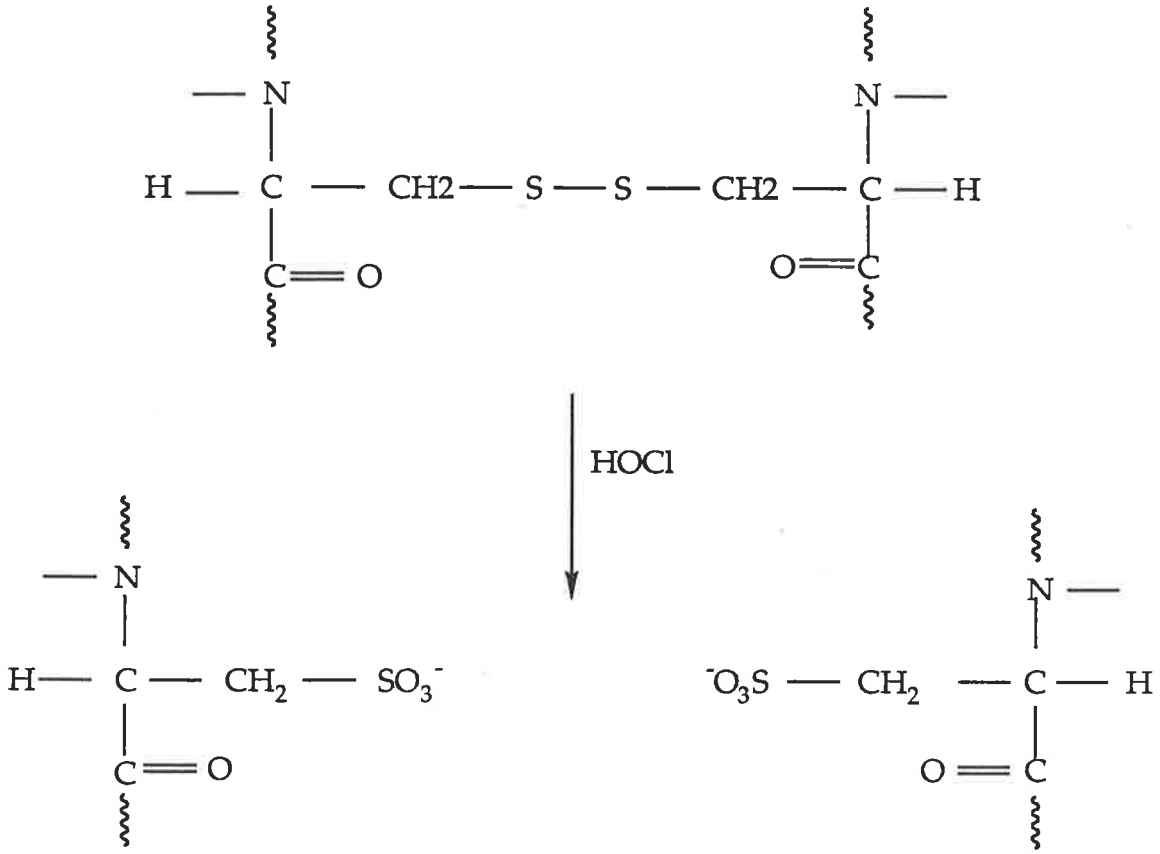
Myeloperoxidase is another enzyme that is localized to the neutrophil granules. This enzyme was originally purified by Agner in 1941, and was named verdoperoxidase. Triggered cells can release this enzyme into extracellular fluids. Myeloperoxidase catalyses the oxidation of halide ions to HOCl with the consumption of hydrogen peroxide. Chloride, bromide, iodide are possible halides in this system. As Cl^- has the highest concentration in the plasma (more than a thousand times that of the other halides), it is thought to be the preferred halide in this reaction.

$$H_2O_2 + X^- + H^+ \xrightarrow[(X^- = Cl^-, Br^-, I^-, SCN^-)]{\text{Myeloperoxidase}} HOX + H_2O$$

HOCl exists in equal concentration to the conjugate base hypochlorite (OCl^-). $HOCl/OCl^-$ generated from this reaction is perhaps the most powerful biologically relevant oxidant, as it is a very potent electron acceptor. It is capable of destroying a variety of microorganisms and mammalian cell targets as well as causing damage to proteins, lipids, carbohydrates, and nucleic acids by oxidizing their functional groups. These groups include amino, imidazolium, phenolic ring, guanidium, indol residue, the

amide nitrogen of peptide bonds and disulfide bonds. For polypeptide chains that are covalently linked by disulfide bonds, as in insulin, oxidation with HOCl can cleave the disulfide bonds, yielding cysteic acid residues (Weiss 1989).

Cysteine



Cysteic acid

Finally the reaction of $HOCl/OCl^-$ with free amino acids generate long-lived N-chloramines. These N-chloramines have less oxidizing potential than $HOCl/OCl^-$, however they have a longer effective lifetime. These long lived N-chloramines are probably able to diffuse away from their site of generation (ie. adjacent to the neutrophil) and cause damage at distant sites, while $HOCl/OCl^-$ is believed to react close to its site of production (Davies et al, 1993) . HOCl has the ability to rapidly react with the β amino acid taurine to form the stable oxidant, taurine chloramine.

$$\underset{\text{Taurine}}{HOCl + H_2N\text{-}CH_2CH_2SO_3H} \longrightarrow \underset{\text{Taurine chloramine}}{ClNH\text{-}CH_2CH_2SO_3H + H_2O}$$

The generation of chloramine in neutrophils was inhibited by catalase, the myeloperoxidase inhibitors, azide, cyanide, and by chloride free conditions, but not by superoxide dismutase or hydroxyl radical scavengers (Wiess et al, 1982).

1.5. Cytokines

1.5.1. Introduction

Cytokines are small, extracellular glycoproteins acting on the cells via surface receptors to influence the proliferation, migration and behaviour of many cell types (Stewart et al, 1995). They play an important pathological role in RA by mediating connective tissue destruction and inflammation. They are released by cells in response to specific signals, and exert either a positive or negative effect on the expression of genes to affect the function or response of target cells. The pro-inflammatory cytokines, IL-1 and TNF-α can stimulate the production of proteases by fibroblasts, synoviocytes, and chondrocytes. Elevated levels of IL-1α and IL-1β mRNA are found in both synovial fluid and the tissues of the inflamed joint. Anti-inflammatory cytokines such as IL-4, IL-10, and TGF-β show inhibitory effects on protease production. TGF-β stimulates the production of collagen, proteoglycan, fibronectin and TIMP, which form part of the repair mechanism. Each of the above cytokines binds to specific receptors on target cells in order to exert their biological activities.

1.5.2. Origin of cytokines

The key pro-inflammatory cytokines IL-1, TNF, IL-6 and IL-8 are produced by monocytes/macrophages and other cells involved in RA such as synoviocytes and chondrocytes. These cytokines cause destruction of cartilage and bone by releasing degradative enzymes such as collagenase and stromelysin. IL-1, TNF and IL-6 are

produced by monocytes during the acute phase of RA. There is a direct relationship between the number of monocytes and the level of cytokine production (Dayer and Burger 1994)

The age of the pro-inflammatory cytokine producing cell directly affects the level of production. Once monocytes have differentiated into macrophages less cytokines are produced. Anti-inflammatory cytokines like IL-4 and granulocyte-macrophage colony stimulating factor (GM-CSF), reduce production of pro-inflammatory cytokines by increasing monocyte differentiation into macrophages.(Duff et al, 1994)

1.5.3. IL-1 and TNF-α as mediators of inflammation in joint

IL-1 and TNF-α activate chondrocytes and synoviocytes to produce metalloproteinases such as stromelysin and collagenase. They also inhibit the synthesis of proteoglycans (Duff 1994; Bodden et al, 1994). IL-1 can induce synovitis by releasing enzymes and other inflammatory mediators, and eventually leads to the destruction of cartilage and bone which is characteristic of RA (Makino et al, 1994). Specific immunoassays for IL-1 have shown raised levels of IL-1 in synovial fluid from RA joints (Rooney et al, 1990) and in blood of patients with active RA (Eastgate et al, 1991). Several studies using target cells such as dermal fibroblasts, articular chondrocytes, trabecular bone cells and almost all mesenchymal cells have shown that IL-1 has similar stimulatory effects on the production of collagenase and prostaglandins (for a review see Dayer and Burger 1994). Three forms of IL-1 are known. IL-1α, a protein of 17.5 KDa including 159 amino acids, is essentially cell-associated. IL-1β is a protein of 17.3 KDa with 153 amino acids, released into the extracellular environment after stimulation. IL-1 receptor antagonist (IL-1Ra) is a 22-25 KDa protein that shares approximately 26% and 19% amino acid identity with IL-1β and IL-1α respectively (Stewart et al, 1995). Synovial fluid IL-1β concentration more closely reflects local inflammation while whole blood IL-1β concentration reflects systemic inflammation. IL-1 plays a pivotal role in the process as leading to joint damage. This is illustrated by studies using neutralising antibodies

against IL-1β and IL-1α in patients with RA, which show inhibition of cartilage resorption of human cartilage by RA synovial fluids (Suzuki et al, 1990; Bendtzen et al, 1990). These three members of IL-1 family are each capable of binding to membrane receptors, but only IL-1α and IL-1β are biologically active. IL-1Ra acts as a competitive IL-1 inhibitor, it binds to the receptor without activating signal transduction (Dinarello 1991).

There are two types of IL-1 receptors. Type I (IL-1RI) has a higher affinity for IL-1α and IL-1Ra and mediates signalling activity. Type II (IL-1RII) has a higher affinity for IL-1β and lacks signalling capacity. Various known mechanisms by which IL-1 transmits its signal have been proposed. One effect of IL-1 is to activate the transcription factor NF-$_k$B in several cell types. NF-$_k$B is thought to reside in latent form in the cytoplasm complexed with its inhibitor, I$_k$B. It is thought that inactivation of inhibitor, I$_k$B by phosphorylation release NF-$_k$B, which then enters the nucleus where it binds to its target enhancer motif to activate transcription (Stylianou et al, 1992).

Similarly TNF-α has many biological activities in common with IL-1. TNF-α has extensive properties affecting the inflammatory cells by stimulating the production of collagenase and PGE_2 by synovial cells (Dayer and Burger 1994). It also synergizes with IL-1 or other cytokines to increase or inhibit their biological activities. TNF-α is produced as a propeptide of 26 KDa. It is associated with the cellular membrane where it is biologically active. Its active form consists of 3 subunits of 17 KDa making a trimeric form. TNF-α exerts its biological activities by binding to specific receptors located on the cell surface. There are two types of TNF-α receptors, including type 1 or p55 and type 2 or p75, which mediate signal transduction. They can be cleaved from the cell membrane yielding soluble forms (sTNFR). The sTNFR can bind TNF-α, acting as endogenous antagonists and prevent TNF-α from attaching to the membrane bound receptors (Barrera et al, 1996). The number of total receptors in normal and malignant cells, has been estimated to be between 10^3 and 10^4/cell. This protein was found in the synovial fluid of more than 50% of rheumatoid patients. It is also synthesised by rheumatoid synovial

tissue. It has been shown that, the combination of TNF-α and IL-1 triggers a more serious inflammatory response than that resulting from any of them separately (Wagge and Espevik 1988). There is now little doubt about the pathological significance of IL-1 and TNF-α in diseases such as RA, since IL-1, IL-1Ra, and TNF-α are present in the rheumatoid synovial membrane and can be detected in plasma and in synovial exudates of RA patients together with soluble forms of both TNFR and IL-1RII (Stewart et al, 1995). Also clinical studies using monoclonal antibodies to TNF have shown promising effects in inhibiting mortality in animal models of sepsis, however preliminary human studies have not been as encouraging (Wherry et al, 1993). It has also been noted that IL-1 and TNF-α induce each other at least in vitro as well as many other pro inflammatory cytokines, including granulocyte-macrophage colony stimulating factor (GM-CSF), IL-6, leukemia-inhibitory factor, IL-8 and other chemotactic factors. Therefore ongoing anticytokine strategies for RA are primarily focused on suppressing the actions of IL-1 and TNF.

1.5.4. Transforming growth factor-β (TGF-β)

For the first time in 1981 Harold Moses and Michael Sporn independently discovered TGF-β in their laboratories (Moses et al, 1981; Roberts et al, 1981). Three isoforms of TGF-β (TGF-β1, β2 and β3) have been found to exist in mammals. They are mainly involved in three biological activities. Firstly, the TGF-β inhibit the growth of most cells, but stimulates the growth of some mesenchymal cell types (Schwann cells, Osteoblasts, Chondrocytes). Secondly, They have anti-proliferative properties (inhibition of T- and B-lymphocytes), which leads to their immunosuppressive effects. Thirdly, they enhance the formation of extracellular matrix components (collagen, fibronectin, glycosaminoglycans and proteoglycans). TGF-β is also useful in another way by opposing the effects of other cytokines. The potent immunosuppressive effects of the TGF-β also suggest that this cytokine may be valuable in the treatment of disease like RA which is characterized by aberrant function of the immune system (Ruscetti and Palladino

1991). It also antagonizes the action of certain inflammatory cytokines such as IL-1 and TNF-α (Wahl 1992). In a number of studies, TGF-β has been shown to be a negative regulator of the degradation of synovial cells and articular chondrocytes (Edwards et al, 1987, Overal et al, 1991; Lum et al, 1996). An increase in TIMP-1 biosynthesis in response to TGF-β is accompanied by concomitant decreases in the synthesis of both MMPs. IL-1 increases the degradation of matrix components and reduces the synthesis of new matrix, which results in an overall loss of cartilage matrix. TGF-β on the other hand increases the biosynthesis of the structural components and effectively rescues the cartilage from the catabolic effects of the inflammatory cytokines. Lum et al have determined the efficiency of TGF-β to reverse the catabolic effects of IL-1β on human articular chondrocytes (Lum et al, 1996).

1.6. Neutrophil activation by chemotactic factors

Infiltration of tissues with large numbers of neutrophils is observed in a variety of pathological conditions. Neutrophils are able to scavenge damaged tissue and kill and digesting micro organisms. Neutrophils phagocytose particulate material and this is accompanied by release of proteolytic enzymes, superoxide, H_2O_2, and variety of bioactive lipids from granules. Several of these products, themselves are capable of inducing inflammation and tissue damage, which is normally observed after neutrophil accumulation.

In recent years, several neutrophil chemoattractants have been characterized. Neutrophils exposed to chemotactic stimuli marginate, adhere to the endothelial cells, and migrate into the extravascular space. After stimulation in vitro, several functional responses, including shape change, adherence, directed movement, enzyme secretion, and the respiratory burst are observed. To initiate each response, first the agonist binds to its receptor and the agonist-receptor complex interacts with a GTP-binding protein, followed by stimulation of phosphatidyl inositol hydrolysis by a phospholipase C. This yields inositol phosphate and diacylglycerol and finally the entire reaction leads to cytosolic free

calcium rise and the activation of protein kinase C, which is part of the signal transduction process (Baggiolini et al, 1989).

Several neutrophil chemoattractants with different origins and modes of formation have been characterized in recent years.

1.6.1. The family of pro inflammatory supergene "intercrine"

During the past 20 years, numerous members of a new family of cytokines have been identified. These 8-10 KDa cytokines are basic, heparin-binding polypeptides. They exhibit 20-45% homology in their amino acid sequence, and have pro inflammatory and reparative activities. Many of these cytokines are produced by various cell types therefore using the term macrophage inflammatory peptide (MIP) for some of them is inappropriate. This family of cytokines have been called the "intercrine" family and classified in two subsets, based on their chromosomal location and amino acid sequence. The cDNA for intercrine α subfamily is located on human chromosome 4, which includes: interleukin-8 (IL-8), platelet factor-4 (PF-4), IP-10, β thromboglubin (βTG), and GRO/MGSA. Those for intercrine β subfamily are on human chromosome 17 including: LD-78, ACT-2, RANTES, and macrophage chemoattractant and activating factor-MCAF. All the intercrines have four cysteine residues making two disulfide bridges. In the α subfamily the first two cysteines are separated by only one amino acid which is called "C-X-C", whereas in β subfamily it is called "C-C" because their first two cysteines are adjacent. Structural analysis reveals that these cysteines are important for the tertiary structure and for binding of the intercrines to their receptors. (Oppenheim et al, 1991)

1.6.2. Neutrophil-activating peptide-1 (NAP-1)/IL-8

In 1985 Luster et al reported the gene expression for a peptide named "IP-10" in the interferon gamma (IFN-gamma) stimulated macrophages. (Luster et al, 1985) In 1987

Yoshimora and his group were the first to identify and separate a novel monocyte cell-derived neutrophil chemoattractant peptide from IL-1 and TNF. This peptide was initially named monocyte-derived neutrophil chemotactic factor (MDNCF) (Yoshimura et al, 1987). Since then various investigators have referred to this peptide as a MDNCF, neutrophil activating protein (NAP), and the most recently IL-8.

IL-8 is produced in vitro by a wide variety of cell types including monocytes, neutrophils and dermal fibroblasts. IL-8 activates a number of functions of human neutrophils, including increasing their adhesion to unstimulated human umbilical cord vein endothelial cells (HUVECs), leading to directional transendothelial migration. Figure 1.8 shows the migration of neutrophils from the blood at the inflammatory site (Mukaida et al, 1995).

1.6.3. Molecular properties of IL-8

The precursor of human IL-8 contains 99 amino acids. At the C-terminal region, residues (91-95), Lys-Phe-Leu-Lys-Arg are most probably the heparin binding site. There are 14 basic amino acids (lysine and arginine) in the 72 amino acid form of mature IL-8, giving basic characteristics to this protein. Intermediate forms (79 and 77 amino acids) as well as truncated (69 amino acids) forms of IL-8 have also been purified from the cultured media of LPS-stimulated human PBMC, TNF stimulated fibroblasts, and endothelial cells. Nuclear magnetic resonance (NMR) and x-ray crystallography techniques have been performed to analyse three-dimensional structure of human IL-8, using purified recombinant IL-8 expressed in E coli. (Clore et al, 1990; Baldwin et al, 1990).

Figure 1.9 shows the overall topology and hydrogen-bonding pattern for IL-8 (Oppenheim et al, 1991).

Figure 1.8

Neutrophil infiltration and IL-8. Local production of IL-8 by infiltrating leukocytes and tissue fibroblasts induces neutrophil migration to the inflammatory site and activates neutrophils. At the inflammatory site, neutrophils attach to the blood-vessel wall by binding to selectin, an adhesion molecule present on the cell surface of endothelial cells. The neutrophils then extravasate into the tissue through gaps between the endothelial cells, following the concentration gradient of IL-8. Neutrophils play an important role in host defence through various mechanisms, including phagocytosis of the pathogen, increased respiratory burst and release of lysosomal enzymes. Figure adapted from Harada et al, 1996.

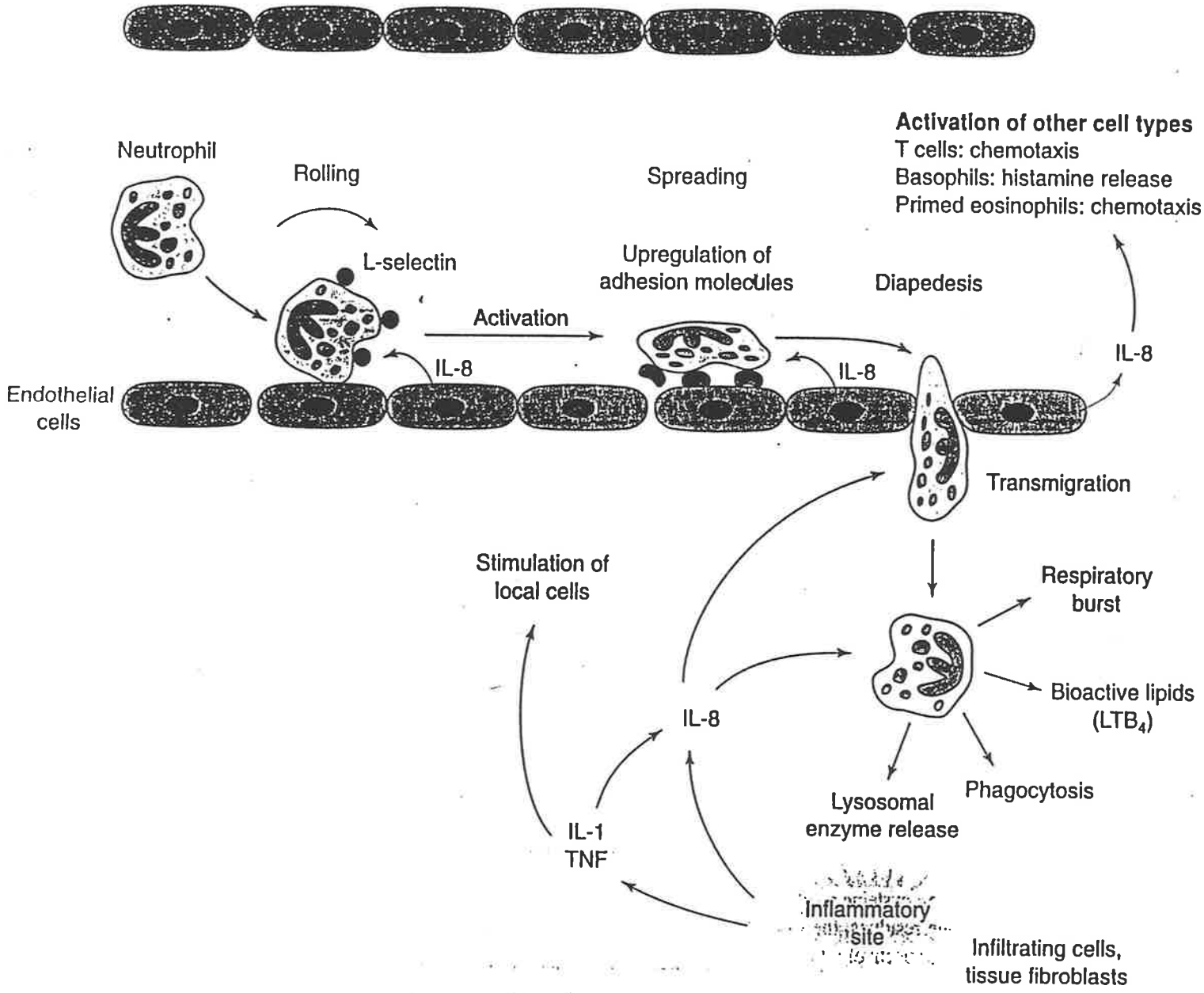
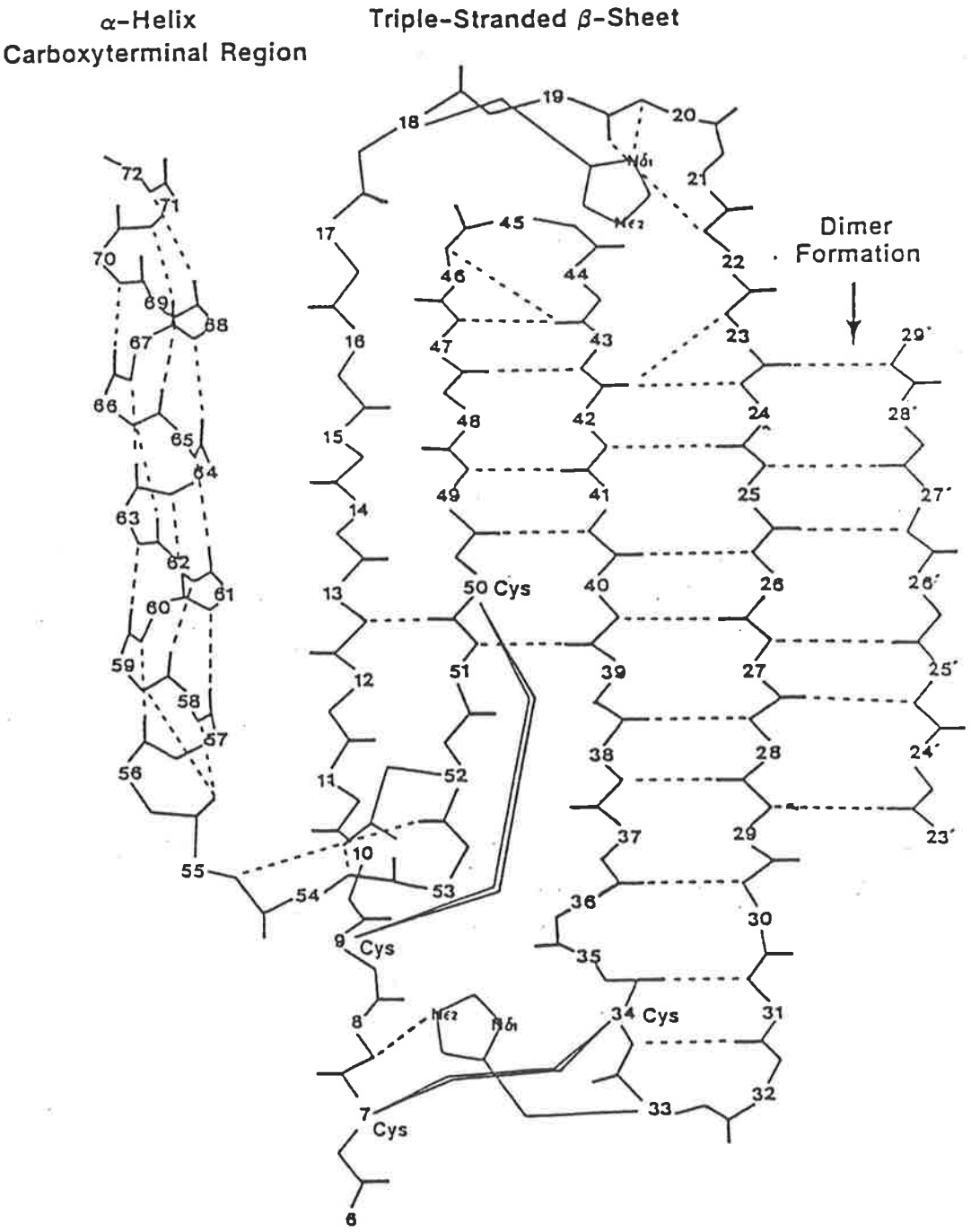

Neutrophil
Rolling
Spreading
Activation of other cell types
T cells: chemotaxis
Basophils: histamine release
Primed eosinophils: chemotaxis
L-selectin
Upregulation of
adhesion molecules
Diapedesis
Activation
IL-8
IL-8
IL-8
Endothelial
cells
Transmigration
Stimulation of
local cells
Respiratory
burst
Bioactive lipids
(LTB_4)
IL-8
Lysosomal
enzyme release
Phagocytosis
IL-1
TNF
Inflammatory
site
Infiltrating cells,
tissue fibroblasts

Figure 1.9

Schematic presentation of the overall topology and hydrogen-bonding pattern of IL-8.

Figure adapted from Oppenheim et al, 1991.


α-Helix
Carboxyterminal Region
Triple-Stranded β-Sheet
Dimer
Formation
50 Cys
9 Cys
34 Cys
7 Cys

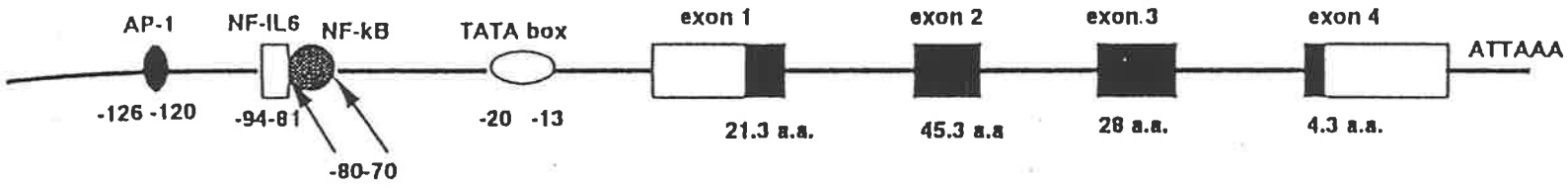
1.6.4. Mechanism of IL-8 gene expression

IL-8 is produced by numerous types of cells upon stimulation with pro inflammatory cytokines such as IL-1 (Mukaida et al, 1992). In response to inflammatory stimuli, IL-8 mRNA is expressed within 1 hr and after 3 hrs it reaches a maximum. Studies utilizing the nuclear run off assay (Oliveira et al, 1992) have revealed that, in several cell types IL-1 or TNF are partly responsible for the IL-8 mRNA induction by activation of transcription. However in U373 cells, these cytokines increase the stability of IL-8 mRNA (Kasahara et al, 1991).

IL-8 genomic DNA consists of four exons and three introns with a single TATA and CAT like structure, collectively 1.5 Kb 5'-flanking region. The 5'-flanking region contains several potential binding sites for transcription factors such as AP-1, NF-IL-6, and NF-$_k$B (Figure 1.10).

Figure 1.10

Schematic structure of human IL-8 gene. Binding site regions for the transcription factors including AP-1, NF-IL6, and NF-$_k$Bare shown. The number of amino acids encoded by each exon are shown below the line. Figure adapted from Mukaida et al, 1994.


AP-1
NF-IL6
NF-kB
TATA box
exon 1
exon 2
exon.3
exon 4
ATTAAA
-126 -120
-94-81
-80-70
-20 -13
21.3 a.a.
45.3 a.a
28 a.a.
4.3 a.a.

In a resting state NF-$_k$B is associated with its inhibitor I$_k$B, whereas upon activation it dissociates from inhibitor followed by nuclear translocation and binding to its *cis* elements (Baeuerle 1992). This dissociation is explained by phosphorylation of I$_k$B (Baeuerle and Baltimore 1988) and protein degradation (Toledano et al, 1991), but it is still not clear how IL-1 treatment modifies IL-8 expression. NF-$_k$B appears to be the most crucial factor in IL-8 gene transcription, although it needs to cooperate with NF-IL6 and AP-1, respectively as the first and second choice (Mukaida et al, 1994).

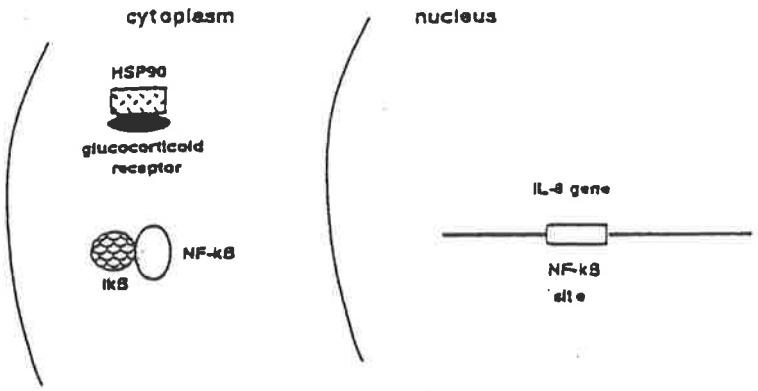
1.6.5. IL-8 gene repression

The molecular mechanism of IL-8 gene repression, is proposed, in a human glioblastoma cell line, T98G, stimulated with IL-1. The immunosuppressant, FK 506 and a synthetic glucocorticoid, dexamethasone, have been used. According to this proposed mechanism, FK 506 suppressed the activation through the IL-8 AP-1 and NF-$_k$B sites induced by the combination of phorbol ester and Ca^{2+}-mobilizing agents. Whereas glucocorticoids suppress the IL-8 gene transcription by interfering with the most essential transcription factor, NF-$_k$B (Figure 1.11) (Mukaida et al, 1992 & 1994).

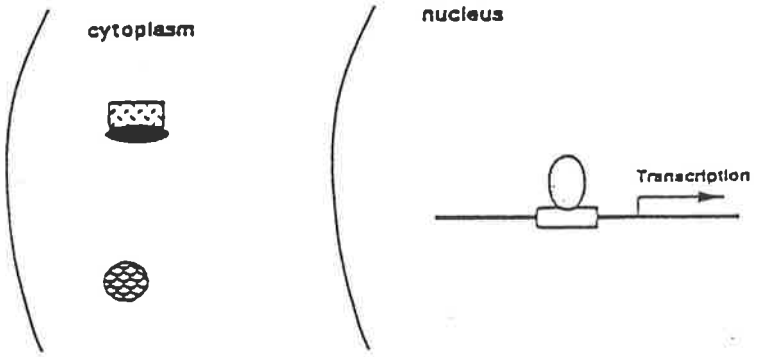
Figure 1.11

Proposed mechanism of a glucocorticoid-mediated IL-8 gene repression in a human glioblastoma cell line, T98G, stimulated with IL-1. Figure adapted from Mukaida et al, 1994.

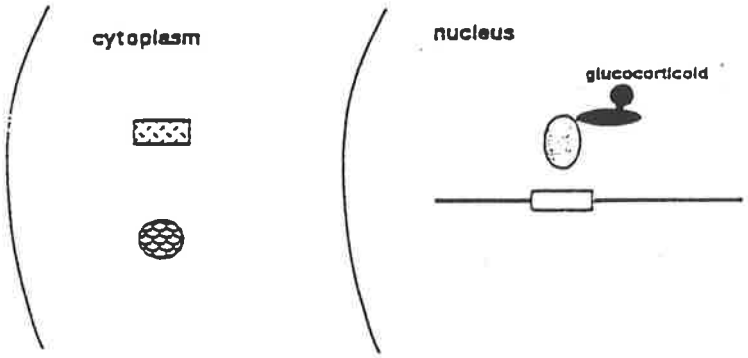
I) Resting



II) Stimulation with IL-1



III) Stimulation with IL-1 and a glucocorticoid



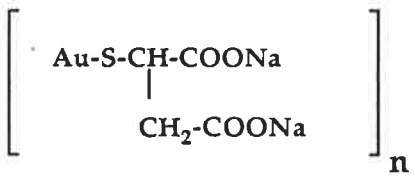
1.7. Anti-rheumatic drugs

1.7.1. Introduction

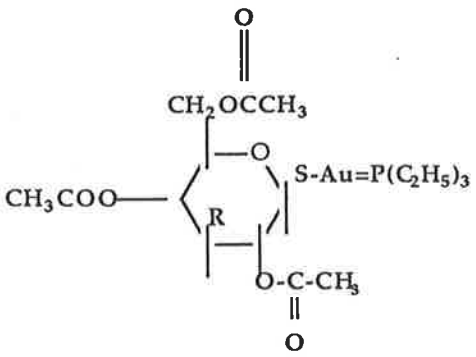
Management of rheumatoid arthritis (RA) and OA relies on both drugs and several biologic agents, although the mode of action of these agents needs to be carefully evaluated. Three classes of drugs are used for the treatment of RA: nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and the slow acting agents. New drugs , which have disease modifying or immunomodulating properties are also under investigation. These include three types of agents: tenidap, a compound with cyclooxygenase inhibition and cytokine modulating activity properties. Monoclonal antibodies against intracellular adhesion molecule-1 (ICAM-1), and antibodies to TNF-α, which are likely to provide useful therapeutic approaches to the treatment of RA. These new agents are designed according to the recent understandings about the molecular basis of chronic inflammation and tissue degradation and synthesis (Blackburn, 1996). Also successful inhibition of metalloproteinases might open new ways to formulate "designer therapies" (Vincenti et al, 1994).

1.7.2. Gold containing drugs

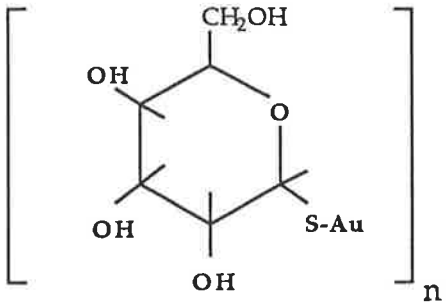
Gold compounds have long been used as therapeutic agents for rheumatoid arthritis (chrysotheraphy). The compounds used are, gold(I) gold sodium thiomalate (GSTM), gold thioglucose (GTG) and auranofin (AF) (tetra acetyl thioglucose) (triethyl phosphine) gold(I).



Aurothiomalate



Auranofin



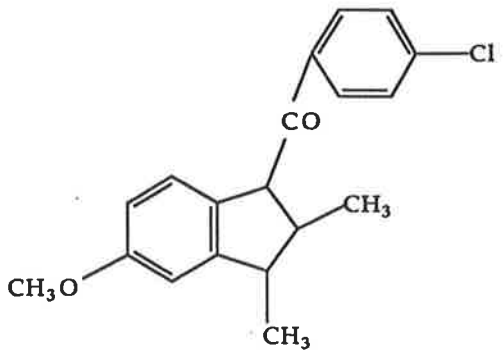
Aurothioglucose

These drugs are distributed throughout the body via the blood stream after administration. The sulfur containing components of these gold containing drugs reach the blood stream rapidly and are then almost completely eliminated via the kidneys. This rapid cleavage of the gold-sulfur bond, in the blood stream suggests that the thiolate part of the drug is not important in long term effect of the drug. The suggestion that the therapeutic component in these compounds is gold, implies that the organic ligands provides a delivery system and stabilize the gold against decomposition in storage. Gold is in either the +1 or the +3 oxidation state. The +3 is toxic, it oxidises tissue with which it comes into contact, and becomes reduced to +1 oxidation state. All of the gold containing drugs used in chrysotheraphy contain the element in the +1 oxidation state (Parish 1992). Gold(I) forms its most stable compound with thiol (sulfur) ligands. However it has other ligands such as phosphorus, nitrogen and oxygen. It appears that these compounds can undergo ligand exchange very readily since there is plenty of space around the gold atom to let attacking groups approach and bind. Thus one ligand may be displaced with a new

ligand. A detailed study in rats have shown that, the level of gold in the joints is 2-3 times greater than those in the surrounding bone, suggesting some accumulation of gold in the synovial fluid. Based on various effects of gold compounds noted in different model systems, a number of mechanisms have been proposed to explain the mechanism of gold compounds in RA. GSTM inhibits various enzymes in human synovial cells, and several human epidermal enzymes. Although the effects demonstrated in vitro are broad, it is not known whether these actions are relatively important. Further evaluation of the mechanism of action of these agents may lead to improvements in therapy. Our study was undertaken to answer the question whether gold compounds may interfere with the pathways responsible for the imbalance between the MMPs and their inhibitors in vitro as well as in cultured human synovial cells.

1.7.3. Nonsteroidal Anti-inflammatory Drugs (NSAIDs)

NSAIDs, such as indomethacin are widely used in the treatment of patients with RA. NSAIDs are distinguished from slow-acting antirheumatic drugs by their faster action and less profound effect on the disease (Day 1988). These drugs act by preventing the conversion of arachidonic acid to intermediate and terminal prostanoids such as prostaglandin E_2 (PGE_2). This may relate to the fact that, in inflammatory rheumatic diseases, pain responds faster to NSAID treatment than the other indices of inflammation such as swelling and erythema. However there is increasing evidence that additional biochemical effects may be important especially in the control of inflammation. These drugs have been regarded as not being able to slow the progression of RA, ie. there are not disease-modifying agents (Harris 1986). Whether there is such a sharp distinction between anti-inflammatory drugs (eg. NSAIDs) and disease-modifying agents is a matter of controversy particularly as there are now agents (eg tenidap) which appear to bridge this gap (see Chapter 4).



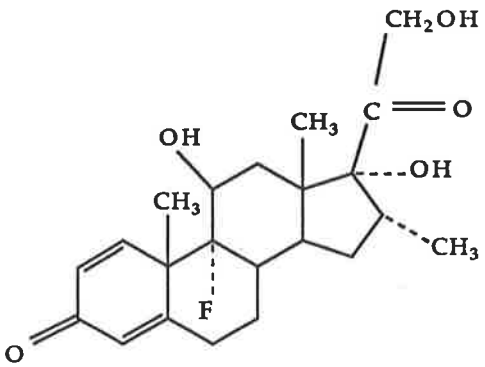
Indomethacin

The terminal prostaglandins are pro-inflammatory. Prostaglandin endoperoxidase synthases (cyclooxygenase, COX) catalyzes the first step in the conversion of arachidonic acid into the prostanoids and related metabolites. It is now recognized that this enzyme has two isoforms COX-1, which is constitutive and COX-2 which is inducible (for a review see Griswold and Adams 1996). Cyclooxygenase inhibition and the subsequent

inhibition of prostaglandin synthesis is likely to be the unifying mechanism for the anti-inflammatory effects of common NSAIDs. Indomethacin is widely used as a potent inhibitor of COX-1 and COX-2 activity. It has also been shown that treatment with indomethacin increases synovial fibroblast cell growth in the presence of IL-1 or TNF (Gitter et al, 1989). Clinically, indomethacin is used in the treatment of rheumatoid and osteoarthritis.

1.7.4. Glucocorticoids

Glucocorticoids are potent immunosuppressive and anti-inflammatory drugs, used successfully in the treatment of RA. Glucocorticoids such as dexamethasone are able to affect a variety of intracellular metabolic pathways. These include a reduction of prostanoid synthesis, through inhibition of phospholipase A_2 and cyclooxygenase (Kohler et al, 1989 &1990). Dexamethasone can also inhibit TPA-induced TNF-α expression as well as IL-1α/β production (Knudsen et al, 1987). It can also diminish the synthesis of IL-1 and IL-6 by mononuclear cells (Lyson and McCann 1992). Therefore it is likely that the effects of glucocorticoids are mainly due to inhibition of cytokine release by immune cells.



Dexamethasone

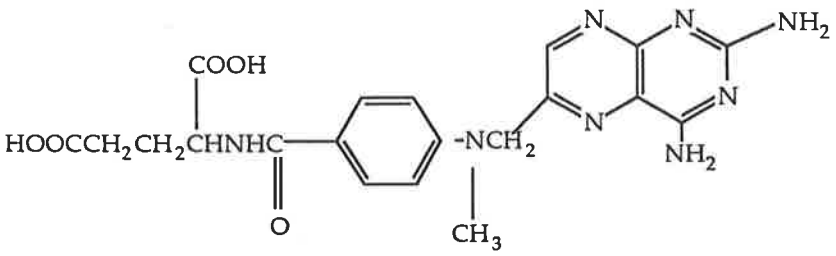
Glucocorticoids form complexes with their specific cytoplasmic receptors, called glucocorticoid receptor (GR) which bind to regulatory elements of the DNA. Therefore

the receptor density of the target cells and the receptor affinity of the glucocorticoid, appeared to be among the factors which determine the magnitude of the biologic effects of glucocorticoids. However in a study by Schlaghecke it was shown that, diminished receptor density in RA patients did not result in glucocorticoid resistance (Schlaghecke et al, 1994).

Glucocorticoids are also known as potent inhibitors of collagenase induction by inflammatory cytokines. The target for this effect appears to be the AP-1 site within the collagenase promoter region, which also mediates its induction. This negative regulation is mainly due to repression of AP-1 activity by the GR.

1.7.5. Methotrexate

Methotrexate has been approved as an effective second-line agent to treat rheumatoid arthritis. However mechanisms of its action on the disease process are largely unknown.



Methotrexate

Therefore more studies are needed to complete the understanding of its action (Furst and Joel 1988).

Experimental studies have suggested that, methotrexate is effective in inhibiting IL-1 production in adjuvant arthritis in rats (Segal et al, 1990). It can also inhibit certain activities of IL-1 in human mononuclear cells without interfering with the production or secretion of IL-1 (Segal et al, 1990; Songsirideg and Frust 1990). Whereas different

studies indicated that methotrexate had no effect on arachidonic acid metabolism. Phospholipase A2 activity, cyclooxygenase and the 5-lipoxygenase metabolic pathways were not affected by methotrexate (Bannwarth et al, 1994). Furthermore, methotrexate appeared to have no effect on IL-1 stimulated production of prostaglandin E_2, hyaloronic acid and collagenase from human synovial fibroblasts in culture (Meyer et al, 1993). In another study by Firestein (Firestein et al, 1994), the synovial gene expression was determined by quantitative in situ hybridiization using computer-assisted image analysis. They have indicated that collagenase gene expression significantly decreased after methotrexate therapy without any change in cell density. However, methotrexate did not directly inhibit the induction of collagenase gene expression by IL-1, in cultured human synoviocytes (Firestein et al, 1994).

1.7.6. Cyclosporin-A

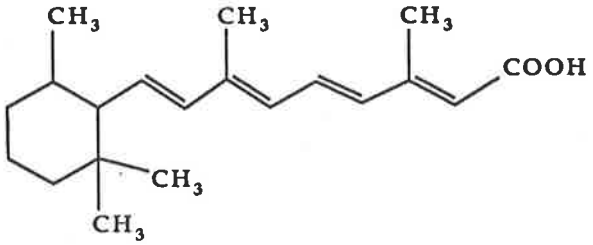
Cyclosporin-A (CyA) is a widely used immunosuppressive drug used in a number of auto immune disorders. One of the most important known properties of CyA is the ability to inhibit the production of cytokines involved in the regulation of T cell activation. CyA has also direct effects on B cells, macrophages, bone and cartilage cells. Recent studies have described the mechanism of action for CyA. Generally lipophilic, the CyA molecule diffuses across the membrane and binds to specific proteins known as cyclophilins. Binding of CyA to cyclophilins appears to be a close link to the immunosuppressive properties of the drug (Russell et al, 1993). Cyclophilins are a group of intracellular proteins, that are enzymes that act as peptidyl-prolyl cis-transisomerases (rotamases). This activity results in changes in the proline residues within proteins. Selective inhibition of cytokine gene transcription may occur where CyA blocks this activity by binding to the cyclophilins. Specifically inhibition of T-cell growth factor (IL-2) production appears to be an important action of CyA (Brauer et al, 1993 ; Elliott et al, 1984).

In a study by Lohi (Lohi et al, 1994), CyA potently enhanced the expression of collagenase in dermal fibroblasts. In this study the increased level of AP-1 activity, was suggested to be the possible reason for this effect of CyA. In another study by Brauer, CyA has effectively decreased the level of IL-6 in the synovial fluid and serum of rats with antigen-induced arthritis (Brauer et al, 1994).

In order to estimate the degree of efficacy of CyA a series of clinical trials were undertaken. The trials data indicated that CyA is potentially a disease modifying antirheumatic drug (DMARD), since it is able to reduce the two important parameters-synovitis and acute phase reactants. Moreover CyA is likely to slow the progression of cartilage and bone damage caused by RA (Forre 1990). Comparative controlled trials have also suggested that CyA has similar effects to those observed with D-penicillamine or azathioprine (Kurki P, 1993).

1.7.7. Retinoic acid

Retinoic acid has immunomodulatory properties, and is effective on proliferation and differentiation. Retinoic acid binds to nuclear receptors, the retinoic acid receptors, and retinoic X receptors (RARα,β,γ and RXRα,β,γ). These receptors belong to the superfamily of steroid and thyroid hormone receptors, and are able to bind to specific DNA sequences, which act as ligand-inducible transcription factors. In a study by Brinckerhoff, the production of collagenase by rheumatoid synovial cells was inhibited by retinoic acid (Brinkerhoff et al, 1980). In the case of the collagenase promoter, retinoic acid can act as a negative regulator of AP-1 responsive genes by interaction of RARs with c-Jun, resulting in prevention of AP-1 binding to its response element (Schule et al, 1991). It is also proposed that retinoic acid regulation of the collagenase gene, in a rabbit synovial fibroblast cell line (HIG82), depends on the availability and interaction of specific RARs with multiple DNA elements within the promoter and with transcription factors, including AP-1 related proteins (Pan et al, 1992).

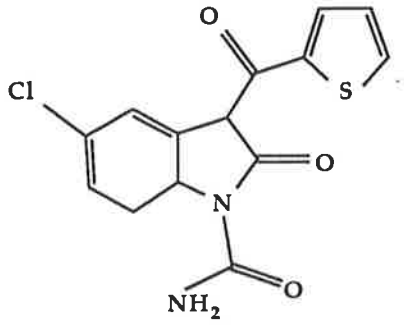


Retinoic acid

The expression of TIMP-1 has also been shown to be regulated by retinoids (Clark et al, 1987). Since the retinoid compounds have been shown to coregulate the expression of collagenase and TIMP-1 in an inverse manner, this could prove these agents to be therapeutically important.

1.7.8. Tenidap

Tenidap is the first representative of a new chemical family of anti arthritic agents, the oxindoles. Tenidap sodium acts as an anti arthritic agent by modulation of the production and action of pro inflammatory cytokines and inhibition of cyclooxygenase.



Tenidap

Several anti arthritic properties of this novel drug includes, its ability to protect cartilage integrity, modulate cytokine synthesis, and its potent inhibitory action on the release of activated neutrophil collagenase (Blackburn et al, 1991). In addition a number of studies suggest the ability of tenidap to down-regulate levels of IL-1 receptor on culture human (normal or OA) chondrocyte and RA synoviocytes (Pelletier et al, 1996). These findings suggest that, the functional significant of this down-regulation may result in decreased mRNA and protein levels of collagenase and stromelysin in response to stimulation with IL-1.

1.8. Summary

Inflammation of the joint is characterised by the selective infiltration of inflammatory cells into the synovium, e.g. neutrophils. Oxygen metabolites have been consistently identified as the most destructive toxins released from the neutrophils in inflamed tissue. The inflammatory cells also release a number of cytokines which act as mediators of pathology in inflammatory diseases. These include, proinflammatory cytokines, such as IL-1α, IL-1β and TNF-α as well as anti-inflammatory cytokine TGF-β.

Members of the family of MMPs such as collagenase and stromelysin are expressed in large amount from connective tissue cells, in inflamed joint. The expression of these proteolytic enzymes is stimulated with the present cytokines, IL-1β and TNF-α. Because these enzymes have fundamental role in the pathophysiology of RA and because the destruction of the extracellular matrix is largely irreversible, their inhibition would be an important way to prevent the tissue destruction. According to the established treatments, which are thought to influence levels of enzyme, there are 2 principle ways to decrease levels of metalloproteinases: inhibition of enzyme activity or inhibition of enzyme synthesis. A local imbalance between activated enzymes and their inhibitors such as TIMP, will lead connective tissue destruction.

The other mediator of the inflammation, IL-8 can mediate the recruitment of inflammatory cells into the joint. IL-8 gene and IL-8 protein are rapidly produced in the presence of stimulants IL-1β and TNF-α.

Taking together, these inflammatory mediators push the balance towards degradative processes in inflamed joint.

Antirheumatic drugs have been used widely to modulate connective tissue degradation, although the mechanism of action of these drugs are poorly understood. Therefore, in this thesis I have been trying to answer to the following questions:

1. Do oxidants, released by activated neutrophils, inhibit the activity of, TIMP-1 against collagenase? Do antirheumatic drugs interfere with the oxidative inactivation of TIMP-1?

2. Do antirheumatic drugs affect the expression of the mRNA for MMPs and TIMP-1 in human synovial fibroblasts? Do they antagonise the catabolic effects of the pro inflammatory cytokines such as IL-1β and TNF-α in these cells? Do anti-inflammatory cytokines, such as TGF-β, antagonize the stimulatory effects of these pro inflammatory cytokines?

3. Do antirheumatic drugs prevent inflammation by inducing the expression and/or production of IL-8, in cells that are present in inflamed joint, human synovial fibroblasts? Do antirheumatic drugs antagonise the catabolic effects of the pro inflammatory cytokines such as IL-1β and TNF-α, which enhance IL-8 expression?

Chapter 2

The oxidative inactivation of tissue inhibitor of metalloproteinases-1 (TIMP-1) by hypochlorous acid (HOCl) is suppressed by anti-rheumatic drugs

2.1. Abstract

The fact that TIMPs prevent uncontrolled connective tissue destruction by limiting the activity of MMPs, have been conclusively explained in chapter 1. The complex tertiary structure of TIMPs which is dependent upon 6 disulphide bonds suggests that they should be susceptible to oxidative inactivation. We examined the oxidative inactivation of human recombinant TIMP-1 (hr TIMP-1) by HOCl and the inhibition of this process by anti-rheumatic agents.

hrTIMP-1 was exposed to HOCl in the presence of a variety of anti-rheumatic drugs. hrTIMP-1 activity was measured by its ability to inhibit BC1 collagenase activity as measured by a fluorimetric assay using the synthetic peptide substrate (DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg) specific for MMP-1.

The neutrophil derived oxidant HOCl, was able to inactivate hrTIMP-1 at concentrations of these oxidants reported to be achieved at sites of inflammation. However, the long lived N-chloramine derivative, N-chlorotaurine (NCT), was not able to inactivate hrTIMP-1 at concentrations achieved in the pericellular environment of the neutrophil. We have also determined that, anti-rheumatic drugs have the ability to protect hrTIMP-1 from inactivation by HOCl. For D-penicillamine, this effect occurs at plasma levels achieved with patients taking the drug but for other agents tested this occurs at relatively high concentrations that are unlikely to be achieved in vivo except may possibly be relevant in a micro-environment.

These results are in keeping with the concept that biologically derived oxidants can potentiate tissue damage by inactivating key but susceptible protein inhibitors such as TIMP-1 which form the major local defence against MMP induced tissue breakdown.

2.2. Introduction

Collagenase can cleave native collagen molecules and allow the resultant denatured collagen to be digested by a range of other enzymes including other MMPs. In this way collagenase regulates fibrillar collagen turnover (Murphy et al, 1991, Mallya et al 1990).

TIMPs regulate the activity of MMPs by tightly binding to the active site of the enzyme and forming inactive complexes with a 1:1 stoichiometry (Williamson et al, 1993). The balance between the activities of MMP and TIMP is a crucial factor in regulating extracellular matrix breakdown in vivo (Denhardt et al, 1993). Average concentrations of MMPs and TIMP fragments are significantly elevated in the joint fluid of patients with osteoarthritis (OA) as compared with volunteers with healthy knees (Lohmander et al, 1993). Structural studies of TIMP-1 suggest a highly conserved secondary structure. There are 12 cysteine residues which form six conserved disulfide bonds, giving a protein structure of six loops and two domains. TIMP activity is dependent upon this elaborate tertiary structure (Birkedal-Hansen et al, 1993). The high density of disulfide bonds and relatively complicated tertiary structure, which is a functional requirement, would suggest that this proteinase inhibitor is susceptible to oxidants (Denhardt et al, 1993).

HOCl is a major product of the oxidative burst of neutrophils (Weiss et al, 1989). There is accumulation of polymorphonuclear leukocytes (PMN) in the synovial fluid of patients with rheumatoid arthritis (RA) which, after stimulation, may release inflammatory mediators (Laurindo et al, 1995). The oxidative burst of neutrophils generates superoxide anion ($O_2\cdot^-$), which is rapidly dismutated to form hydrogen peroxide (H_2O_2). The neutrophil enzyme, myeloperoxidase, catalyzes the reaction of H_2O_2 with chloride ions to form the highly reactive and cytotoxic agent hypochlorous acid (HOCl) (Weiss and Peppin 1986). In aqueous solution HOCl exists in essentially equal concentration as the conjugate base hypochlorite (OCl^-) and together form a

powerful biologically relevant oxidizing agent capable of causing damage to proteins (Weiss et al, 1982).

HOCl will react with amino acids such as taurine to generate a derivative group of oxidants known as the chloramines (Zglicznski et al, 1971). Generally, chloramines are less powerful oxidants than HOCl itself (Wiess et al, 1989). Taurine, whose biological role remains unclear, is found at concentrations approaching 20 mM in neutrophils and macrophages (Grisham et al, 1984). NCT has been reported to be essentially unreactive with major cellular components. It has been suggested that taurine may act as a "sink" for the oxidizing potential of $HOCl/OCl^-$, generating a chlorinated species of lower reactivity and thereby minimizing indiscriminate damage (Grisham et al, 1984). Since taurine is the most abundant endogenous amine in the pericellular environment of the neutrophil, we have used NCT as an example of chloramines in our model. In many cases oxidation appears to result in a partial unfolding or rearrangement of target proteins (Okada et al, 1988, Davis et al, 1993).

Previous reports have shown that latent neutrophil collagenase (MMP-8) can be activated by neutrophil derived oxidants, principally HOCl. This has highlighted the way that oxidant generation and enzymatic mechanisms can interact to lead to tissue breakdown. Oxidative inactivation of enzyme inhibitors is an indirect mechanism of interaction between oxidant generation and the equilibrium between proteases and their inhibitors. The most well known instance where this occurs is the oxidative inactivation of plasma proteinase inhibitors, such as α_1-proteinase inhibitor (α_1-PI), where oxidation of the methionine at the active site of α_1-PI results in loss of affinity of this inhibitor for its target enzyme (Matheson et al, 1981). In another study by Frears the inactivation of TIMP-1 by peroxynitrite ($ONOO^-$) has been demonstrated (Frears et al, 1996). We have reasoned that since hrTIMP-1 has an elaborate 6 loop structure, each loop maintained by interchain disulfide bonds, that oxidative cleavage of these disulfide bonds would result in major structural disintegration and consequence loss of affinity for matrix metalloproteinases.

Thiol-containing antirheumatic drugs, such as D-penicillamine, sodium aurothiomalate, and aurothioglucose, are widely used in treatment of RA (Cuperus et al, 1985). Some studies have investigated the effects of antirheumatic drugs, including thiol-containing drugs, on the oxidative activity of myeloperoxidase (Pekoe et al, 1982, Matheson R, 1982). It has been demonstrated that D-penicillamine effectively scavenges HOCl formed by myeloperoxidase and inhibits the enzyme itself (Cuperus et al, 1985).

In this study, we have examined the possibility that oxidants may inactivate hrTIMP-1 and thus allow MMPs to be unfettered in areas of HOCl generation, as for example the pericellular environment of the activated neutrophil. We have also examined the hypothesis that anti-rheumatic drugs, particularly those shown to act as anti-oxidants, may protect hrTIMP-1 from oxidative inactivation (Bannwart et al, 1994).

2.3. Materials and methods

2.3.1. Materials

Chemicals and biologicals were purchased from the following sources: Chemicals used in the operation of acid-soluble type I collagen and the collagenase assay, Sigma Chemical Company, St.Louis, MO., USA or Ajax Chemicals, Sydney, Australia. Penicillin/streptomycin and tissue culture plates, Flow Laboratories, Virginia, USA. Assay wells (Nunc-Immunomodule maxisorp), for coating with purified type I collagen were from Nunclon, Denmark.

D-Penicillamine, gold sodium thioglucose (aurothioglucose), chloroquine, taurine Trypsin, soy bean trypsin inhibitor (SBTI), and Tris (hydroxymethyl aminomethane), were purchased from the Sigma Chemical Company, St.Louis, MO., USA. Gold sodium thiomalate (aurothiomalate) was from Aldrich, U.K. Reagent grade chemicals were purchased from BDH Chemicals Australia Pty. Ltd., Kilsyth, Victoria, Australia. Dulbecco's Modified Eagle's Medium (DMEM) was from Cytosystems, Castle Hill, New South Wales, Australia. Auranofin was from Smith, Klein and French (Pty Ltd). Methotrexate was from David Bull Laboratories, Melbourne, Australia. Aurothiomalate and aurothioglucose were stored desiccated as powder at -20°C. D-penicillamine, methotrexate, chloroquine and auranofin were stored desiccated at 4°C.

Human recombinant TIMP-1 (hrTIMP-1) was a gift from Dr. Andrew J. Docherty (Celltech Research Slough, Berkshire, U.K). The rat mammary carcinoma cell line BC-1 was a gift from Professor Robert L. O'Grady, University of Technology, Sydney, Australia (Stevenson et al, 1985).

NCT was made by the addition of a solution of NaOCl to taurine buffer (50 mM Taurine, 10 mM $CaCl_2$, 100 mM NaCl, pH 7.5) according to the method of Weiss (Wiess et al, 1982).

2.3.2. Collagen preparation

Tendons were extracted from 12 medium rat tails and placed in ice cold sodium chloride solution (0.2 M). After rinsing them three times with NaCl (0.2 M), they were damp dried. A pre-cooled stainless steel mortar and pestle was used for smashing the tendons. To allow thorough freezing and fracturing, the mortar was only filled to one quarter capacity with tendons. Liquid nitrogen was used to freeze the tendons which were then hammered with a sledge hammer. This process was repeated four times to produce finely ground tendons. Shattered tendons were stirred in 0.5 M Acetic Acid for 48 hours. Then the solution was filtered through a metal sieve. The solution was centrifuged in a pre cooled TY 65 rotor for 2 hours at 28 K, 4° in a Beckman L8-70 M Ultracentrifuge. The supernatant was dialysed against 0.02 M phosphate buffer pH 7.8. After dialysis the collagen was dissolved in 0.5 M acetic acid overnight. The collagen was then lyophilised in a pre-chilled glass vessel using a vacuum freeze drier at -40° with the pressure < 1 Torr for 48 hours, to make a white, light shell inside the vessel. The white powder was collected and dissolved in 0.5 M acetic acid to give 2 mg/ml concentration. At this step collagen molecules were dissolved in acetic acid. By the addition of high concentrations of an electrolyte (NaCl), the solvent molecule (acetic acid) is unable to solvate the solute macromolecules (collagen) resulting the collagen molecules to come out of solution. This is called salting out. For salting out the collagen, 30% NaCl solution in 0.5 M acetic acid was used to adjust salt concentration of collagen solution into 6%. In order to aviod high local concentration 450 mls of NaCl in 0.5 M acetic acid was added dropwise on ice and stirred over night. Then it was Centrifuged at 5000g for 1 hour at 4°C in a Beckman high speed centrifuge with a JA14 rotor in 250 mls buckets. The pellet was collected and dissolved in 200 mls of 0.5 M acetic acid, then dialysed out the salt and the salt concentration was readjusted to 3%, centrifuged at 5000g for 1 hour. The supernatant containing collagen solution was collected and its salt concentration was adjusted to 4%. The pellet contains collagen

which was dissolved in 0.5 M acetic acid and the excess salt was dialysed out against 0.1 M acetic acid (Chandrakasan et al,. 1976) .

2.3.3. Hydroxy proline assay

There is a standard relationship between the molarity of hydroxy proline and the concentration of collagen in solution. Thus collagen concentration was measured using the hydroxy proline assay performed at a diagnostic laboratory (Endocrine Laboratory (IMVS)) according to the method of Bergman and Loxley (Bergman and Loxley 1970). 1/10, and 1/100 dilutions of collagen was made up in neutralising buffer (100 mM Tris-Cl, 200 mM NaCl, 0.04% NaN3, pH = 7.8). According to this assessment, the molarity of hydroxy proline for the 1/100 dilution was reported 43 μM. The concentration of the purified collagen was calculated to be 3.92 mg/ml using the molarity of hydroxy proline in this preparation.

2.3.4. Standard curve of the purified collagen

Collagen coated plates were prepared according to the method of Nethery (Nethery et al, 1986). Briefly, a stock solution of collagen was dissolved in a neutralising buffer on ice. Then a range of collagen concentrations was made using the same neutralising buffer. 50 μl aliquots of each concentration was gelled in each row of microwells (Nunc-Immuno Module, MaxiSorp F16, DK 4000 Roskilde, Denmark) giving a range of concentrations between 0-30 μg/well. The collagen containing microwells were incubated at 30°C in a humidified container for 24 hrs, followed by another 24 hrs incubation in dry conditions at the same temprature. After which any remaining salt was removed by rinsing the wells 3 times with millipore filtered water. Microwells were then stained using 25% (w/v) Coomassie Blue (Coomassie blue R250, LKB, Reactifs IBF Villeneure-la Garenne, France), in 10% acetic acid, 50% methonol and 40% filtered water, for 25 mins at room temprature (RT). After the incubation time the stain was tipped out of the wells, rinsed 3 times and dried at RT.

The absorbance at 595 nm was determined on the Bio Rad Model 3550-uv Micro plate Reader. An absorbance (595 nm) versus concentration curve was constructed from the above information (Nethery et al, 1986). This standard curve of collagen enables us to determine the amount of remained collagen in each micro well in the collagenolytic experiments. (Figure 2.1)

2.3.5. BC-1 collagenase preparation and purification

A neoplastic rat epithelial cell line, BC1 was grown under a serum-free medium (HAMS F-12 10.65 g/2L, DMEM 18.42 g/2L, $NaHCO_3$ 2g/2L, Pen/Strep 5U/ml, insulin 5µg/ml, transferrin 1mg/ml, L-glutamine 0.292 mg/ml, BSA 5mg/ml) in 75 cm^2 flasks at 37°C in a humidified atmosphere containing 5% CO_2, according to the method of Stevenson (Stevenson et al, 1985). These cells have the ability to produce large quantities of latent collagenase3 (MMP-13), viz. BC1 collagenase (Guy Lyons et al, 1989; Freije et al, 1994). Every 3 days the conditioned media was collected and centrifuged at 3000g for 15 mins to get ride of the unwanted particles. The enzyme, BC1 collagenase was then purified by passing the clarified conditioned media pH = 7.5 through a Heparin-Sepharose affinity column. Bound protein was eluted from the column using an eluting buffer consisting of Tris-Cl 50 mM, NaCl 800 mM, $CaCl_2$ 10 mM, 0.2% NaN_3 (Stevenson et al, 1985). Column eluates were assayed for protein by using a Bio-Rad protein assay according to (Bradford et al, 1976). This method involves the binding of the Bio-Rad dye to proteins. The dye was added to the serial concentrations of the bovine serum albumin (BSA) as standard curve, and the absorbance was measured at the wavelength of 595 nm.

2.3.6. Activation of BC1 collagenase

Enzyme BC1 collagenase was incubated at 35°C for 15 mins with Trypsin at a final concentration of 2.5 µg/ml in the assay buffer (Tris 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM, NaN_3 0.02%, pH 7.5) . The reaction was terminated by using a 5-fold molar excess of soya-bean trypsin inhibitor (SBTI) 12.5 µg/ml. A time course for this assay of collagenase activity showed that the maximum activity was achieved between 5-15 mins incubation of the collagenase with trypsin (Fig 2.2).

2.3.7. Microtiter spectrophotometric collagenase assay

This assay is based on collagen digestion by trypsin-activated BC1 collagenase. Collagen (20 µg/well) was gelled onto the bottom of micro wells at 30°C for 24 hours in humidified conditions, and another 24 hours in dry conditions. These collagen coated wells were stored under the assay buffer for up to 2 weeks.

Trypsin activated collagenase was incubated in these wells for 15 mins at 35°C to digest the substrate over night. After assay incubation the wells were washed and stained with Coomassie Brilliant blue R-250 for 20 mins and rinsed. Absorbance at 595 nm quantified on a BIO RAD model 3550-UV micro plate reader. The enzyme activity units/ml was determined by reference to the standard curve of the collagen (Nethery et al, 1986). 1 unit of collagenase activity equals to 1 µg of collagen fibrils degraded per min at 35°.

2.3.8. Fluorimetric assay of the BC1 collagenase activity.

This assay was performed according to the method of Netzel-Arnett (Netzel-Arnet et al, 1991). The assay relies on cleavage of the substrate, DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg at the Ala-Leu bond, thus releasing Trp fluorescence from quenching by the DNP group at the N-terminal region of the heptapeptide substrate. The substrate used, is best cleaved by collagenase (MMP-1). The heptapeptide substrate was synthesized using the

solid phase method with Nα-Fmoc-amino acids. The synthetic peptide was coupled with dinitrofluorobenzene prior to cleavage from the resin. It was purified by HPLC using a Waters C18 Novapak column and subsequently recovered by lyophilization. The heptapeptide was solubilized in 4% DMSO.

Samples, after dialysis to the appropriate buffer, were activated and then equilibrated at 35°C for 30 seconds in a 5mm path length quartz cuvette (Starna type 3-5) and the assay started with the addition of the heptapeptide substrate to give a final concentration of 12 mM. The reaction was monitored over time at 35°C in a Perkin-Elmer LS-5 fluorimeter (excitation l = 280nm, slit width: 10nm; emission l = 360nm, slit width: 10nm). Collagenolytic activity was calculated from the initial linear slope of the curve being expressed as the rate of increase in fluorescence at 360 nm per second.

2.3.9. Collagenase inhibition assay

The inhibitory capacity of hrTIMP-1 was determined using the same flourimetric assay as above. In this assay, trypsin activated BC1 collagenase was used as the indicator of the hrTIMP-1 inhibitory activity. To establish the dose curve with hrTIMP-1, different concentrations of the hrTIMP-1 between 1 to 50 nM was applied to the active enzyme to make the inactive complex in 30 seconds. Synthetic peptide, 12 μM, was then added to show the % inhibition of the enzyme with the inhibitor. In order to examine the effects of HOCl on the activity of hrTIMP-1, we initially incubated hrTIMP-1 with different concentrations of HOCl, between 0.1 and 200 μM at 35°C for 1 hour. The HOCl pre-exposed hrTIMP-1 was then used in the next experiments, to inhibit the activity of BC1 collagenase. Subsequently reactants were added to the cuvette in the following sequence: assay buffer, oxidant-exposed hrTIMP-1, enzyme (BC1 collagenase), substrate (synthetic peptide).

Results were expressed as change in fluorescent intensity per unit time. For experimental samples this was expressed as a percent of control, which was the reaction of activated BC1 collagenase and substrate alone.

2.3.10. Drugs

For experiments involving anti-rheumatic agents, a range of these drugs was applied to the hrTIMP-1 prior to the addition of HOCl. The drug and oxidant pre-incubated hrTIMP-1 was then used in the next experiments to inhibit the activity of BC1 collagenase. Fresh solutions of drugs were prepared on a daily basis.

2.3.11. Statistical analysis

Each experiment was repeated three times. All results were expressed as the mean ± standard error of the mean (SEM). Where error bars are not shown, they are smaller or the same size as markers.

2.4. Results

2.4.1. Standard curve of the collagen

Collagen coated micro wells, stained with Coomassie Brilliant blue, gave a 4-5 mm diameter circle of uniformly stained collagen. The intensity of the blue colour indicates the amount of remaining collagen. Figure 2.1 shows that A_{595} is proportional to the amount of collagen per micro well over the range 10-25 μg collagen. Thus solubilization of collagen may be measured directly from the final absorbance in the range A_{595} = 1-2.9.

2.4.2. Activation of BC1 collagenase, using spectrophotometric collagenase assay

Enzyme BC1 collagenase was incubated at 35°C for times between 0-30 mins with trypsin at a final concentration of 2.5 μg/ml in assay buffer (Tris 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM, NaN_3 0.02%, pH 7.5). The reaction was terminated using 12.5 μg/ml SBTI. The time course curve shows that maximum collagenase activity was achieved between 5-15 mins pre-incubation of the collagenase with trypsin (Figure 2.2).

2.4.3. Inhibition of BC1 collagenase by hrTIMP-1

For subsequent experiments, activation of BC1 collagenase was achieved by pre-incubation for 10 mins with trypsin. This reaction was then stopped by addition of a 5 fold molar excess of SBTI as above. As shown in Figure 2.3, hrTIMP-1 was able to inactivate BC1 collagenase that had been activated by pre-exposure to trypsin. The inhibition was investigated for a maximum hrTIMP-1 concentration of 35 nm, at which the BC1 collagenase activity was reduced to 10% of the control. There was a sigmoidal relationship between the concentration of inhibitor and enzyme activity.

Figure 2.1.

Dose curve of the collagen.

Ice cold collagen was diluted with neutralizing assay to give a range of collagen concentrations. 50 µl aliquotes of each collagen solution were gelled in microwells, washed and stained, then A_{595} was measured as described in methods section. Each data point represents the mean of quadruplicate wells ± SEM. Where SEM is not shown, it is less than or equal to the size of the markers.

Figure 2.1

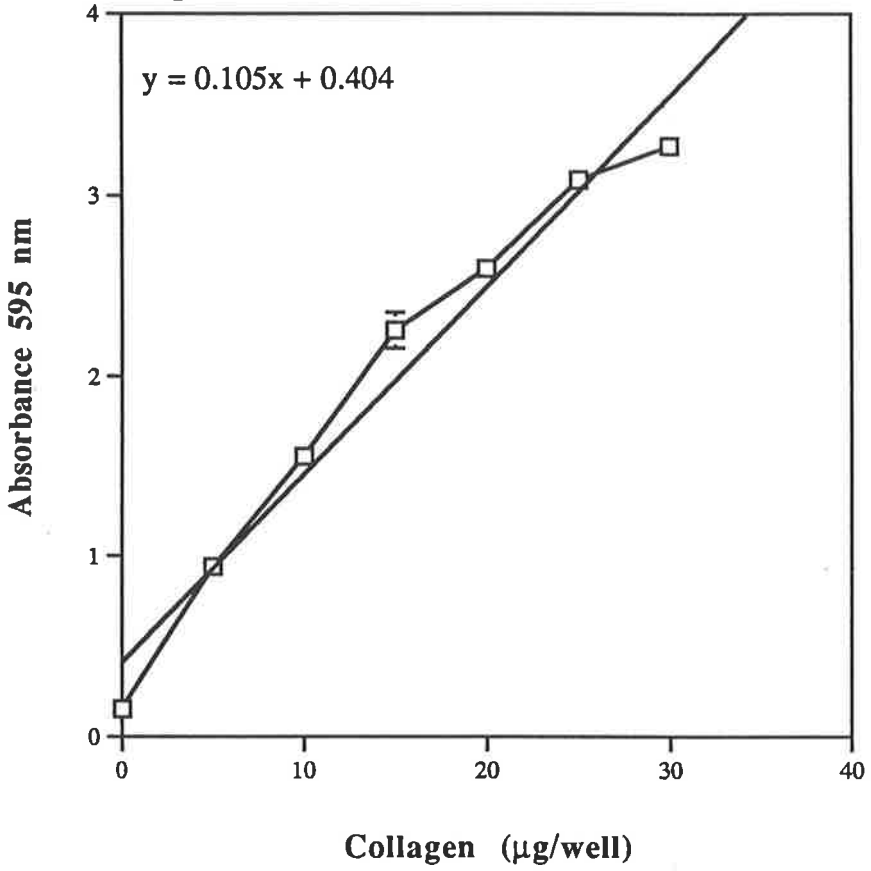

y = 0.105x + 0.404
Absorbance 595 nm
0
1
2
3
4
0
10
20
30
40
Collagen (µg/well)

Figure 2.2.

Time dependent activation of collagenase with trypsin.

BC1 collagenase was incubated with trypsin (2.5 μg/ml) for indicated time points. After each incubation time point , the reaction was stopped using SBTI (12.5 μg/ml). The data represent mean value ± SEM of triplicate measurments.

Figure 2.2

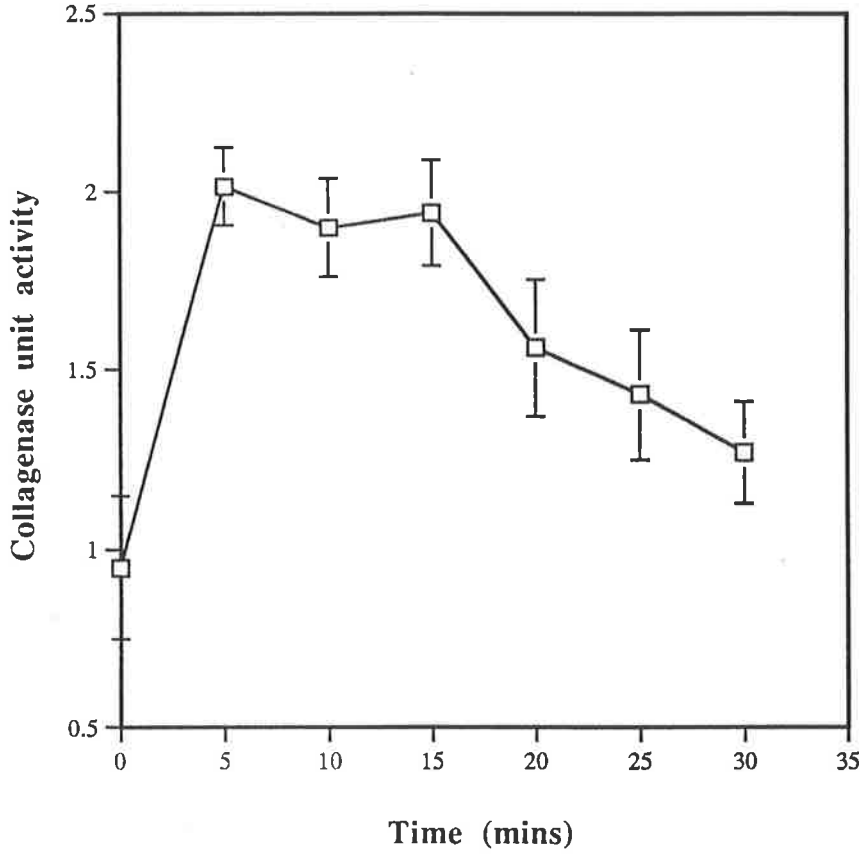
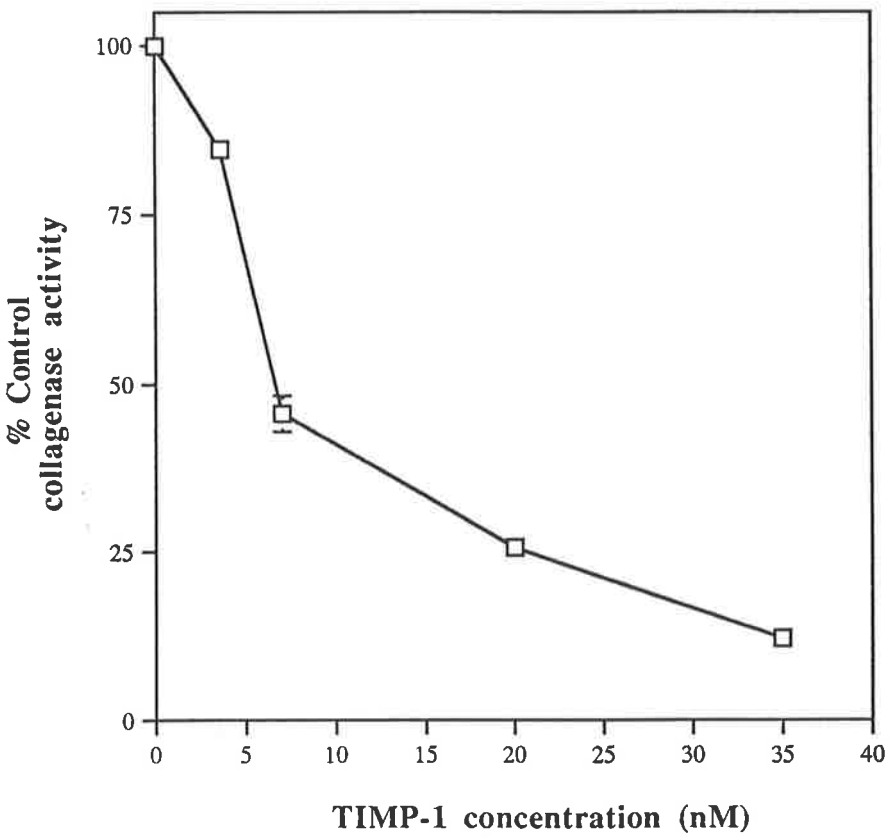

2.5
2
1.5
1
0.5
Collagenase unit activity
0
5
10
15
20
25
30
35
Time (mins)

Figure 2.3.

Inhibition of trypsin activated BC1 collagenase by TIMP-1.

Serial concentrations of TIMP-1 applied to the active enzyme at 37°C. A synthetic peptide was used as the substrate to determine the level of the BC1 collagenase activity. 100% control collagenase activity was the level of BC1 collagenase activity, when this enzyme was not treated with any concentration of TIMP-1. 50% inhibition, of the BC1 collagenase activity, was achieved using 7 nM TIMP-1. Values represent means ± SEM for three separate experiments expressed as % control collagenase activity. Where SEM is not shown, it is less than or equal to the size of the markers.

Figure 2.3


100
75
50
25
0
% Control
collagenase activity
0
5
10
15
20
25
30
35
40
TIMP-1 concentration (nM)

2.4.4. Oxidative inactivation of TIMP-1

We examined for the loss of activity of hrTIMP-1 to inhibit BC1 collagenase, after pre-exposure to HOCl. TIMP-1 was diluted in PBS to give the final concentration of 1.1 μM. Figure 2.4 shows the inactivation of TIMP-1 activity after pre-exposure to HOCl between concentrations of 0.1-200 μM. 100% control TIMP-1 activity is when no HOCl was applied therefore the inhibitor has its maximum activity to inhibit the active enzyme. 50% inactivation was achieved using 175 μM HOCl, while 200 μM HOCl caused compelete inactivation of the inhibitor. This represents an oxidant to inhibitor molar ratio of 200:1. In order to show that this was not due to a direct effect of HOCl on enzyme, HOCl was exposed to BC1 collagenase at the final concentration achieved in this experiment (ie. 200 μM) and no change in collagenase activity was observed. When NCT was used as an oxidant in concentrations of NCT between (0.1-10 mM) no inactivation of hrTIMP-1 was seen (Figure 2.4).

2.4.5. Effects of anti-rheumatic drugs on BC1 collagenase activity

Figure 2.5 shows the direct effect of several anti-rheumatic agents on BC1 collagenase activity. Auranofin has no inhibitory effect up to a concentration of 500 μM, whereas chloroquine, aurothiomalate and aurothioglucose give moderate inhibition at 500 μM but not 100 μM. Methotrexate and D-penicillamine have significant effects at 100 μM. Subsequent experiments to examine oxidative inactivation of TIMP-1 were therefore designed such that BC1 collagenase used in the collagenase inhibition assay was not exposed to concentrations of each drug greater than 30 μM, where there is no significant effect of the drug on BC1 collagenase activity.

Figure 2.4.

Oxidative inactivation of human recombinant TIMP-1 by HOCl and NCT.

TIMP-1 was incubated with increasing concentrations of HOCl (100-200 μM) or NCT 100-500 μM) at 37° for 1 hour. 100% control TIMP-1 inhibitory activity was the level of TIMP-1 activity when this inhibitor was not incubated with any oxidant. 175 μM HOCl caused 50% inactivation of TIMP-1, whereas no inactivation of TIMP-1 was achieved using NCT up to 500 μM. Using HOCl 200 μM completely abolished the inhibitory activity of TIMP-1. Values represent means ± SEM for three separate experiments expressed as % control TIMP-1 inhibitory activity.

Figure 2.4

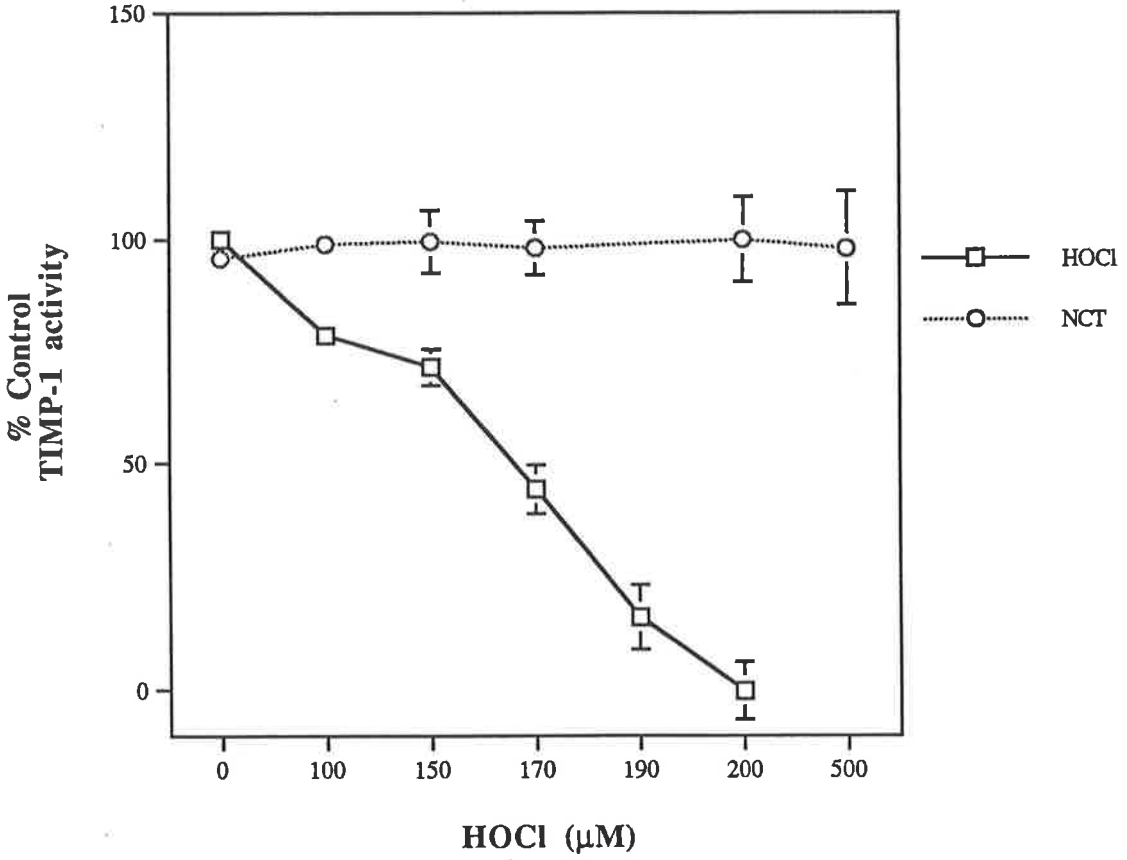
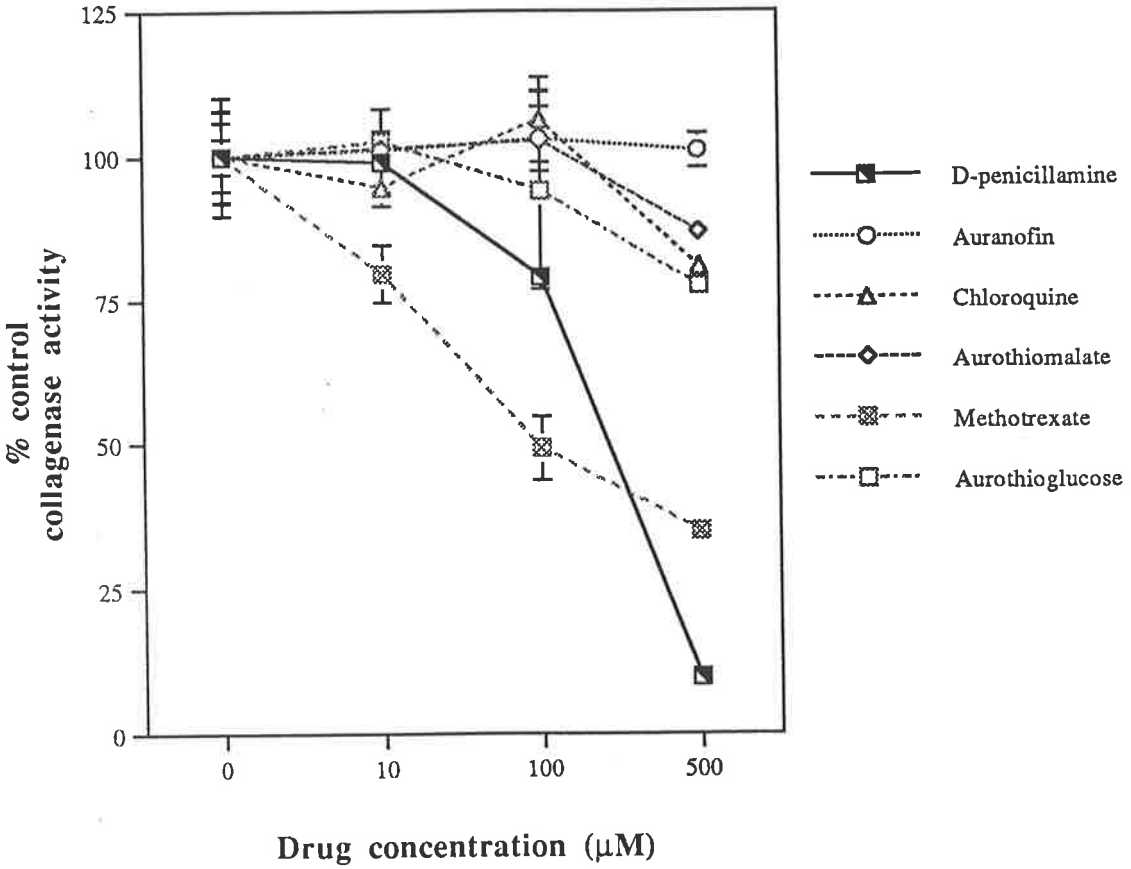

150
100
50
0
% Control
TIMP-1 activity
0
100
150
170
190
200
500
HOCl (μM)
HOCl
NCT

Figure 2.5.

Direct effect of several anti-rheumatic agents on MMP-1 activity.

Trypsin-activated BC1 collagenase was incubated with serial concentrations of different anti-rheumatic drugs including, Aurothioglucose (10-500 μM), Aurothiomalate (10-500 μM), Auranofin (10-500 μM), Chloroquine (10-500 μM), D-penicillamine (10-500 μM), and Methotrexate (10-500 μM) for 1 hour at 35°C. 100% control collagenase activity, indicates the level of BC1 collagenase activity, when this enzyme was not treated with any drug. Values represent means ± SEM for three separate experiments expressed as % control collagenase activity.

Figure 2.5


125
100
75
50
25
0
% control collagenase activity
0
10
100
500
Drug concentration (μM)
D-penicillamine
Auranofin
Chloroquine
Aurothiomalate
Methotrexate
Aurothioglucose

2.4.6. Effects of anti-rheumatic drugs on the inhibitor oxidative inactivity

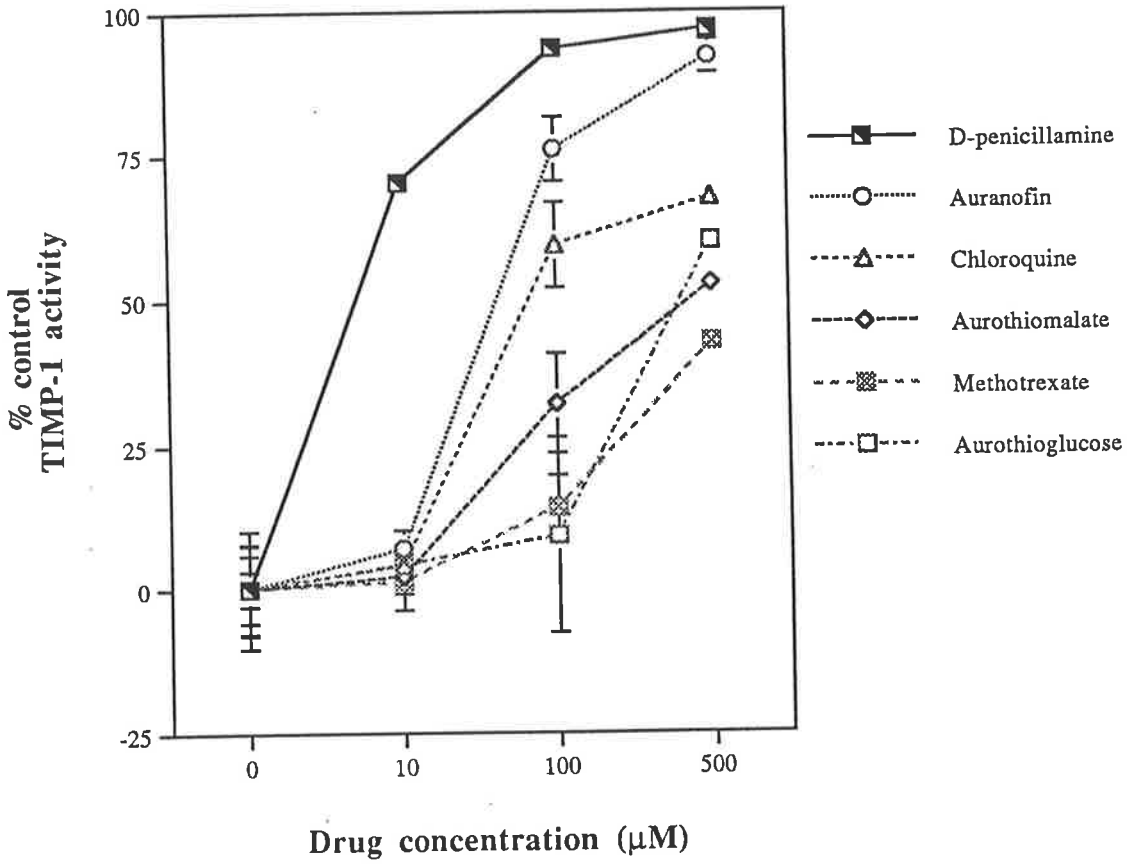
Figure 2.6 shows the results of experiments where hrTIMP-1 is exposed to HOCl in the presence of a variety of anti-rheumatic drugs. At 0% TIMP-1 activity, the inhibitory effect of TIMP-1 is completely suppressed by HOCl, while no oxidative inactivation of TIMP-1 is evident at 100%. First experiment is mainly designed for the treatment of TIMP-1, where pre-treatment with drugs can protect TIMP-1 from HOCl inactivation. In the second experiment the pretreated TIMP-1 is exposed to BC1 collagenase, where the samples are diluted, and therefore BC1 collagenase avoids exposure to drug concentrations over 30 μM. D-penicillamine shows the greatest ability to protect hrTIMP-1 from oxidative inactivation by HOCl, this effect being evident at a concentration of 10 μM. With other drugs tested, auranofin, chloroquine and aurothiomalate had the ability to protect TIMP-1 from oxidative inactivation at a concentration of 100 μM in descending order of potency. All drugs were partially effective at the 500 μM concentration. Note that this is about 20 fold higher than the biological drug levels.

Figure 2.6.

Activity of hrTIMP-1 in the presence of drugs and oxidant.

Human recombinant TIMP-1 was pretreated with a variety of anti-rheumatic drugs followed by exposing to HOCl for 1 hour at 35°C. At 0% control TIMP-1 activity, where this inhibitor was not pretreated with drugs, the hrTIMP-1 inhibitory effect against collagenase, was completely suppressed by HOCl. Whereas, at 100% control TIMP-1 activity, the oxidative inactivation of hrTIMP-1 was completely abolished by anti-rheumatic drugs. Values represent means ± SEM for three separate experiments expressed as % control TIMP-1 activity.

Figure 2.6


100
75
50
25
0
-25
% control
TIMP-1 activity
0
10
100
500
Drug concentration (μM)
D-penicillamine
Auranofin
Chloroquine
Aurothiomalate
Methotrexate
Aurothioglucose

2.5. Discussion

In the present study we have shown that HOCl can directly inactivate TIMP-1 in vitro. Our preliminary experiments with enzyme alone were undertaken using the collagen fibril digestion assay, however using inhibitors in these micro wells caused certain limitations of this assay. These limitations include, lowering the sensitivity of this assay, and also TIMP-1 was active in phosphate containing buffer whereas by using this buffer with calcium containing assay buffer, there was the risk of forming insoluble calcium phosphate. Therefore subsequent experiments with enzyme and inhibitor together were designed using the fluorimetric assay.

First, TIMP-1 was shown to inhibit the cleavage of the synthetic peptide by active enzyme. However TIMP-1 looses this ability after being pretreated by HOCl. This suggests that neutrophils, which infiltrate inflammatory sites, may contribute to TIMP-1 inactivation by releasing their major product of oxidative burst, HOCl. Quantitative analyses demonstrated that 10^6 maximally triggered neutrophils produced approximately $2x10^{-7}$ mol of HOCl during a two-hour incubation (Test and Weiss, 1986). The quantities of oxidant generated by the neutrophil are impressive. Because TIMP-1 has a 6 loop structure, made by the disulfide bond, the oxidative cleavage of these disulfide bonds may result in lack of inhibitory activity in this inhibitor.

Previous reports have shown that latent neutrophil collagenase (MMP-8) can be activated by neutrophil derived oxidants, principally HOCl (Weiss, 1989). This has highlighted the way that oxidant generation and enzymatic mechanisms can interact to lead to tissue breakdown. Direct oxidative inactivation of enzyme inhibitors is a more direct mechanism of interaction between oxidant generation and the equilibrium between proteases and their inhibitors. The most well known instance, where this occurs, is the oxidative inactivation of plasma proteinase inhibitors, such as α_1–proteinase inhibitor (α_1–PI), where oxidation of the methionine at the active site of α_1–

PI results in loss of affinity of this inhibitor for its target enzyme (Matheson et al, 1981). In another study by Frears the inactivation of TIMP-1 by peroxynitrite ($ONOO^-$) has been demonstrated (Frears et al, 1996). The structure of TIMP-1 has also been modified using the chemical reagent, diethylpyrocarbonate (DEPC), which is a potent inactivator of human TIMP-1. Exposure to DEPC specifically modify histidine residues, resulting in loss of human TIMP-1 activity (Williamson et al, 1993).

We have reasoned that since hrTIMP-1 has an elaborate 6 loop structure, each loop maintained by interchain disulfide bonds, that oxidative cleavage of these disulfide bonds would result in major structural disintegration and consequent loss of affinity for matrix metalloproteinases.

In this study, we have demonstrated that pre-exposure of hrTIMP-1 to HOCl resulted in loss of its capacity to inhibit activated collagenase. Our results are in agreement with the other study by Stricklin and Hoidal, indicating TIMP degradation with hypochlorite ion (Stricklin and Hoidal 1992). HOCl released from neutrophils rapidly reacts with readily available amines and is thus unlikely to diffuse away from the immediate pericellular environment of the neutrophil. The most abundant amine in the pericellular environment of the neutrophil is taurine, thus most HOCl is converted to NCT (Weiss 1989). We have shown that NCT does not inactivate hrTIMP-1. Thus, oxidative inactivation of hrTIMP-1 is likely to occur only in the pericellular environment of the neutrophil, or in a cellular micro-environment, where HOCl is found.

We have also shown that a spectrum of anti-rheumatic agents have the ability, at sufficient concentrations, to prevent the oxidative inactivation of TIMP-1. For D-penicillamine it is evident at concentrations approaching those reported in patients taking this agent (ie. about 100 μM). With the other agents studied however, suppression of HOCl induced inactivation of hrTIMP-1 occurs at concentrations significantly higher than plasma concentrations in patients. However, this in itself does not dismiss a potential therapeutic effect since selective cellular uptake of drugs may result in intracellular concentrations higher than plasma levels. For example, in vivo

uptake of gold occurs over days or weeks, and the final distribution of gold in cells may be very different from simple serum levels (Hurst et al, 1989, Jelum and Munthe 1992).

The inactivation of protease inhibitors by oxidants leading to enhanced proteolytic activity has been suggested as a model for neutrophil mediated matrix degradation (Weiss 1989). Our study enhances this model by demonstrating that the neutrophil mediated matrix degradation may also apply to TIMP-1 oxidative inactivation. Furthermore we showed that therapeutic agents may interfere with this balance between oxidants and enzyme inhibitors.

Our next approach would be answering the question, whether these therapeutic agents may overcome tissue destruction in another way by modulating MMP-1 and/or TIMP-1 expression from the cells which are present in inflamed area, synoviocytes. This area of action for different anti rheumatic agents has been explained in chapter 3.

Chapter 3

Effects of gold sodium thiomalate on MMP-1 and TIMP-1 gene expression in the absence or presence of glucocorticoids

3.1. Abstract

The inflammatory cytokine, interleukin-1 (IL-1), which is present in inflamed joints, enhances collagenase gene expression (Mc Neul et al, 1990). Gold sodium thiomalate (GSTM) is a well established disease modifying anti-rheumatic agent used in the treatment of RA (Walz, 1984). Glucocorticoids have potent anti-inflammatory effects including the suppression of collagenase gene expression (Jonat et al, 1990). There is also evidence that GSTM may interfere with the DNA binding and transactivation activity of the glucocorticoid receptor (GR) (Makino et al, 1993).

We examined the effect of GSTM, Gold sodium thioglucose (GSTG), and thiomalate (TM) on collagenase and TIMP-1 gene expression in cultured human synovial fibroblasts. Steady-state mRNA levels for these genes and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were determined by Northern analysis. The level of mRNA for collagenase and TIMP-1 genes, expressed under experimental conditions was quantified by the slot-blot technique.

GSTM inhibited the IL-1 stimulated expression of collagenase mRNA, however it did not significantly change the expression of TIMP-1 mRNA under the same conditions. GSTM 500 μM resulted in 50% inhibition of IL-1β stimulated collagenase gene expression. TM alone did not alter collagenase gene expression at the same concentrations. GSTG produced the same effect as the effect of GSTM at equimolar concentrations.

These observations suggest that gold compounds have the capacity to regulate collagenase, but not TIMP-1 gene expression after stimulation by IL-1β. This capacity is likely to be dependent on the presence of the aurous ion, as it was not seen with ligand (TM) alone. Furthermore, GSTM counteracted the repression of collagenase gene expression by glucocorticoids.

3.2. Introduction

Gold in the form of the gold (I) compounds, such as GSTM has been advocated for the treatment of a number of human diseases, especially inflammatory polyarthritis such as rheumatoid arthritis (RA). GSTM is a water soluble salt of gold thiol complexes. The clinical efficacy of gold compounds in the treatment of RA has been clearly established, however their mechanism of action is still uncertain. Recently it has been found that GSTM acts as an immunosuppressive agent which inhibits protein kinase C (PKC) (Hashimoto et al, 1992). This effect was attributed to the interaction of gold with the sulfhydryl moiety in the enzymatic domain of PKC. It has been demonstrated that GSTM significantly down-regulated expression of adhesion molecules, including intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), on human umbilical cord vein endothelial cells (HUVECs) (Koike et al, 1994).

The synovial membrane consists of a thin layer of synovial cells that rapidly merges into the subsynovial layers. The normal synovial lining layer is made of Type A cells, believed to stem from monocytes. Type A cells are macrophage-like, and they exhibit phagocytic functions. Type B cells have fibroblast-like morphology, and synthesize hyaluronan which is responsible for synovial fluid viscosity.(Schumacher et al, 1985; Wiessmann et al, 1982). In joint diseases such as RA, the cellular phenotype and architecture of the synovial tissue is greatly altered (Harris, 1990). In situ studies of rheumatoid synovium have demonstrated that the fibroblastoid-like synovial lining cells can cause matrix degradation by increased expression of stromelysin and

collagenase (McCachren et al, 1991). Cytokines released by monocytes and lymphocytes infiltrating the tissue beneath the lining layer might initiate the expression of these metalloproteinases.

Fresh and cultured fibroblastoid cells from rheumatoid synovium are capable of producing a number of cytokines including IL-1, IL-6 and transforming growth factor β (TGF-β) on their own (Ritchlin et al, 1994). Several studies on cultured fibroblasts from rheumatoid and osteoarthritic synovium have shown that synovial cells from patients with RA differ from those with non-inflammatory synovitis like OA. For example, in fibroblasts cultured from RA synovium up to at least the tenth passage, (representing 5-6 months of tissue culture), the net amounts of MMP-3, MMP-1 and TIMP-1 were still significantly greater than those expressed in third passage synovial cells from patients with OA, although for both, the level decreased with increasing passage number (Murphy et al, 1981). Since type A (macrophage-like cells) do not persist for this long in culture, this persistently altered gene expression involved the type B (fibroblast-like) cells. Therefore the main focus in this study was the fibroblast-like synoviocytes.

Cytokines are important mediators of inflammation, playing a major role in the physiologic regulation of connective tissue metabolism. The levels of IL-1, tumour necrosis factor-α (TNF-α), IL-6 and other cytokines are elevated in the synovial fluid and the serum of patients with RA (Barrera et al, 1996). There is also evidence that serum levels of IL-1 and IL-6 correlate with disease activity (Schlaghecke et al, 1994).

Both IL-1 and TNF-α up-regulate collagenase gene expression in synovial cells in culture (Mackay et al, 1992; Unemori et al, 1991). These two cytokines have also been shown to increase TIMP expression in skin fibroblasts (Dayer et al, 1986), which forms another level of regulation of activity. In a study on the post-receptor signalling pathways through which IL-1β exerts its effects, it has been indicated that IL-1β stimulation of MMP expression and synthesis in human synovial fibroblasts involves the activation of PKC (DiBattista et al, 1994).

Dexamethasone treatment of the fibroblasts, reduces PMA-induced steady state levels of the collagenase mRNA (Jonat et al, 1990). Repression of metalloproteinase synthesis by glucocorticoids occur transcriptionally. Briefly, in glucocorticoid mediated repression, AP-1 (one enhancer element, located in the promoter region of collagenase gene site) is the best understood region. In the presence of hormone (h), the glucocorticoid receptor (GR)-hormone (GRh) complex interacts with the AP-1, causing repression of transcription (Vincenti et al, 1994). The possibility that GSTM may interfere with the DNA-binding and trans-activation activity of the GR was reported by Makino and his colleagues (Makino et al, 1993). They (Makino et al, 1994) have demonstrated this antiglucocorticoid action of GSTM, using phorbol ester-treated human fibroblasts. Although in their study, GSTM did not prevent phorbol ester-mediated induction of collagenase gene expression, in contrast, GSTM paradoxically derepressed effects of glucocorticoids on collagenase gene expression and restored collagenase mRNA levels. They suggested that the molecular target of that paradoxical GSTM action was likely to be the glucocorticoid receptor.

Given this background, In order to elucidate the effects of GSTM on collagenase activity, we have examined the levels of mRNA for MMP-1 and TIMP-1 in cultured human synoviocytes exposed to drug and IL-1β. Because our main interest was to examine different effects of gold containing therapeutic agents on MMPs and TIMP expression, we felt it important to examine the effect of GSTM on glucocorticoid-mediated repression of collagenase gene expression in IL-1β pre-treated human synovial fibroblasts.

3.3. Materials and methods

3.3.1. Reagents and drugs

Dulbecco's Modified Eagle's Medium (DMEM) was from Cytosystems Pty Ltd, Castle Hill, NSW, Australia. Human recombinant interleukin 1-Beta (IL-1β), BSA , Sephadex G-50 spin column, HEPES and salmon sperm DNA, gold sodium thioglucose, thiomalate and dexamethasone were all obtained from the Sigma Chemical Company, St. Louis, Mo 63178 USA. RNAzol was from TEL-TEST INC. USA. Gigaprime DNA labelling kit and (^{32}P) dCTP were both from Bresatec Ltd, Thebarton, South Australia. Positively charged nylon hybridization transfer membrane (Hybond TM-N+) was from Amersham Australia, PO Box 99, North Ryde, Sydney, New South Wales, Australia. Gold sodium thiomalate was from Aldrich Chemicals, Castle Hill, New South Wales, Australia. All drugs were stored desiccated as powder at -20°C. To prepare fresh solutions of drugs on a daily basis, each of them was dissolved in DMEM except for thiomalate, which was dissolved in PBS and filtered through a sterile filter unit (MILIEUX-GOV 0.22 μm Filter Millipore Products Division, Bedford, MA 01730).

3.3.2. Fibroblast cultures

Synovial tissue was obtained from the patients with RA during hip replacement surgery or arthroscopy. Patients were asked to participate in this study and informed consent form was obtained. The study protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital. Cultured synovial fibroblasts were prepared according to the method of Tessier (Tessier et al, 1993). Briefly, the tissues were minced into 1-3 mm^3 pieces, suspended in 3 ml of DMEM, supplemented with 15% heat inactivated foetal calf serum (FCS) and antibiotics in a 25 mm^2 flask, and incubated for 2 weeks at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested from stock cultures by trypsinization with 0.5% trypsin at 37° for 5 min

and subcultured into 25-mm^2 or 75 mm^2 flasks. By the third passage, all of the cultured cells exhibited the typical fibroblast-like morphology. Fibroblasts in the 3-9 passage were inoculated into 60 x 15 mm sterile polystyrene tissue culture dishes (LUX Contur style, Nunc, Inc., Naperville USA) at 5 x 10^5 cells/well, and grown to confluency. In general, before experimentation, cells were exposed to serum free media containing 0.2% BSA for 24 h, since FCS contains a variety of growth factors and cytokines that may modulate collagenase and TIMP-1 synthesis by fibroblasts. All experiments with cytokines and drugs were performed between the third and ninth passage in serum free media. The viability of cells was always examined with trypan blue exclusion.

3.3.3. Plasmids

Plasmids containing the cDNA sequences of interest were obtained from a number of sources:

Human collagenase cDNA- 1.25 Kb fragment of the human collagenase cDNA in SP6 plasmid (vector circle), between the Hind III and Bam HI restriction sites of the polylinker region (constructed by Mark I Cockett - Celltech Ltd)

Human stromelysin cDNA- 1.58 Kb human stromelysin cDNA in SP6 plasmid (circle vector), between two Eco RI restriction sites of the polylinker region (constructed by Mark I Cockett - Celltech Ltd)

Human TIMP-1 cDNA- An approximately 650 bp fragment of the human TIMP-1 cDNA in vector pSP65 inserted between HindIII to BamHI restriction sites (Docherty et al, 1985). These plasmids were kind gifts from Dr. A Docherty, Celltech Ltd.

Human GAPDH cDNA- 1.007 Kb fragment of the human epithelium GAPDH cDNA in the ampicillin resistance vector, pStII inserted between EcoRI to BamHI restriction sites. This plasmid was a kind gift from Dr. Trapnell (Arcari et al, 1984).

3.3.3.1. Amplification and purification of plasmid cDNA

Small quantities of plasmids were obtained, therefore cloning and growth in competent bacteria was required to generate adequate amounts for the following experiments. All plasmids used, contained genes for ampicillin resistance. All manipulations involving bacteria were conducted in a laminar flow cabinet in a specially designated 'PC2' class room. E. coli bacteria cells, DH5a from a glycerol stock that was stored for several months at -70°C, were grown on an L-broth (Tryptone 10 g, yeast 5 g, NaCl 10 g in 1 litre H_2O, pH = 7.5, autoclaved) LB-Agar plate at 37°C overnight. One of the colonies grown on the LB-Agar plate over night was inoculated into a 10 ml sterile tube containing 5 mls of L-Broth and incubated in an agitating incubator at 37°C to grow overnight. The best time to introduce the plasmid into the bacteria is at its logarithmic phase. To keep the bacteria at its logarithmic phase, 1 ml of the above mixture containing bacteria was placed into a 500 ml autoclaved flask containing 50 mls of L-Broth. An OD_{600} absorbance between 0.35 and 0.45 was indicative of the logarithmic phase of bacterial growth. Rate of growth was periodically checked by analysing an aliquot of the culture suspension with a spectrophotometer. Using a glass 1 ml cuvette, the instrument was blanked with L-broth. 1 ml aliquots of the culture were periodically removed and the absorbance at 600 nm was read. After 2 hrs incubation, the OD_{600} was 0.35 and bacterial growth was stopped by setting the culture (flask) on ice for 30 mins. The total volume of the culture suspension, containing bacteria at its logarithmic phase, was then transferred to a precooled sterile 50 ml centrifuge tube and centrifuged at 1500 g at 4°C in a Beckman JB 20 rotor for 15 minutes. The supernatant was gently tipped off and the pellet was gently resuspended in 17 ml of precooled simple frozen storage buffer [FB buffer: KCl 7.4 g, $CaCl_2.2H_2O$ 7.5 g, glycerol 100 g, potassium acetate ($KC_2H_3O_2$) 10 mls of a 1 M stock (0.01 M), pH = 7.5] using a pipette, then kept on ice for 30 mins. This buffer facilitates subsequent transformation (plasmid incorporation) and then the suspension was centrifuged at 1500 g at 4°C for 15 minutes. The final pellet was resuspended in 2 mls of FB buffer. The bacteria was

dispensed to pre-cooled 10 ml sterile tubes on ice, enough tubes to have three tubes per plasmid being prepared, plus an extra to act as a negative control. For each plasmid the three tubes were prepared by adding 10, 20, or 50 ng of plasmid stock. This dose range was used to help ensure that following subsequent growth on agar plates, at least one of the concentrations would yield a plate from which a single colony could easily be picked. No plasmid was added to the negative control tube. After the appropriate amounts of plasmid were added to the bacteria, the tubes were kept on ice for 30 mins. Then the cells were heat shocked by partially immersing the tubes into a water bath at 42°C for 1 minute and 15 seconds to introduce the plasmid into the bacterial cells, then returned to ice. The transformed bacteria were grown in 800 ml of L-broth at 37°C for 30 minutes in an agitating incubator. Ampicillin agar plates 0.1% were prepared by adding tryptone 10 g, yeast 5 g, NaCl 10 g, and agar 15 g in 1 litre H_2O, pH = 7.5, autoclaved, cooled to 60°C, then ampicillin (100 mg), was added. This solution was then poured into 100 mm sterile Petrie dishes in a laminar flow cabinet, and allowed to set. In order to collect the plasmid containing bacteria, 200 µl of these transformed cells, from each 10 ml tube, were grown on the 0.1% ampicillin agar plate, then the plates were incubated overnight at 37°C overnight. Only bacteria that contain the antibiotic resistance plasmid could grow on this plate, whereas the untransformed negative control bacteria failed to grow. This indicated successful transformation by plasmids as evidenced by the imparting of ampicillin resistance to the transformed cells.

3.3.3.2. Small scale preparation of plasmid cDNA

A mini-prep plasmid purification was performed to confirm the identity of the transforming plasmids.

Single colonies of transformed cells were picked with a sterile wire loop and inoculated into 10 ml tubes containing 2 mls L-broth, then incubated at 37°C, shaking, overnight. 4 colonies were picked and put into a separate tube, for each plasmid. Next day 1.6

mls was transferred from each tube to a microcentrifuge tube. Glycerol stocks were made with the remaining 400 µl and stored at -20°C. 1.6 mls of the suspension of transformed bacterial cells were spun at 8000 g for 30 seconds at 4°C in a microfuge. The medium was removed by aspiration and the remaining pellet was resuspended in 50 µl TES buffer (Tris 25 mM, EDTA 10 mM, SDS 0.1%) to cause lysis of cells. Then 200 ml of 0.2 M NaOH, 1% SDS solution was added to the mixture of the lysed cells and mixed, then 150 µl of ice-cold Na Acetate 3 M, pH 4.6 was added to the above mixture and frozen at -20°C for 60-90 minutes. The cold mixture was subsequently spun at 8000 *g* for 15 mins at room temperature (RT).

Supernatant containing plasmids was removed into a sterile Eppendorf tube. The plasmid DNA was recovered from the lysate by extraction with phenol-chloroform then ethanol precipitation as follows. An equal volume (400 µl) of a mixture of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the tube, vortexed, and centrifuged at 13000 rpm for 3 min, then the upper, aqueous phase removed to a new tube. To precipitate the cDNA plasmid, 1 ml of 100% cold ethanol was added to the supernatant and left for 5 minutes at -20°C. The DNA was pelleted by centrifuging at 13000 rpm for 15 min, then the pellet was washed again with 70% ethanol and centrifuged at 7000 *g* for 15 minutes. To remove any contaminating bacterial RNA, the cDNA plasmids were pelleted and resuspended in TE/RNase buffer (Tris, EDTA, RNase A 20 µg/ml) and stored at -20°C.

3.3.3.3. Digestion of cDNA plasmid

To confirm the presence of the appropriate plasmid a restriction enzyme digestion reaction was designed to separate the cDNA insert from the plasmid backbone, then agarose gel electrophoresis to determine the size of the cDNA fragment was performed. For each digestion, 1 µl of the selected plasmid was digested using the appropriate restriction enzymes for that specific plasmid. For example for SP64 human collagenase plasmid, 1 µl of plasmid containing MMP-1 cDNA was added to 2 µl of

one-phor-all buffer. Then 1 µl of each of the two restriction enzymes Hind III and Bam HI which are specific for the SP64 plasmid were added to the above mixture, with 15 µl H_2O. The digestion process was done at 37°C for 1 hour. A final volume of 10 µl of the cleaved product was loaded onto a 1% agarose gel in TAE buffer containing ethidium bromide. The samples were electrophoresed at 70 volts for 1 hour, using a Bio-Rad Model 1000/500 power supply. A polaroid photograph of the gel under UV illumination was taken to show desired bands, the cDNA products and the remaining part of the plasmid after being cut. The high molecular weight marker next to the products, indicates the actual size of each product which is in agreement with the size of each insert, for human recombinant TIMP-1 (hrTIMP-1, 0.9 Kbp), human recombinant MMP-1 (hrMMP-1, 1.25 Kbp), for human recombinant stromelysin (hrMMP-3, 1.58 Kbp). Once the plasmid identity had been confirmed, the glycerol stocks made earlier from the 'mini-prep' transformed bacterial cultures were used for a large scale plasmid purification (maxi-preparation).

3.3.3.4. Maxi preparation of cDNA plasmids

For large scale plasmid preparation, the cDNA containing bacterial cells, 20 µl, were incubated in 250 ml of T-broth containing 0.1% ampicillin in a large conical flask and incubated in an agitating incubator at 37°C overnight. Bacteria from overnight culturing were collected after 10 minutes centrifugation at 1500*g*, then resuspended in TES buffer. These cells were lysed by adding 10 mls of NaOH (200 mM) in SDS (1%) and 10 mls of K-Acetate (3 M). The mixture was kept at -20°C for 15 minutes. To remove the lysed cells, the above mixture was centrifuged in 1500*g* at 4°C for 15 minutes. Plasmid containing supernatant was removed into the falcon tubes and each tube was filled with cold isopropanol and kept on ice for 10 minutes. The above mixture was centrifuged at 1500*g* at 24°C for 15 minutes to precipitate the cDNA plasmids. The pellet was washed in 70% ethanol and left to dry for 10 minutes. The dried pellet was resuspended in 2.5 ml TE buffer, containing ethidium bromide. Then 100 mg of Cesium chloride was added to each tube and fully dissolved by vortex

mixing. The solution was centrifuged at 12000*g* at 25°C for 18 hrs in a Beckman Optima TAX Ultracentrifuge, using a 100.2 rotor.

After ultracentrifuging, the cDNA plasmid containing layer appeared as a thin pink layer in the middle of each tube. This layer was carefully aspirated from each tube using a 19 gauge needle and a 1 ml syringe. TE buffer was added to make up the final volume to 2 mls. Then 2 mls of iso-amyl alcohol was added and the mixture was vortex mixed. After settling this mixture for a few minutes, the ethidium bromide containing layer which was on top was aspirated. The remained solution was washed again with another 2 mls of isoamyl alcohol, to remove the remaining ethidium bromide. The washing step was repeated several times until all of the ethidium bromide was removed. To precipitate the plasmids, 5 mls of cold 100% ethanol was added and the mixture was left at -20° for 30 mins. The pellet was collected by centrifuging the mixture at 4000*g* for 30 mins. After removing the supernatant and aspirating the excess fluid, the remained pellet was left to dry for 5 mins. The pellet was resuspended in 500 µl TE buffer by vortexing. A mixture of 50 µl of 3 M sodium acetate pH 4.6 and 1 ml ethanol 100% was added to the plasmids and stored at -20° for 30 mins. Then the mixture was centrifuged for 15 mins at 4°C. The pellet was washed twice with 1 ml of 70% ethanol and re dissolved in 200-500 µl of diethyl pyrocarbonate (DEPC) treated water and stored at -20°C. The OD_{260} for the plasmid products was measured using the UV lamp on a spectrophotometer.

3.3.3.5. Isolation of cDNA sequence

The cDNA sequence was separated from the plasmid sequence, for further use as a template for generating radio labelled probes. Restriction enzymes were used to cut the plasmid DNA in the polylinker regions at either end of the cDNA. The restriction sites for each cDNA are indicated above. A typical digestion reaction consisted of combining reagents in the following proportions- 100 µg plasmid, 10 µl restriction enzyme 1, 10 µl restriction enzyme 2, in One-Phor-All buffer (made to a final

concentration of 1x by adding an appropriate amount of sterile water). The reaction was performed in a microcentrifuge tube incubated at 37°C for 4 hours, then heated to inactivate the restriction enzymes, depending on the enzymes used. A small aliquot was run in a 1% agarose gel to check for completeness of digestion. If satisfactory, the entire reaction volume was loaded in a 1% gel and run sufficiently to clearly separate cDNA band from plasmid bands.

The cDNA band was cut from the gel and the cDNA recovered from the gel by electroelution as follows. The gel slice was placed in a strip of 1.5 cm diameter dialysis tubing (MW<10000), with a small amount of 1 x TAE running buffer, then the tube was clamped at either end and placed transversely in a gel running tank filled with running buffer. Current was applied for sufficient time to ensure cDNA passage out of the gel, then the current direction was reversed briefly (30 sec). The running buffer containing the cDNA was aspirated from the dialysis tubing and concentrated by spinning through a Centricon 30 micro concentrator tube. The tube was topped up and re-spun twice with sterile water to remove salts, then the retentate collected into a microfuge tube and the cDNA quantitated by spectrophotometer (OD_{260} of 1 = 50 mg/ml DNA). A small aliquot was also run in a 1% agarose gel to check the integrity of the cDNA.

3.3.4. Isolation of messenger RNAs

Total cellular RNA was isolated by the method of Chomzynski P (Chomzynski et al, 1987). Briefly RNAzol TM B (TEL-TEST INC. USA) was used to denature the cell membrane and RNA was extracted with phenol-chloroform/isoamylalcohol. Isopropanol was used to precipitate the RNA. Following solubilization of the RNA pellet in DEPC treated sterile water, RNA was quantitated spectrophotometrically at 260 and 280 nm with a spectrophotometer (Beckman DU 640, Beckman instruments), and the OD_{260}/OD_{280} ratio was between 1.7 and 2.0.

3.3.5. Radiolabeling of cDNA probes

Probes were prepared from the 100 ng of cDNA for MMP-1, TIMP-1, or glyceraldehyde phosphate dihydrogenase (GAPDH), that was denatured at 95°C for 10 min, in 25 ml hybridization solution. cDNA were then labelled with the stabilised α-^{32}P deoxycytidine 5'-triphosphate (^{32}P) dCTP, 10 μci/ml using a Gigaprime DNA labelling kit (oligo-labelling or random-priming system). The probe was then purified on a sephadex G-50 spin column. Specific radioactivity of the probes was usually about 2 x 10^8 cpm per mg of cDNA in 25 ml hybridization solution.

3.3.6. Northern blotting

Quadruplicate samples of total cellular RNA (6-7 μg) were denatured and electrophoresed on a formaldehyde gel (Maniatis et al, 1982). RNA bands were stained with ethidium bromide, photographed, blotted onto nylon membranes, baked and crosslinked for 3 mins to UV light. Then each lane was separated and probed for either MMP-1, TIMP-1, and GAPDH-labelled cDNAs. Blots were prehybridised for 3 hours at 42°C in a solution containing 50% formamide, 5 x SSPE (1.5M sodium chloride, 0.1M sodium phosphate, 0.01M ethylene diamine tetra acetic acid (EDTA) pH = 7.4, 100 μg/ml denatured salmon sperm DNA, 2 x Denhardt's solution, and 5% sodium dodecyl sulphate (SDS). Hybridization was performed in the same solution by addition of denatured probe for 22-24 hours at 42°C. Blots were washed sequentially in 2 x SSPE and 0.1% SDS, twice at room temperature. This was followed with a wash in 1 x SSPE/0.1% SDS at 42°C. Membranes were exposed to a Kodak X-Ray film in an autoradiograph cassette for 5 days.

3.3.7. Slot-blot analysis

Purified total RNA (approximately 3 μg) was denatured with 3 x volume of a denaturing mixture (21% formaldehyde, 13% of 10 x MOPS and 66% formamide) and heated for 15 min at 95°C. Positively charged nylon hybridization transfer membrane (Hybond TM-N+) was hydrated for 25 mins in sterilized 20 x SSC (3M NaCl, 0.3M Sodium citrate, PH = 7.0). The membrane was then mounted in a 48-well, slot-blot apparatus (BioRad, 3300 Regatta Boulevard Richmond, California 94804). 1 ml of the corresponding high salt solution cold 20 x SSC was added to each of the denatured RNA samples immediately prior to transfer. Each sample was applied in quadruplicate containing four descending dilutions (1, 1/2, 1/4, 1/16). Samples, were then vacuumed through the membrane and wells were washed once with 500 ml 20 x SSC and twice with 500 ml 10 x SSC. Membranes were removed from the slot-blot apparatus, and RNA was crosslinked to the membrane by exposure for 3 min to UV light.

3.3.8. Hybridization

Membranes were prehybridized in a mixture of sterilized, DEPC treated 10 x SSPE pH = 7.4, 0.5% SDS, 5 x Denhardt's reagent and 0.5 mg denatured salmon sperm DNA for 1 hour at 65°C. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe served as a control of RNA loading as it is constitutively expressed. The prehybridized membrane were then hybridized with the denatured probe in 25 ml of the mixture for 18-24 hours (Maniatis et al., 1982). After hybridization stringent serial post hybridization washes were conducted at 65°C, with the final wash in 0.1 x SSPE, 0.1% SDS, dried, and wrapped in clear Glad Wrap and exposed to X-ray film for 24 hours at -70°C (Bennett et al., 1994). The density of each lane was quantitated by exposing the membrane to a phosphorimager screen (Molecular Dynamics) and the image on the screen was scanned using an Image Quant (IQ version 3.0) soft ware on a Molecular Dynamics PhosphorImager computer.

To re-probe each membrane, the first probe was removed by using the solution of boiled 0.5% SDS for 15 mins at room temperature (Liu et al, 1995).

3.3.9. Morphology and viability of human synoviocytes

Figure 3.1 shows the cellular morphology of nonstimulated and stimulated human synoviocytes with 10 pM IL-1β in the absence and/or presence of GSTM (500 μM). The trypan blue dye exclusion test has indicated that, cell viability was $>98\%$. However, decreased viability of these cells was observed when >500 μM GSTM was used. The inhibition of the collagenase gene by GSTM, in the experimental range, could not therefore be explained by detachment of the cells from the culture plates.

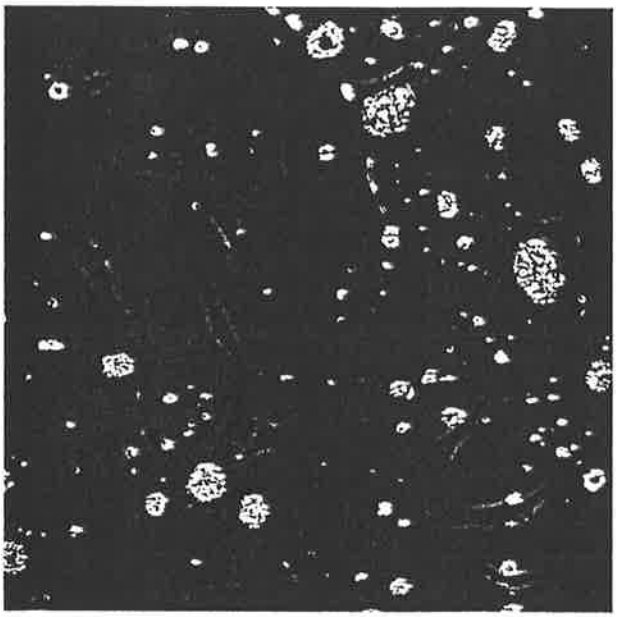
3.3.10. RNA, Protein, and data analysis

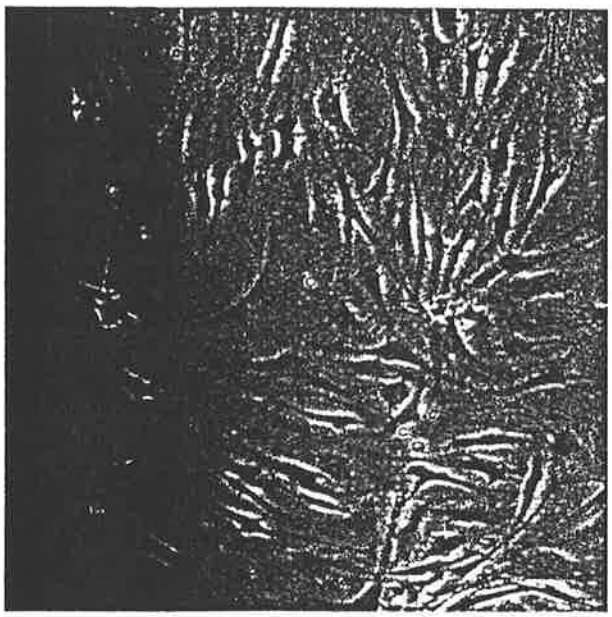
The results are expressed as the relative intensity (RI) of MMP-1 or TIMP-1 mRNA normalised to the level of GAPDH mRNA. Percent control of the RI was calculated as the ratio of the RI of collagenase or TIMP-1 band, to the RI of the GAPDH band. Values were expressed as mean ± the standard errors of the mean (SEM) with "n" refers to number of cell lines (i.e., patients) analysed. Where SEM is not shown, the size of the error bar is the same size or smaller than symbols. Student's t-test was used to test for significant differences between means.

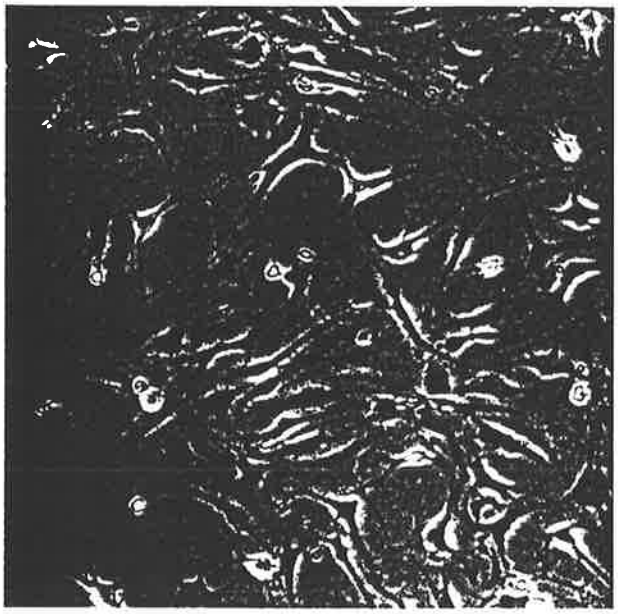
Figure 3.1.

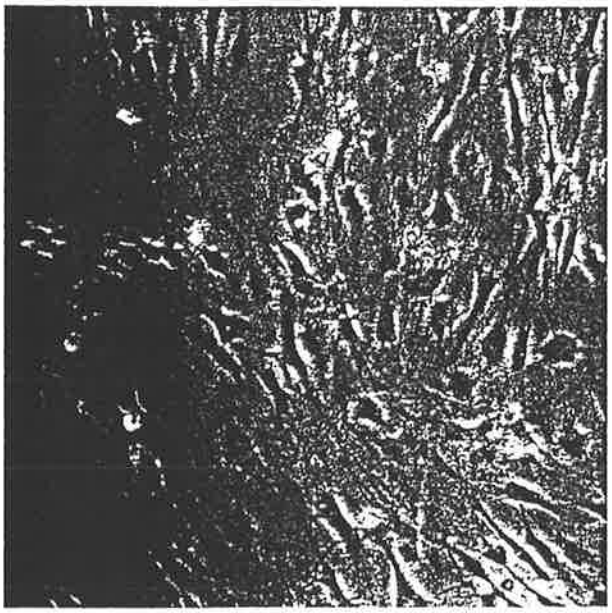
Cellular morphology of primary and passaged cultured synoviocytes.

Photographs of A. primary cultures , B. Synoviocyte cultures in passage 3 , C. IL-1β (10pM) treated synoviocytes in passage 3, D. Third passaged cells treated with IL-1β in combination with GSTM 500 μM









3.4. Results

3.4.1. Specificity of each probe to its complementary mRNA

We initially investigated the expression of the enzyme collagenase and its inhibitor TIMP-1 in our system by cultured human synoviocytes upon stimulation with IL-1β (10 pM) in serum free medium. We used complementary DNA probes corresponding to collagenase, TIMP-1 and the control gene GAPDH. Figure 3.2 shows the autoradiographs of Northern blot analysis using mRNA isolated from confluent cultured human synoviocytes in serum free medium for 24 hours. RNA from these cells showed a detectable level of hybridization to the bands, corresponding to TIMP-1, MMP-1, and GAPDH, ^{32}P-labelled cDNA, indicating the specificity of each cDNA probe to its complementary mRNA.

3.4.2. Human recombinant IL-1β-mediated collagenase and TIMP-1 mRNA expression was time and dose dependent

Dose-response studies of IL-1β mediated collagenase and TIMP-1 mRNA expression were performed using synoviocytes from the same patients. Figure 3.3A shows that there is a dose-dependent increase in cytokine-induced collagenase expression between the concentrations 1-100 pM of IL-1β, yielding the maximal response at 100 pM. TIMP-1 mRNA expression is also up-regulated in a dose-dependent manner reaching a maximum by using IL-1β (10pM) (Figure 3.3B).

Human recombinant IL-1β (10pM) induced collagenase mRNA expression in cultured human synoviocytes from patients with RA in a time-dependent fashion. This was seen in the first 24 hours of incubation and plateaued after that. There is a significant increase in the level of collagenase mRNA expression at 8 hours of incubation with IL-1β (10pM) compared to the control (Figure 3.4).

These results are in accordance with the previous studies and allowed us to establish optimal conditions (IL-1β 10 pM for 24 hrs) to examine the effects of anti-rheumatic agents particularly gold compounds and their ligands on collagenase and TIMP-1 production.

Figure 3.2.

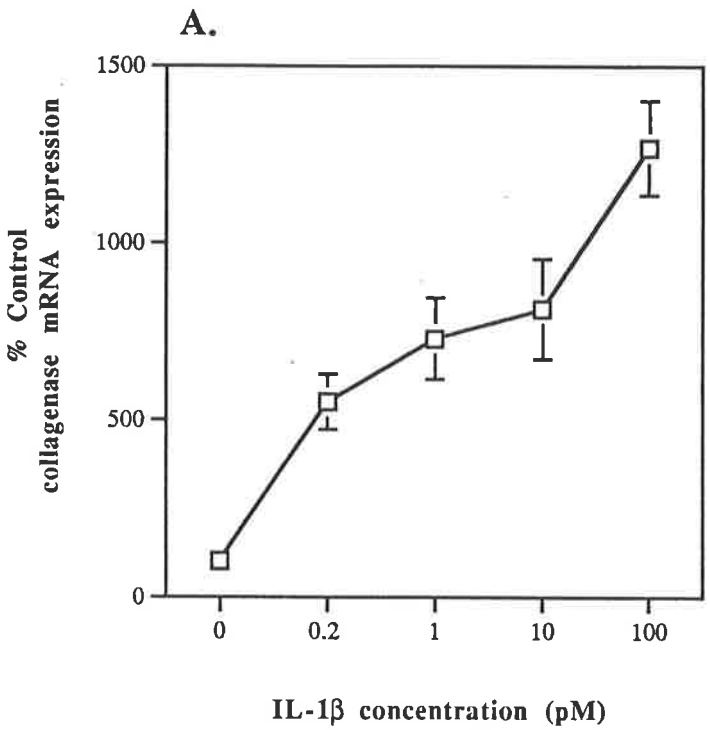
Messenger RNA (mRNA) expression of collagenase, TIMP-1, and GAPDH in cultured human synovial fibroblasts.

Autoradiographs of Northern blot analysis using mRNA isolated from human synovial fibroblast in serum-free medium for 24 hours. The Northern blots were hybridised as described in the methods section. Detectable levels of hybridization to the bands, corresponding to A. TIMP-1, B. MMP-1, and C. GAPDH, ^{32}P-labelled cDNA, indicates the specificity of each cDNA probe to its complementary mRNA.

Figure 3.3.

IL-1β induced a dose-dependent increase in collagenase and TIMP-1 mRNA expression.

Human synoviocytes were cultured with various concentrations of stimulant for 24 hours. After incubation time, (A) collagenase and (B) TIMP-1 mRNA expression was assayed by the slot blotting technique. The vertical axis represent the % control of RI for collagenase and TIMP-1 mRNA expression; bars, SEM.


A.
% Control
collagenase mRNA expression
1500
1000
500
0
0
0.2
1
10
100
IL-1β concentration (pM)

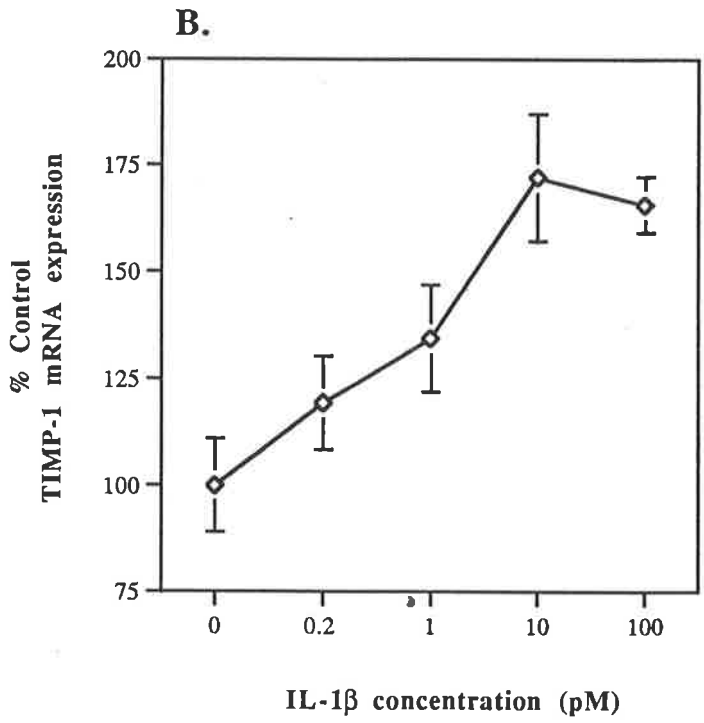
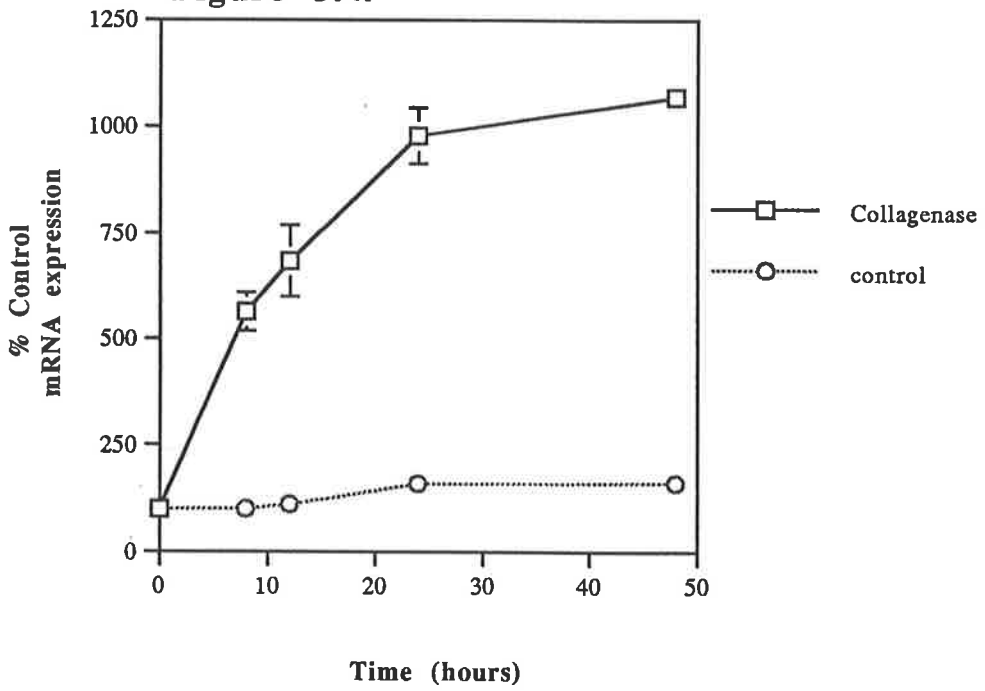

B.
% Control
TIMP-1 mRNA expression
200
175
150
125
100
75
0
0.2
1
10
100
IL-1β concentration (pM)

Figure 3.4.

Time-dependent increase in collagenase mRNA expression by IL-1β induced human synoviocytes.

Human synovial fibroblasts were cultured with 10 pM IL-1β for the indicated time points. After incubation time, collagenase mRNA expression was assayed by the slot blotting technique as described in the methods section. The vertical axis represents % control of the RI for collagenase mRNA expression.

Figure 3.4.


1250
1000
750
500
250
0
% Control
mRNA expression
0
10
20
30
40
50
Time (hours)
Collagenase
control

3.4.3. GSTM significantly suppressed collagenase gene expression on rhIL-1β stimulated cultured human synovial fibroblasts

Pre-treatment of human synovial fibroblasts with GSTM and GSTG down-regulated IL-1β induced collagenase gene expression between the concentrations 100-500 μM, whereas TM was without significant effect at this range (Figure 3.5). Note that drug concentrations used in these experiments were 5 to 10 fold higher than clinically attainable concentrations. Each compound tested had no significant effect on the rhIL-1β induced TIMP-1 expression in the same cells and under the same conditions (Figure 3.6). Figure 3.7 also shows the slot-blot analysis of collagenase and TIMP-1 induction by IL-1β followed by repressive effects of GSTM. IL-1β (10 pM) was added to confluent monolayers of human synovial fibroblasts in serum-free medium in the presence and absence of GSTM at the final concentrations of 100 and 500 μM. RNA was harvested after treatment of the cells and blotted in duplicate onto a blotting membrane. Blots were then hybridized with ^{32}P-labelled cDNA clones for collagenase (A) and TIMP-1 (B). GAPDH ^{32}P-labelled cDNA, was also included as a control.

3.4.4. Glucocorticoid-mediated suppression of collagenase gene expression and effects of GSTM

We then examined the suppressive effects of a synthetic glucocorticoid, dexamethasone on collagenase and TIMP-1 expression. We studied the possibility that GSTM might cooperate with and/or potentiate the negative effect of glucocorticoids on collagenase gene expression.

Dexamethasone treatment of human synoviocytes significantly reduced IL-1-induced levels of collagenase mRNA but not TIMP-1 (Figure 3.8), indicating the presence of functional GR in these cells. Next we pretreated RA synoviocytes with dexamethasone

in the absence or presence of GSTM (1-100 μM), followed by another 24 hours treatment with IL-1β (10pM), cells were then collected and the extracted mRNA was used to determine the relative intensity of the collagenase mRNA expression under treatment conditions. Figure 3.9 shows that, GSTM (100 μM) counteracted dexamethasone-induced repression of collagenase gene expression, although only 30% of the inhibitory effect of dexamethasone was blocked. This effect of GSTM was not evident when lower concentrations of this drug were used.

A

28 S

Collagenase

18 S

B

28 S

18 S

TIMP-1

C

28 S

18 S

GAPDH

Figure 3.5.

Expression of collagenase mRNA on human synovial fibroblasts incubated with IL-1β in the absence and presence of GSTM, TM and GSTG.

Collagenase mRNA expression was measured after 24 hrs using slot blot analysis. Mean ± SEM was calculated from three individual experiments. Where SEM is not shown, it is less than or equal to the size of the markers. *, $p < 0.05$; **, $p < 0.005$.

Fig 3.5.

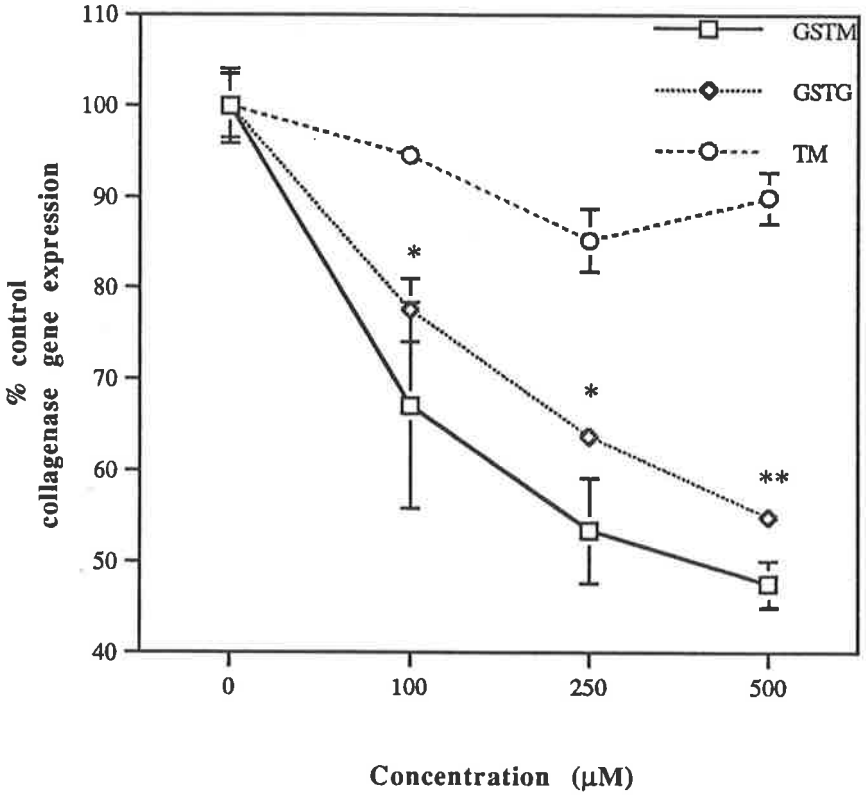

GSTM
GSTG
TM
% control collagenase gene expression
110
100
90
80
70
60
50
40
0
100
250
500
*
*
**
Concentration (μM)

Figure 3.6.

Expression of TIMP-1 mRNA in human synovial fibroblasts incubated with IL-1β in the absence and presence of GSTM, TM and GSTG.

Confluent human fibroblasts between passages 3-9 were cultured for 24 hrs in serum free (0.2% BSA) medium. No addition (control).

Figure 3.6.

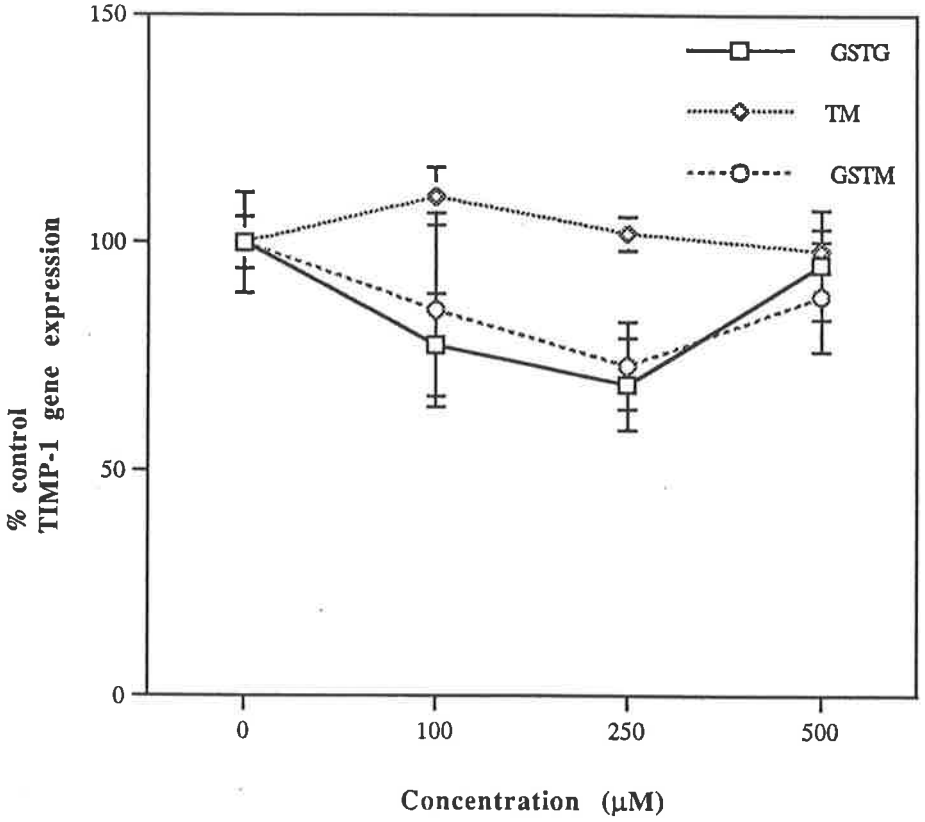
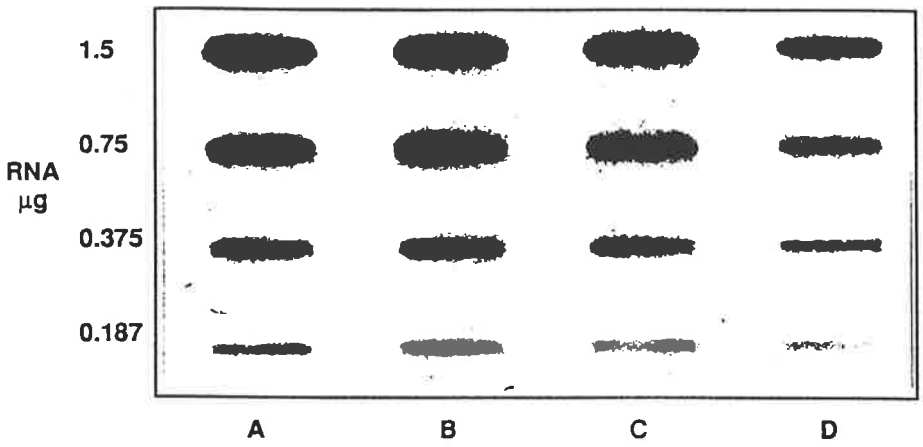

150
100
50
0
% control
TIMP-1 gene expression
GSTG
TM
GSTM
0
100
250
500
Concentration (μM)

Figure 3.7.

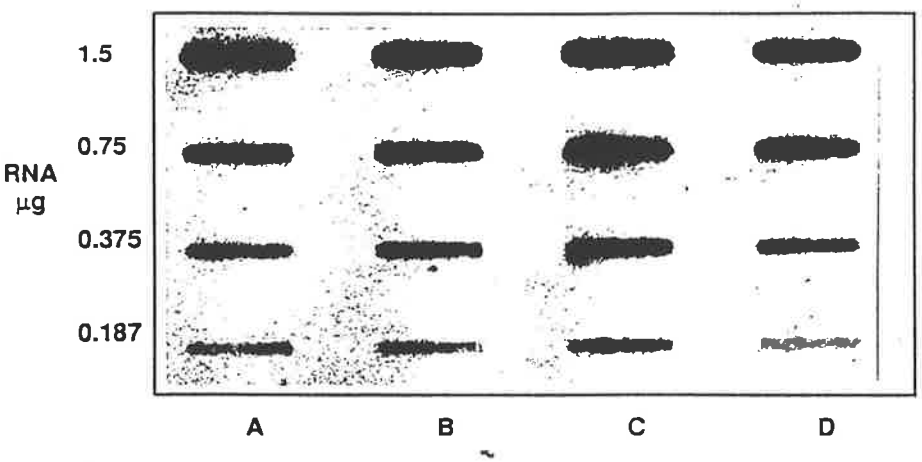
Comparison of cellular mRNA obtained from cultured human synoviocytes in treatment conditions using slot blot protocols.

Cell treatments are as follows, Panel A. standard extraction dissolved in 20 x SSC, B. IL-1β (10 pM), C. IL-1β + GSTM (100 μM) , D. IL-1β + GSTM (500 μM). The RNA samples were applied to a nylon membrane and probed with ^{32}P-labelled complementary DNA (cDNA) for a. MMP-1, b. TIMP-1, c. GAPDH and exposed for 2 days.

a.


1.5
0.75
RNA
µg
0.375
0.187
A
B
C
D

b.


1.5
0.75
RNA
µg
0.375
0.187
A
B
C
D

c.

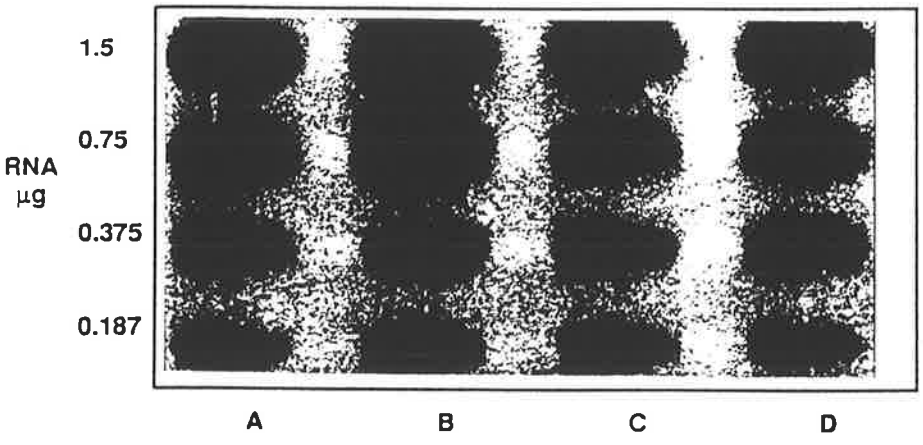

1.5
0.75
RNA
µg
0.375
0.187
A
B
C
D

Figure 3.8.

Effect of dexamethasone on the mRNA expression of human collagenase and TIMP-1.

A. Confluent cultures of IL-1β stimulated human synovial fibroblasts were incubated with the indicated concentrations of dexamethasone for 24 hours. After incubation, mRNA expression was determined using slot blot analysis. *, $p < 0.05$; **, $p < 0.005$.

Figure 3.8.

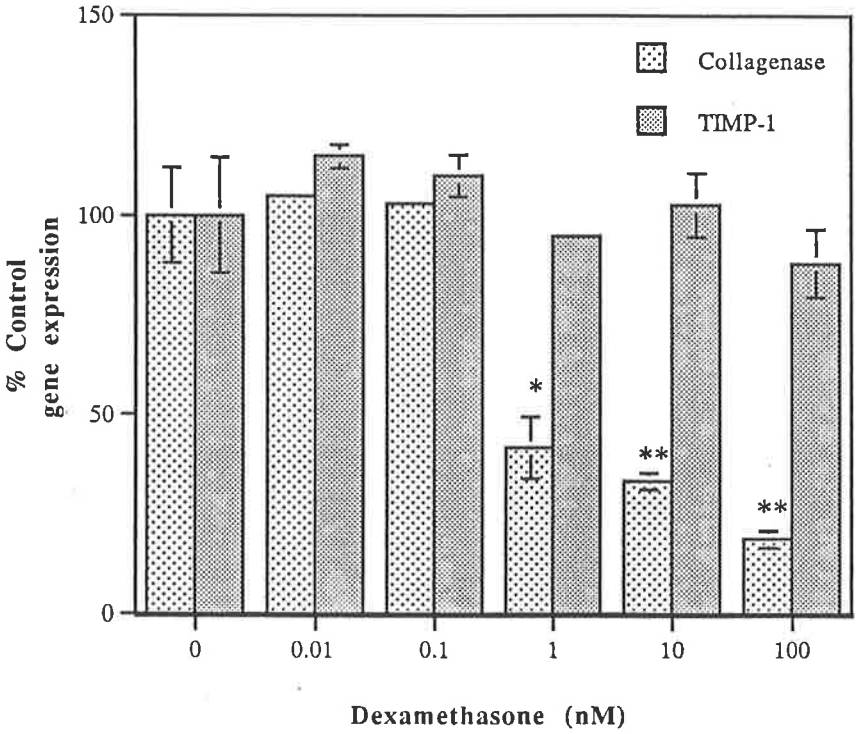
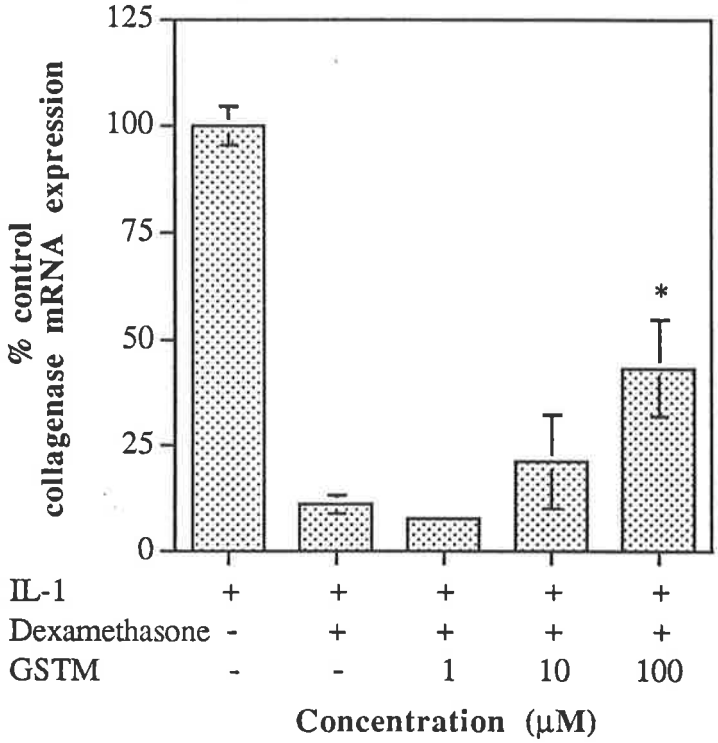

150
100
50
0
% Control
gene expression
Collagenase
TIMP-1
*
**
**
0
0.01
0.1
1
10
100
Dexamethasone (nM)

Figure 3.9.

Reversal of glucocorticoid-induced depression of collagenase mRNA production by synovial cells.

Human synovial fibroblasts were cultured for 24 hours with dexamethasone (10 nM) in the absence or presence of GSTM at the indicated concentrations. Cells were then stimulated with IL-1β (10pM) for another 24 hours. mRNA was extracted and each sample was blotted as described in the methods section. Blots were probed sequentially with ^{32}P-labelled cDNA for collagenase and GAPDH. (*) p value is equal to 0.05 (P = 0.05).

Figure 3.9.


% control collagenase mRNA expression
125
100
75
50
25
0
*
IL-1 + + + + +
Dexamethasone - + + + +
GSTM - - 1 10 100
Concentration (μM)

3.5. Discussion

More than 40 years ago the benefit of gold compounds in the treatment of rheumatoid arthritis was first reported, however the mechanism of their action is still not clearly understood. In vitro studies have shown that gold compounds have suppressive action on the production or activity of monocyte-derived proinflammatory cytokines such as IL-1 (Awad et al 1995). Gold compounds also antagonize some biological effects of IL-1 (Williams et al, 1992). The mechanism of action of gold in RA is an important goal for research on gold therapy, however more understanding of the biochemistry of these drugs will facilitate research on the mechanism of action and therapeutic regimens. A body of evidence indicates that the anti-rheumatic activity of gold compounds is attributed to effects on various inflammatory mediators upstream of collagenase activation. For example, GSTM significantly inhibited intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on vascular endothelial cells (Koike et al, 1994). Prostaglandin E2 (PGE2) synthesis is also inhibited by GSTM used in therapeutic serum concentrations. Because the antierosive effects of gold compounds appear to be particularly important to study, therefore the main purpose of this study was to investigate the effects of GSTM, GSTG and TM on the expression of collagenase and TIMP-1 by RA synoviocytes.

Gold is selectively concentrated within inflamed synovial tissues during gold therapy (Vernon-Roberts et al, 1976). We have first confirmed that RA synoviocytes produce significant amounts of MMP-1 and TIMP-1 upon stimulation with the pro-inflammatory cytokine IL-1β, in our cultured synoviocyte system. Then we studied the modulatory effects of gold containing anti-rheumatic drugs as well as their ligand component (TM) on the induced expression of these genes in cultured RA synoviocytes.

Our study demonstrated that GSTM but not its TM component, down-regulated collagenase gene expression in human rheumatoid synovial fibroblasts exposed to IL-1β. This suggests that after cleavage of gold-thiomalate (gold-ligand) link in the blood stream, the thiomalate (TM) component is unlikely to play a part in effects of this drug.

Our results are in contrast to the work of Newman et al, who have reported that the inhibitory effects of GSTM on production of mRNA for VCAM-1 and E-selectin (Newman et al, 1994). We clearly used a different cellular model. However both results taken together would imply that both components may have independent effects on mRNA expression of various proteins. The ineffectiveness of TM in our system suggests that the relative inhibition of MMP-1 gene expression is possibly mediated by gold rather than its thiomalate component.

TIMP-1 expression was not significantly affected by GSTG, GSTM or its TM salt. This may simply result from different promoter regions on TIMP-1 gene or lack of some responsive elements which might regulate inhibition of MMP-1 gene expression by gold containing drugs. IL-1β stimulates MMP synthesis by activating PKC in human synoviocytes (DiBattista et al, 1994). Immunoregulatory effects of GSTM is attributed to the inhibition of T cell PKC activity (Hashimoto et al, 1992). These suggest that, one possible mechanism through which inhibition of MMP-1 gene by gold drugs occurs, may be related to the inhibition of PKC.

In another report it was claimed that, GSTM did not affect PMA-mediated induction of collagenase mRNA expression in monolayer cultures of normal adult skin fibroblasts (Makino et al, 1994). However different cell types and different stimulants as well as different experimental conditions might simply be the reason for the ineffectiveness of gold in their system.

We have also demonstrated that GSTM has an antiglucocorticoid effect in IL-1 induced collagenase gene expression by human synoviocytes. This is in agreement with the work by Makino, indicating the paradoxical reversal of the repression of collagenase

gene expression by GSTM, using phorbol-ester-treated human skin fibroblasts.(Makino et al, 1994). Glucocorticoids down-regulate the cytokine induced collagenase expression by inhibiting AP-1 activity, which is mainly related to the interaction between the AP-1 and glucocorticoid receptor (GR). The antiglucocorticoid action of GSTM was suggested to be due to the interaction between GSTM, or Au(I), and glucocorticoid receptor (GR). This interaction is likely to be the reason for liberation of collagenase gene expression from glucocorticoid dependent repression. Further studies are clearly needed to investigate the involvement of AP-1 as well as other DNA binding elements in MMP-1 and TIMP-1 genes upon treatment of the cells with gold drugs.

In conclusion, a decrease in the synthesis of collagenase mRNA in the inflamed synovium may be one of the mechanisms by which GSTM exerts its anti-inflammatory actions in diseases such as rheumatoid arthritis. This effect is likely to be dependent on the gold component, rather than the TM component. Simultaneous treatment of the cells with GSTM and dexamethasone, may also result to partially block the suppressive effects of glucocorticoids.

We next planned to determine the regulatory effects of other classes of anti-rheumatic agents on collagenase and TIMP-1 gene expression.

Chapter 4

Regulation of MMP-1, MMP-3, and TIMP-1 gene expression by anti-rheumatic drugs, in cytokine stimulated human synovial fibroblasts

4.1. Abstract

The steady-state levels of extracellular matrix proteins are regulated by the rates of their synthesis and degradation. Activated synoviocytes secrete high concentrations of MMPs in RA and related conditions.

We have examined the effects of anti-rheumatic agents, on MMP-1, MMP-3, and TIMP-1 gene expression by cultured RA and OA synoviocytes exposed to pro-inflammatory cytokines. Both enzyme and inhibitor gene levels were quantified by slot-blot analysis. The NSAID, indomethacin enhanced the level of MMP-1 but not TIMP-1 gene expression, however tenidap effectively reduced the gene level of MMP-1, MMP-3 and TIMP-1 in cultured human synoviocytes from OA patients. Dexamethasone induced a dose-dependent decrease in collagenase and stromelysin mRNA level, whereas TIMP-1 gene expression appeared to be unchanged by glucocorticoids. Cyclosporin-A reduced the expression of MMP-1 dose dependently, whereas methotrexate did not influence the level of MMP-1 and TIMP-1 gene expression.

Taken together, our data suggest that, some anti-rheumatic agents may induce their effects by blocking the synthesis of MMPs but others do not. This effect leads to the inhibition of the tissue degradation which may have therapeutic value in several forms of arthritis.

4.2. Introduction

Synovial membranes play an important role in the pathophysiology of the arthritic conditions. In a number of studies using immunohistochemistry and in situ hybridization, in inflamed synovium, synovial lining cells appeared to express and synthesize a large amount of MMPs (McCachren, 1991; Gravallese et al, 1991).

IL-1 has been demonstrated to be a potent stimulator of MMP synthesis by various cell types. It acts through binding to a plasma-membrane receptor, (IL-1R) (Pelletier et al, 1995). The use of IL-1 treated synovial fibroblast culture is a useful model system for understanding the regulation of MMPs in RA and OA conditions.

NSAIDs are effective in controlling the symptoms of patients with RA and other forms of inflammatory arthritis. Two important mechanisms of their actions are to inhibit tissue cyclooxygenases, which are responsible for the conversion of arachidonic acid to prostaglandins, and interfering with pro-inflammatory cytokine production. Treatment with the NSAID, indomethacin, increases synovial fibroblast cell growth in vitro in the presence of IL-1β or TNF (Gitter et al, 1989). Other cyclooxygenase inhibitors and glucocorticoids have similar enhancing effects on IL-1β induced fibroblast proliferation. Tenidap sodium has combined inhibitory activity against the cyclooxygenase and 5-lipoxygenase (Blackburn et al, 1991a). Tenidap is also reported to inhibit the production of IL-1, IL-6 and TNF and the action of IL-1(Sipe et al, 1992). Inhibition of the release of activated neutrophil collagenase (Blackburn et al, 1991b) is another action of tenidap. It thus has a number of actions in addition to cyclooxygenase inhibition which are not shown by other available NSAIDs. The average therapeutic concentration of tenidap in serum of patients with OA and RA is around 10-20 μg/ml. At therapeutic concentrations tenidap is shown to decrease the levels of IL-1R in chondrocytes and synovial fibroblasts (Pelletier et al, 1993; 1996).

Experimental studies have suggested that methotrexate can effectively inhibit IL-1 production (Segal et al, 1990). It also inhibits certain activities of IL-1 without interfering

with the production or secretion of IL-1 (Segal et al, 1990; Songsiridej and Furst 1990). Inhibition of synovial fibroblast proliferation and the chemotactic activity of neutrophils, might be indirect consequences of suppression of IL-1 activity, by methotrexate (Meyer et al, 1993). Methotrexate has also been reported to increase IL-2 synthesis and potently inhibit IL-6 activity (Bannwarth et al, 1994). In articular tissues, methotrexate decreases the activity of proteolytic enzymes, particularly serine proteases and metalloproteinases (Segal et al, 1990). The anti-inflammatory effect of methotrexate is also attributed to the inhibition of the polymorphonuclear (PMN) cell chemotaxis, monocyte chemotaxis, and superoxide production (Nesher et al, 1991; Segal et al, 1990). Methotrexate also inhibits macrophage activation and the influx of activated macrophages into the synovial tissue (Bannwarth et al, 1994). Furthermore synovial fibroblast proliferation was inhibited by methotrexate treatment in cultured human synovial fibroblasts at concentrations achieved in vivo (Meyer et al, 1993).

Glucocorticoids regulate the expression of a variety of genes, through the repression of AP-1 activity by the GCR (Beato M, 1989). The synthesis of MMPs is also transcriptionally repressed by glucocorticoids. A number of studies have shown the down-regulation of MMPs production by glucocorticoids (for a review see Vincenti et al, 1994).

CyA has beneficial symptomatic effects since it improves pain and reduces functional impairment in RA patients (Wells and Tugwell, 1993). CyA also improves both clinical and biological inflammation in RA patients. Evidence from several laboratories also suggests that CyA directly affects gingival fibroblast functions in vitro. However in these experiments only some individuals were susceptible to CyA gingival enlargement because, the level of production and activity of MMP-1 and TIMP-1 was variable among individuals (Tipton et al, 1991). Furthermore CyA appears to suppress the immune system by the inhibition of T-cell activation. CyA may act mainly by inhibiting the secretion of cytokines from T cells and from other cells (Russell et al, 1993). There is little work on the interaction of CyA with the expression of the genes for MMPs and TIMP.

In the previous study we showed that, collagenase gene expression in human synovial fibroblasts was up-regulated by pro inflammatory cytokines and gold containing anti-rheumatic compounds showed the ability to repress this cytokine stimulation of MMP genes.

Here we investigated the modulation of IL-1 induced MMP-1, MMP-3 and TIMP-1 gene expression, using indomethacin, dexamethasone, CyA, methotrexate and tenidap, in human synovial fibroblasts from patients with OA and RA. Since MMPs are important targets for the pharmacological treatment of these diseases, this study was undertaken in order to obtain more information on possible mechanisms within which these therapeutic agents act. This study also answered the question, whether MMP-1 and TIMP-1 mRNAs are differentially modulated by the action of these different therapeutic agents.

4.3. Materials and methods

4.3.1. Reagents

All chemicals used in this study were provided from the same sources as mentioned in Chapter 3, unless otherwise specified. Indomethacin, cyclosporin-A, and dexamethasone were from Sigma Chemical Company, St. Louis, Mo 63178 USA. Methotrexate was from David Bull Laboratories (DBL) Melbourne, Australia. Tenidap (Pfizer Inc, Groton CT 06340) was kindly provided by Dr. Michael James, Rheumatology Unit, Royal Adelaide Hospital.

4.3.2. Methods

4.3.2.1. Synovial fibroblast cultures

Synovial tissue was obtained from the patients with OA and RA and processed as described in Chapter 3. Briefly, Cells were released from the small pieces of tissue by

enzymatic digestion using collagenase (2 mg/ml), in DMEM supplemented with 15% heat inactivated FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, L-glutamine 2 mM at 37°C for 8 hours. The isolated cells were then incubated in tissue culture flasks, in DMEM supplemented with 15% heat inactivated FCS and antibiotics at 37°C in an humidified atmosphere of 5% CO_2, to allow adherence of fibroblastic and non-fibroblastic cells, present in the synovial preparations. At confluence, cells were passaged by trypsinization (0.05% trypsin in PBS) and seeded in 6-well culture plates at about 5 x 10^4 cells/well and allowed to grow for 3 days in the same medium before the treatments. 24 hours before each experiment, the medium was removed and the cells were incubated in serum-free medium. First passage cells containing macrophage-like fibroblasts, were only used for experiments using tenidap.

Effects of each drug on MMP-1, MMP-3 and TIMP-1 gene expression were evaluated by pre-incubating the cells in the presence of each drug indicated for 24 hours at 37°C, followed by 24 hours stimulation with IL-1β.

4.3.2.2. Collagenase, stromelysin and TIMP-1 mRNA expression

At confluence, first passage OA synovial fibroblasts in 6-well plates were incubated in serum free media containing 0.2% BSA in the absence or presence of tenidap (1-100 µg/ml) for 24 hours, followed by an additional 24 hour incubation with IL-1β (10 pM). Experiments with dexamethasone, indomethacin, CyA, and methotrexate, were performed, under the same conditions, on RA synovial fibroblasts at passages between 3 and 9. Morphology and viability of the cultured cells were always examined and confirmed using an inverted microscope.

At the end of the incubation time, cells were washed with sterile PBS and RNA was extracted as described in methods section in Chapter 3. Radiolabeling of the cDNA probes, slot-blot analysis, and hybridization of the membranes were also performed as described in methods section in Chapter 3.

4.3.3. Data analysis (Statistics)

The results are expressed as the RI of MMP-1 or TIMP-1 mRNA normalised to the level of GAPDH mRNA. Percent control of the RI was calculated as the ratio of the RI of collagenase and TIMP-1 band, to the GAPDH band. Values were expressed as mean ± the standard error of the mean (SEM). Where SEM is not shown, the size of the error bar is the same size or smaller than symbols. Student's t-test was used to test for significant differences between means.

4.4. Results

4.4.1. Effects of indomethacin on IL-1 stimulated MMP-1 and TIMP-1 gene expression in human fibroblast-like synoviocytes

We examined whether indomethacin effects the expression of MMP-1 and TIMP-1. IL-1β stimulation of human RA synoviocytes enhanced the level of MMP-1 and TIMP-1 mRNA expression. Cells were pretreated for 24 hours with increasing concentrations of indomethacin, prior to another 24 hour stimulation by IL-1β (10 pM). At the end of the incubation time, cells were analysed for the expression of collagenase and TIMP-1 gene. As shown in Figure 4.1, indomethacin plus IL-1β induced a significant increase in the level of MMP-1 mRNA expression, whereas the level of TIMP-1 mRNA expression remained unchanged. Results are expressed as % control mRNA expression. 100% control indicates the level of mRNA expression when cells are incubated with IL-1β (10 pM).

Figure 4.2 also shows the photographs of a representative slot blot analysis.

4.4.2. Effects of cyclosporin-A (CyA)

We next analysed the effect of CyA on collagenase and TIMP-1 gene expression from RA synoviocytes. Confluent cultures at passages between 3-9, were pre-treated with increasing concentrations of CyA (1-100 μM) for 24 hours, followed by 24 hours stimulation with IL-1β (10 pM). The basal levels of collagenase and TIMP-1 gene expression were low whereas they were enhanced by IL-1β. CyA reduced the level of collagenase gene expression, while the level of the TIMP-1 mRNA remained unchanged (Figure 4.3). The CyA concentration required to reduce the amount of MMP-1 mRNA by 50% (50% inhibition constant; IC_{50}) was 14 μM. 100% control is the level of mRNA expression of cells stimulated with IL-1β (10 pM).

Photographs of the representative slot-blot analysis are also showed in Figure 4.4.

Figure 4.1.

Effect of indomethacin on IL-1β stimulated collagenase and TIMP-1 gene expression.

RA synovial fibroblasts were pretreated with increasing concentrations of indomethacin for 24 hours followed by further treatment with 10 pM of IL-1β for 24 hours. Slot-blot analysis of the mRNA, extracted from the treated cells, showed that indomethacin plus IL-1β significantly enhanced the level of MMP-1 mRNA expression. Whereas the level of TIMP-1 gene expression was not changed. Mean values ± SEM for cells from 3 RA patients. Significant differences are indicated (*, p value < 0.05).

Figure 4.1

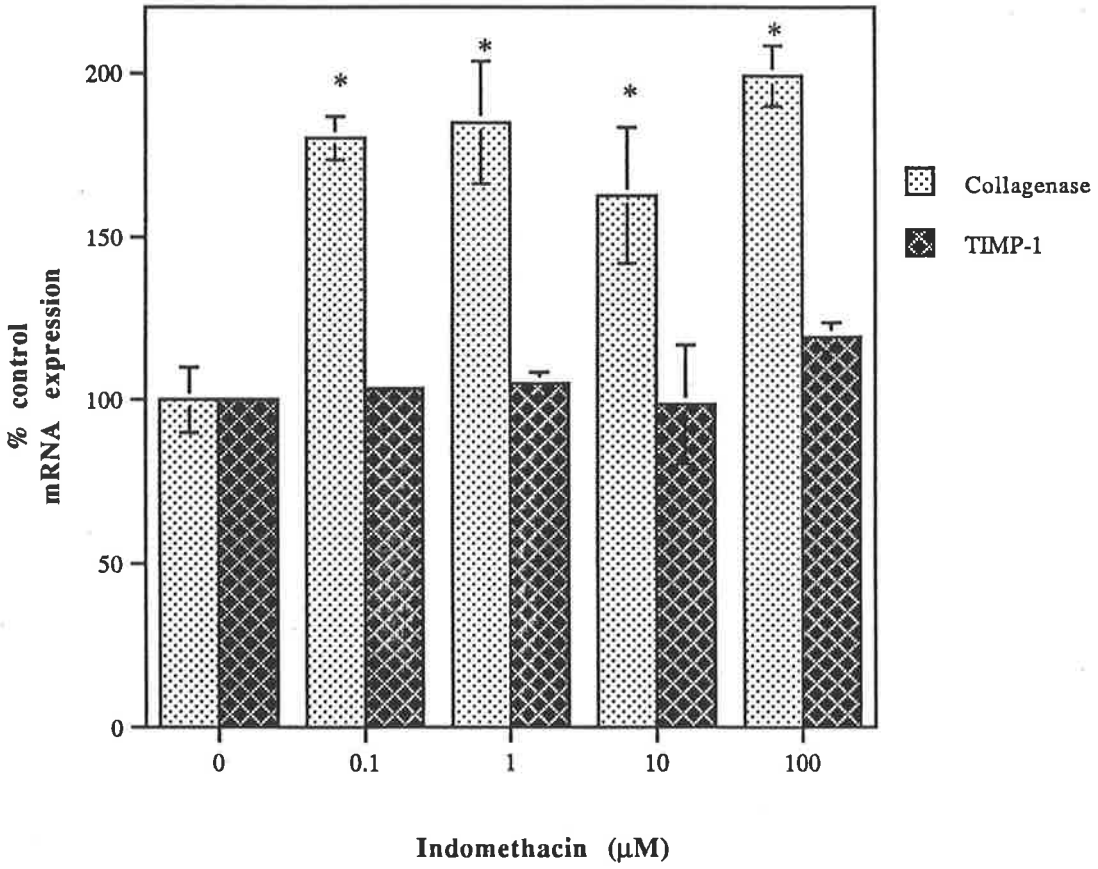

% control
mRNA expression
200
150
100
50
0
*
*
*
*
0
0.1
1
10
100
Indomethacin (μM)
Collagenase
TIMP-1

Figure 4.2.

Comparison of cellular mRNA obtained from RA synovial fibroblasts in treatment conditions using slot-blot protocols.

Cells were pre-incubated for 24 hours with increasing concentrations of indomethacin followed by 24 hours incubation with 10 pM IL-1β. Concentrations of indomethacin used in each lane were as follows; lane 1, 0 μM; lane 2, 1 μM; lane 3, 10 μM; lane 4, 100 μM. mRNA was then extracted and applied into a nylon membrane. The membrane was hybridized with the ^{32}P-labelled cDNA for A. MMP-1, then sequentially stripped and rehybridized to B. TIMP-1, C. GAPDH and exposed for 1 day to a phosphorimager screen. This experiment is representative of 3 others that were performed, all with similar results.

A.

B.

C.

Figure 4.3.

Effect of CyA on IL-1β mediated collagenase and TIMP-1 gene expression.

RA synovial fibroblasts were pretreated with increasing concentrations of CyA for 24 hours followed by further treatment with 10 pM of IL-1β for 24 hours. mRNA was then extracted and fixed onto a nylon membrane by slot-blot analysis. The filter was then hybridized to MMP-1 probe, stripped and rehybridized to TIMP-1 and a GAPDH probe to determine the total amount of RNA loaded into each slot. Relative intensity of collagenase or TIMP-1 mRNA expression was normalised to the GAPDH mRNA in each slot and expressed as % control of the mRNA with respect to control (no drug). Mean values ± SEM for cells from 3 RA patients. Significant differences are indicated (*, $p < 0.05$; **, $p < 0.005$).

Figure 4.3

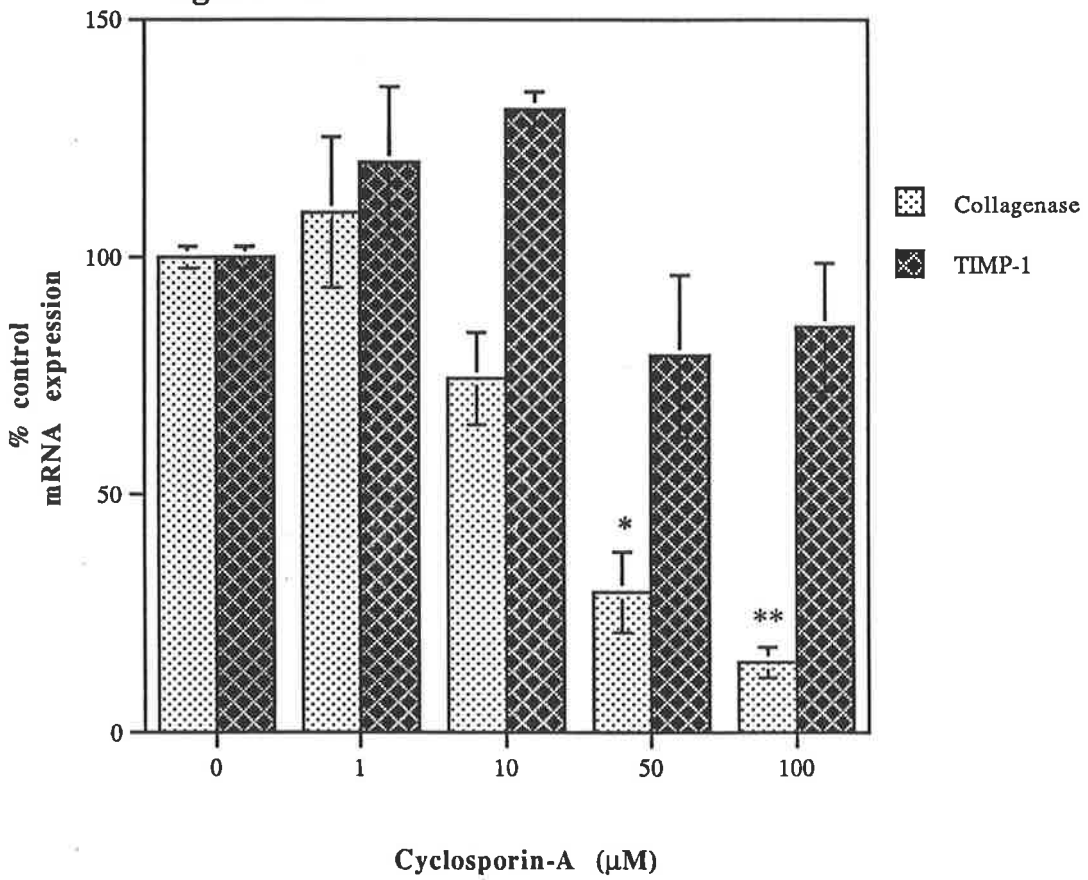

150
100
50
0
% control
mRNA expression
*
**
0
1
10
50
100
Cyclosporin-A (μM)
Collagenase
TIMP-1

Figure 4.4.

Comparison of cellular mRNA obtained from RA synovial fibroblasts in treatment conditions using the slot-blot protocols.

Cells were pre incubated for 24 hours with increasing concentrations of CyA (1-100 μM) followed by 24 hours incubation with 10 pM IL-1β. Concentrations of CyA, used in each lane, were as follows; lane 1, 0 μM; lane 2, 1 μM; lane 3, 10 μM; lane 4, 100 μM. The mRNA samples were applied to a nylon membrane. The membrane was hybridized with the ^{32}P-labelled cDNA for A.MMP-1, then sequentially stripped and rehybridized to B.TIMP-1, C.GAPDH as described in Materials and Methods (Chapter 3). This experiment is representative of 3 others that was performed, with similar results.

A.

B.

C.

4.4.3. Effects of methotrexate

RA fibroblast-like synoviocytes (passage 3-9) were pretreated with methotrexate (0.1-500 μM) for 24 hours, followed by 24 hour stimulation with IL-1β (10 pM). Collagenase and TIMP-1 mRNA levels were assessed by slot blot analysis (n = 3). Methotrexate did not affect the induction of collagenase or TIMP-1 gene expression by IL-1β (Figure 4.5). Methotrexate was tested at concentrations corresponding to serum levels achieved therapeutically (0.01 and 1 μg/ml) (Kremer et al, 1986), as well as concentrations well in excess (10-500 μM). 100% control is the level of mRNA expression achieved when the cells are stimulated with IL-1β (10 pM).

4.4.4. Effects of glucocorticoids

RA fibroblast-like synoviocytes between 3-9 passages were preincubated for 24 hours with dexamethasone 100 nM, followed by 24 hours stimulation with IL-1β. Pretreatment of the cells with dexamethasone significantly inhibited the expression of collagenase and stromelysin mRNA. We next characterized the concentration range over which dexamethasone reduced MMP-1 and MMP-3 mRNA expression in IL-1β stimulated cells. mRNA from the dexamethasone and IL-1β treated cells was extracted and analyzed by slot-blot for the levels of MMP-1, MMP-3, and TIMP-1 gene expression. Stimulation of synovial fibroblasts for 24 hours with IL-1β resulted in the accumulation of these genes. However pre-treatment of synovial fibroblasts with increasing concentrations of dexamethasone (1-100 nM) for 24 hours, resulted in a dose-dependent suppression of MMP-1 and MMP-3 gene expression. In contrast, TIMP-1 gene level was not significantly affected by dexamethasone treatment (Figure 4.6). 100% control is the level of mRNA expression when the cells are stimulated with IL-1β (10pM).

Figure 4.7 shows the representative slot-blots for the expression of MMP-1, MMP-3, TIMP-1, and GAPDH genes. The results of the Figure 4.6 are summarized in a table, comparing the relative expression as well as the % inhibition of MMP-1, MMP-3, and

TIMP-1 mRNA levels (Table 4.2).

Figure 4.5.

Effect of methotrexate on IL-1β stimulated collagenase and TIMP-1 gene expression.

RA synovial fibroblasts were pretreated with increasing concentrations of methotrexate for 24 hours followed by stimulation with 10 pM of IL-1β for 24 hours. The mRNA was extracted from the treated cells and applied into a nylon membrane using slot blot analysis. The filters were then hybridized to collagenase then stripped and rehybridized to TIMP-1 and GAPDH probes. Histograms show the percent relative mRNA levels detected, with respect to control (no methotrexate). Mean values ± SEM for cells from 3 RA patients. Significant differences are indicated (*, $p < 0.05$; **, $p < 0.005$).

Figure 4.5

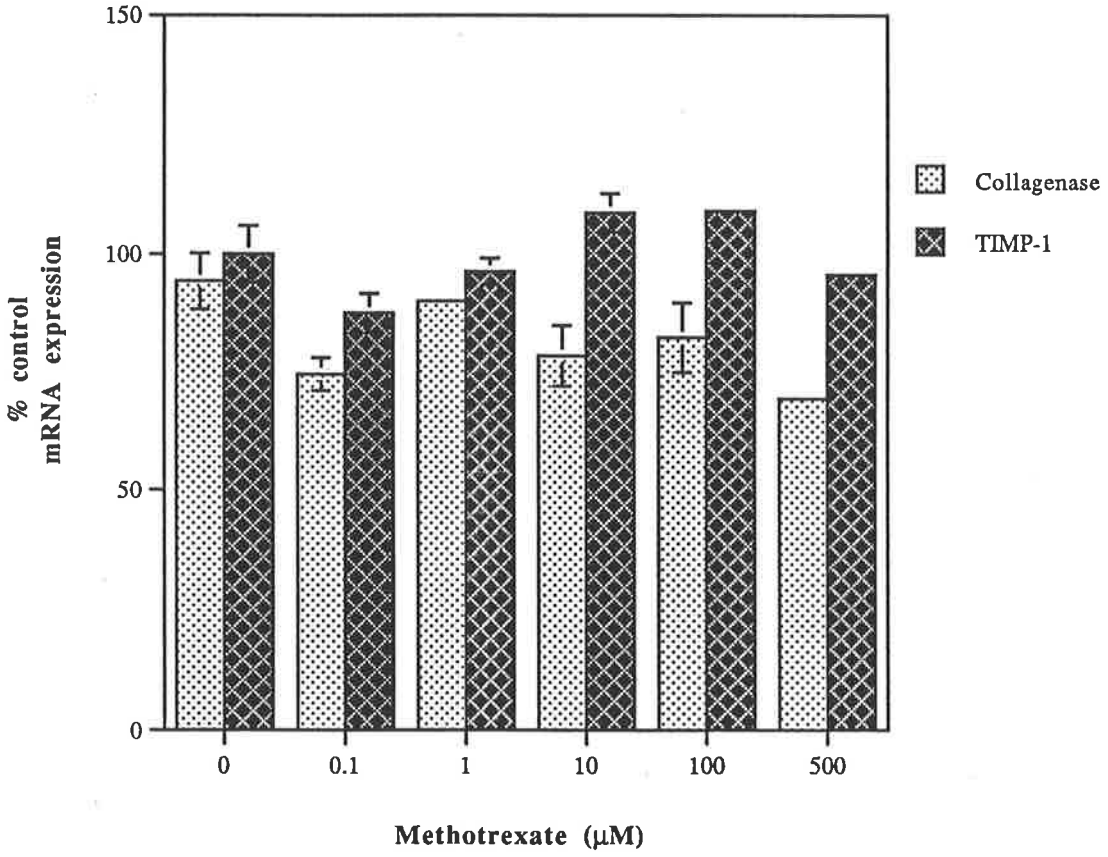

150
100
50
0
% control
mRNA expression
0
0.1
1
10
100
500
Methotrexate (μM)
Collagenase
TIMP-1

Figure 4.6.

Effect of dexamethasone on IL-1β stimulated collagenase and TIMP-1 gene expression.

RA synovial fibroblasts were pretreated with increasing concentrations of dexamethasone (1-100 nM) for 24 hours followed by stimulation with 10 pM of IL-1β for 24 hours. The mRNA was extracted from the treated cells and applied into a nylon membrane using slot blot analysis. The filters were then hybridized to collagenase, stripped and rehybridized sequentially to TIMP-1 and GAPDH probes. The graph shows the percent relative mRNA levels detected, with respect to control (no dexamethasone). Mean values ± SEM for cells from 3 RA patients. Significant differences are indicated (*, $p < 0.05$; **, $p < 0.005$).

Figure 4.6

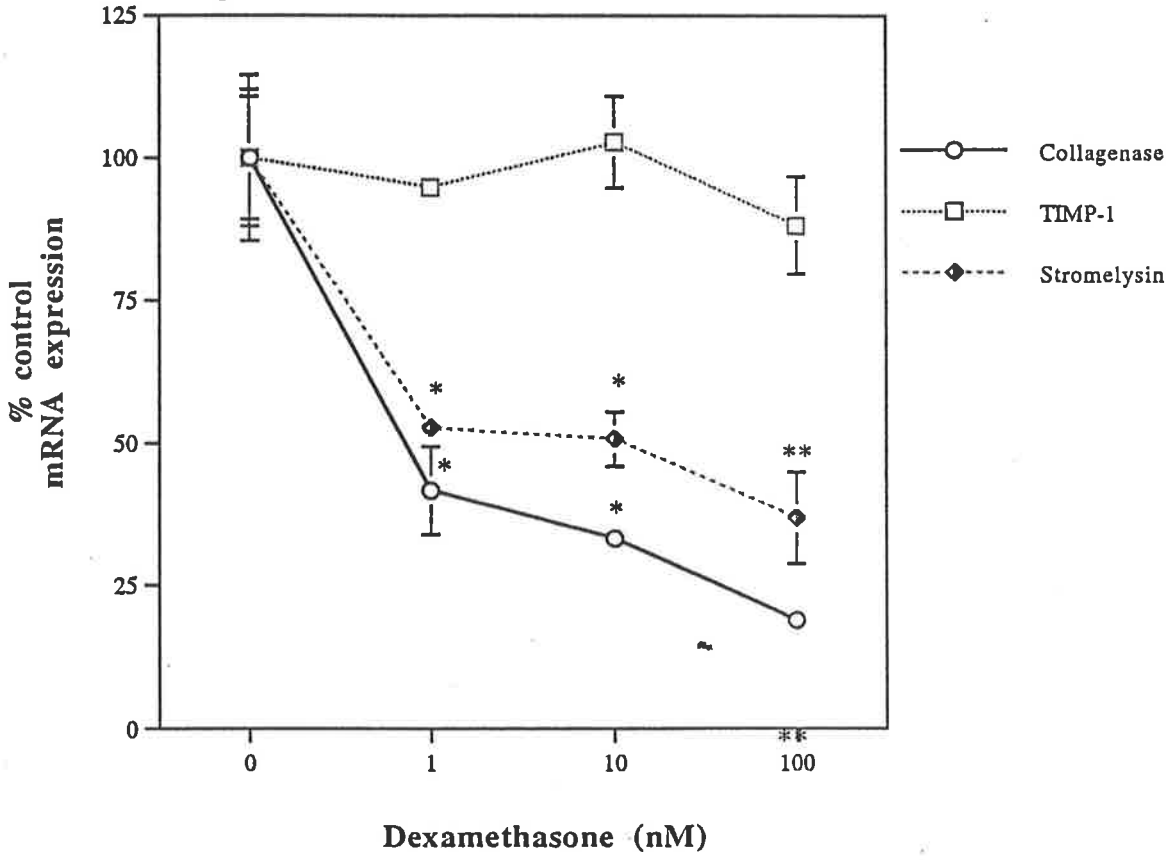

125
100
75
50
25
0
% control
mRNA expression
0
1
10
100
Dexamethasone (nM)
Collagenase
TIMP-1
Stromelysin

Figure 4.7.

Comparison of cellular mRNA obtained from RA synovial fibroblasts in treatment conditions using standard procedure of the slot-blot analysis.

Cells were pre incubated for 24 hours with increasing concentrations of dexamethasone, followed by 24 hours stimulation with 10 pM IL-1β . mRNA was extracted and applied into a nylon membrane. Concentrations of dexamethasone used in each lane were as follows; lane 1, 0 μM; lane 2, 1 μM; lane 3, 10 μM; lane 4, 100 nM. The membrane was hybridized with the ^{32}P-labelled cDNA for A. MMP-1, then sequentially stripped and rehybridized to B. MMP-3, C. TIMP-1, D. GAPDH and exposed to a phosphorimager screen for 24 hours. This slot-blot is representative of 3 others that were performed, all with similar results.

A.

B.

C.

D.

Table 4.2. Relative expression of MMP-1, MMP-3, and TIMP-1 mRNA, normalised to GAPDH, in RA synoviocytes treated with dexamethasone and recombinant human IL-1β.

Dexamethasone treatment concentration (nM)	% mRNA normalised to GAPDH	% dexamethasone inhibition
MMP-1		
Dexamethasone 0	100	0
Dexamethasone 1	41.68	58.32
Dexamethasone 10	33.25	66.75
Dexamethasone 100	18.83	81.17
MMP-3		
Dexamethasone 0	100	0
Dexamethasone 1	52.67	47.34
Dexamethasone 10	50.65	49.5
Dexamethasone 100	36.76	63.24
TIMP-1		
Dexamethasone 0	100	0
Dexamethasone 1	94.87	5.13
Dexamethasone 10	102.68	-2.68
Dexamethasone 100	87.99	12.01

Synovial fibroblasts were preincubated for 24 hours with increasing concentrations of dexamethasone (1-100 nM), followed by 24-hour incubation with rhIL-1β. Membranes with mRNA were hybridised with MMP-1, MMP-3, TIMP-1, and GAPDH probes. Density of the hybridized bands were quantified with an image quantity (IQ) program. Values are expressed as the relative expression of mRNA normalised to GAPDH (mean from 3 independent RA patients).

4.4.5. Effects of tenidap on IL-1 stimulated MMP-1, MMP-3 and TIMP-1 mRNA expression

Cells from OA synoviocytes at their first passage, were pretreated with tenidap 10 μg/ml for 24 hours followed by IL-1β stimulation at 37°C for another 24 hours. Tenidap induced a marked reduction in collagenase mRNA expression after incubation time. Therefore we characterized the concentration range over which tenidap reduced MMPs and TIMP-1 mRNA expression in IL-1β treated synoviocytes. The IC_{50} for MMP-1 gene expression was 5 μg/ml. IC_{50} for MMP-3 and TIMP-1 was about 1 μg/ml (Figure 4.8). 100% control is the level of mRNA expression when the cells are stimulated with IL-1β (10pM).

Representative slot-blot analysis of MMP-1, MMP-3, TIMP-1, and GAPDH genes are shown in Figure 4.9.

Next we used RA synoviocytes, under similar experimental conditions, 24 hours pretreatment of the cells with tenidap followed by another 24 hours stimulation with IL-1. In 3 RA patients used in our experiments (Figure 4.10) there was no significant effect of tenidap on MMP-1, and TIMP-1 mRNA expression, at concentrations corresponding to therapeutic levels, (10-20 μg/ml). IC_{50} for MMP-1 and TIMP-1 in these cells was about 100 μg/ml. Note that this is 10 fold higher than the concentration achieved in serum levels of patients treated with this agent.

Using OA synoviocytes, while IL-1β induced an increase in mRNA levels of MMPs and TIMP-1, the combination of tenidap with IL-1β decreased their message levels as compared with controls (100% stimulation with IL-1β alone), with maximum inhibition of 87.28% for MMP-1, 87.3% for MMP-3, and 90.49% for TIMP-1 (Table 4.3). However, using RA synoviocytes, maximum inhibition was lowered to 43% for MMP-1, and 57% for TIMP-1.

Figure 4.8.

Effect of tenidap on IL-1β mediated collagenase, stromelysin, and TIMP-1 gene expression.

OA synoviocytes first passage, were pretreated with increasing concentrations of tenidap for 24 hours followed by further treatment with IL-1β (10 pM) for 24 hours. The mRNA expression, from cells under different treatments, was detected using the standard protocols for slot-blot analysis. The level of mRNA in each band was normalized to the GAPDH mRNA. Graph shows the percent relative mRNA levels detected with respect to control (no tenidap). Mean values ± SEM for cells from 3 OA patients. Significant differences are indicated (*, $p < 0.05$; **, $p < 0.005$).

Figure 4.8

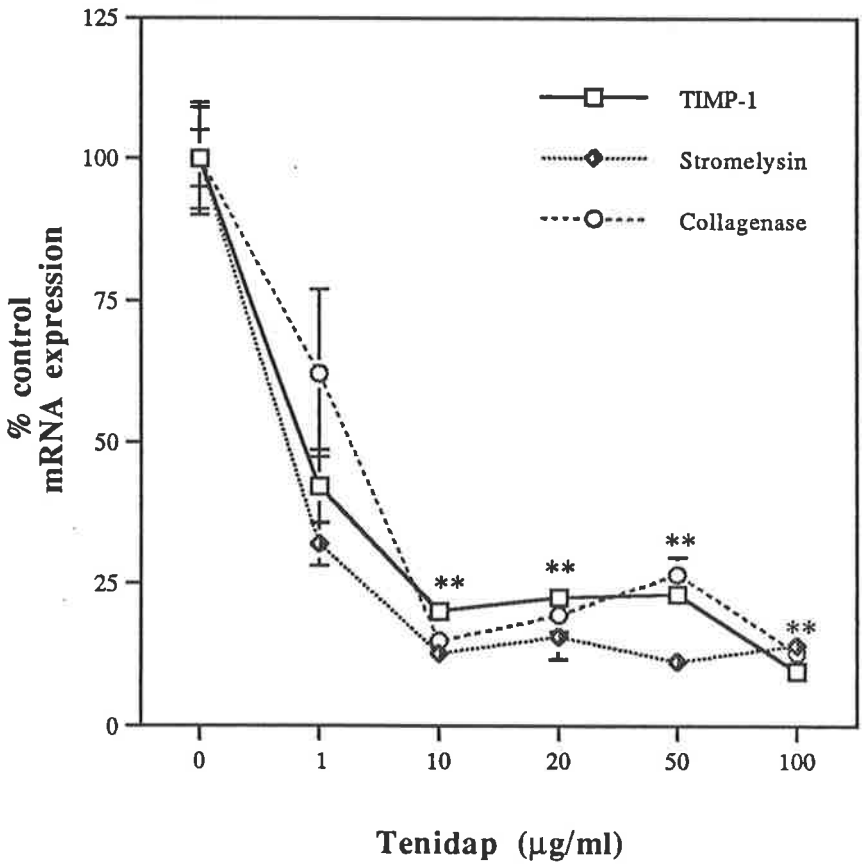

125
100
75
50
25
0
% control
mRNA expression
TIMP-1
Stromelysin
Collagenase
**
**
**
**
0
1
10
20
50
100
Tenidap (μg/ml)

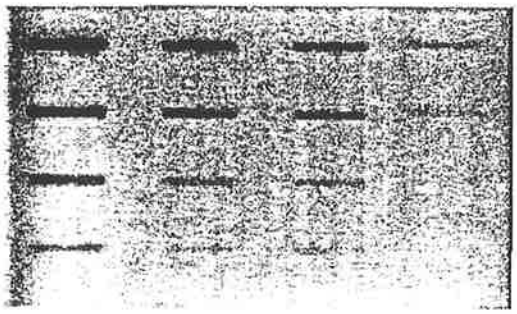
Figure 4.9.

Comparison of cellular mRNA obtained from OA synovial fibroblasts in treatment conditions using standard procedure for slot-blot analysis.

Cells were pre incubated 24 hours with increasing concentrations of tenidap followed by 24 hours incubation with 10 pM IL-1β. mRNA was extracted and applied into a nylon membrane. Concentration of tenidap in each lane was as follows; lane 1, 0 μg/ml; lane 2, 1 μg/ml; lane 3, 10 μg/ml; lane 4, 100 μg/ml. The membrane was hybridized with the ^{32}P-labelled cDNA for A. MMP-1, B. MMP-3, C. TIMP-1, D. GAPDH and exposed for 1 day to a phosphorimager screen. This slot-blot is representative of 3 others that performed, with similar results.

A.

B.



C.

D.

Figure 4.10.

Effect of tenidap on collagenase and TIMP-1 mRNA expression in IL-1β stimulated RA synoviocytes.

RA synoviocytes were pretreated with increasing concentrations of tenidap for 24 hours followed by stimulation with 10 pM of IL-1β for 24 hours. The mRNA was extracted from the treated cells and used in a slot blot analysis. The filters were then hybridized to collagenase and TIMP-1 probes, stripped and rehybridized to a GAPDH probe. The level of mRNA in each band was normalized to the GAPDH mRNA. Histograms show the % control mRNA levels detected, with respect to control (no tenidap). Mean values ± SEM for cells from 3 patients. Significant differences are indicated (*, $p < 0.05$; **, $p < 0.005$).

Figure 4.10

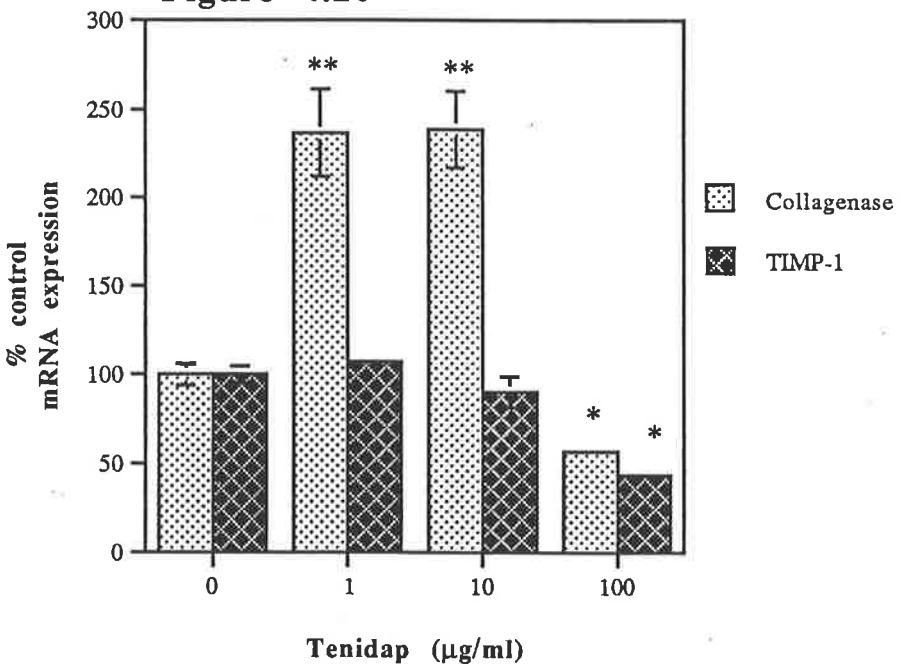

300
250
200
150
100
50
0
% control
mRNA expression
**
**
*
*
0
1
10
100
Tenidap (µg/ml)
Collagenase
TIMP-1

Table 4.3. Relative expression of MMP-1, MMP-3, and TIMP-1 mRNA, normalised to GAPDH, in RA and OA synoviocytes treated with tenidap and IL-1β (10 pM).

Tenidap treatment	% mRNA normalised to GAPDH		% inhibition	
	(OA)	(RA)	(OA)	(RA)
MMP-1				
Tenidap 0	100	100	0	0
Tenidap 1	58.67	237	41.3	-137
Tenidap 10	23	239	77	-139
Tenidap 100	20	57	80	43
MMP-3				
Tenidap 0	100		0	
Tenidap 1	32.05		67.95	
Tenidap 10	12.7		87.3	
Tenidap 100	14.83		85.17	
TIMP-1				
Tenidap 0	100	100	0	0
Tenidap 1	42.21	106	57.79	-6
Tenidap 10	20.04	97	79.96	3
Tenidap 100	9.51	43	90.49	57

Cultured synoviocytes were preincubated for 24 hours with increasing concentrations of tenidap (1-100 μg/ml), followed by a 24-hour incubation with rhIL-1β. Membranes with mRNA were hybridised with MMP-1, MMP-3, TIMP-1 or GAPDH probes. Density of the hybridized bands were quantified with an image quantity (IQ) program. Values are expressed as the relative expression of mRNA normalised to GAPDH (mean from 3 independent experiments).

4.5. Discussion

Several pharmacological agents have been used in an attempt to treat RA. Despite documentation of clinical efficacy, there is still no clear explanation for the mechanism of action of these compounds in RA. Because the ultimate cause of the disease is unclear, suppression of the inflammatory process, remainss the therapeutic aim to prevent the damage to articular cartilage and connective tissue. Our studies were carried out to determine a biochemical basis for the action of these compounds in RA.

Because the imbalance between the MMPs and TIMP are likely to play a critical role in the pathogenesis of this disease, our studies were designed to examine the effects of a range of the therapeutic agents on the expression of MMPs and TIMP-1 on pro-inflammatory cytokine stimulated human synovial fibroblasts.

According to the results obtained in Chapter 3, we have first confirmed that human synoviocytes express substantial amounts of MMP-1, MMP-3, and TIMP-1 upon the stimulation with pro-inflammatory cytokines, IL-1β and TNF-α.

We found that indomethacin, as a representative NSAID, significantly enhanced the level of MMP-1 gene expression in RA fibroblast-like synoviocytes. Whereas TIMP-1 mRNA expression was not changed. This is in contrast to the another previously reported study, which suggested that indomethacin did not change the induction of collagenase mRNA by IL-1 or TNF in rheumatoid human synovial fibroblasts (MacNaul et al, 1990). However DiBattista et al have shown that, prostaglandins inhibit IL-1β induced MMP expression and synthesis in human synovial fibroblasts (DiBattista et al, 1994). Indomethacin is a potent inhibitor of prostaglandin production. These two facts taken together, support the enhancement of collagenase gene expression by indomethacin in our system. On the other hand, prostaglandins do not seem to be the only mediators of collagenase gene expression by IL-1 and TNF. Studies demonstrating that protein synthesis inhibitors can

prevent the IL-1 induction of collagenase (McCachren et al, 1989), have supported the requirement for an intermediate gene product in IL-1 induction of collagenase expression. Since indomethacin increases synovial fibroblast cell growth in the presence of IL-1β (Gitter et al, 1989), this would be another explanation for elevation of the MMP-1 gene expression in our study.

Our results also demonstrate that CyA inhibits IL-1β induced MMP-1, but not TIMP-1, mRNA, from RA synovial fibroblasts in a dose dependent manner. However, the precise mechanism by which CyA inhibits collagenase mRNA expression remains unresolved. The important property of CyA (for a review, see Furst 1995) is its ability to inhibit the production of cytokines involved in the regulation of T cell activation (Russell et al, 1993). CyA has also been found to slow the formation of type I collagen triple helix, resulting in reduction of type I collagen production by cultured fibroblasts (Steinmann et al, 1991). In a study by Lohi et al, CyA enhanced collagenase but not stromelysin-1, gelatinase A, gelatinase B and TIMP-1 mRNA expression in human lung fibroblasts and dermal fibroblasts from systemic sclerosis patients (Lohi et al, 1994). However their assay conditions differ from our work in many respects. In their study, combination of CyA with PMA resulted in a persistent and prolonged induction of c-jun, which was attributed to the persistence of high level of AP-1 activity which could explain the increased collagenase mRNA levels. However the induction of stromelysin-1 and gelatinase is also partially mediated by AP-1 transcription factor, whereas CyA had no enhancing effect on their mRNA levels in that study. This suggests that the effects of CyA are not entirely mediated by AP-1.

The results of our study suggests that CyA treatment results in collagenase mRNA repression. Since there have also been reports of CyA interference or competition with the binding of IL-1 (Bendtzen and Dinarello 1984), suppression of collagenase gene expression in our system may simply be explained by CyA inactivation of IL-1 as a result of the IL-1 receptor blockage or IL-1 receptor gene suppression on the RA synoviocytes. CyA collagenase gene repression might not be necessarily within the same DNA

sequences that participate in the induction of transcription. Further studies are required to determine whether using the specific mutations within the collagenase promoter that abolish transcription activation, may or may not abolish repression by CyA. Another possibility would be, that collagenase mRNA half-life may be affected by CyA, resulting to the decreased level of collagenase mRNA in our system.

The synthetic glucocorticoid, dexamethasone, suppressed the expression of MMP-1 and MMP-3 significantly, but did not have any effect on TIMP-1 gene expression. Similarly glucocorticoids are known as potent inhibitors of the induction of the collagenase family of metalloproteinases by phorbol esters and inflammatory mediators (Brinkerhoff et al, 1986; Frisch and Ruley, 1987; Offringa et al, 1988). According to a number of studies, the transcription activation of collagenase, stromelysin and TIMP-1 genes is mediated by an AP-1 recognition site (TRE) within their promoter. This suggests that TIMP-1, collagenase and stromelysin may be coordinately regulated through the actions of a shared set of cis elements and trans-acting factors (Edwards et al, 1992; DiBattista et al, 1995). The AP-1 site is also the target for repression by glucocorticoids and provides a mechanism that explains how the inhibition of MMP genes occurs (Angel et al, 1987a, 1987b; Brenner et al, 1989). But the fact that TIMP-1 gene expression was not coordinately regulated by dexamethasone in our study, would suggest, the presence of other promoter elements in each of these genes that may explain the differences between regulation of MMPs and TIMP-1 genes by glucocorticoids. The other reason would simply be the lack of a glucocorticoid response element (GRE) at the AP-1 site of the TIMP-1 promoter, which does not allow the glucocorticoid receptor-hormone complex (GRh) to interact with the AP-1 and repress the transcription.

Several effects of tenidap on human synoviocytes and chondrocytes have been reported. They include the suppression of IL-1 stimulated metalloprotease synthesis and expression in OA and normal chondrocytes (Pelletier et al, 1993). This effect was likely to be related to a decrease in the level of IL-1 receptors (IL-1R). Tenidap also was shown to be a potent inhibitor of IL-1R level in OA and RA synovial fibroblasts (Pelletier et al, 1996).

However the exact mechanism responsible for the decreased IL-1R is unknown. In this study we have examined the effects of this drug on OA and RA synoviocytes. Our results determined that, tenidap (10 μg/ml) effectively repressed the expression of MMP-1, MMP-3, and TIMP-1 genes in OA synoviocytes. This is in agreement with the study by Pelletier and his colleagues (Pelletier et al, 1996), who showed the suppressive effect of tenidap on IL-1 induced collagenase synthesis in synoviocytes. They have also suggested that this indirect effect of tenidap on MMP expression occurs via the decreased level of the IL-1 receptor. The reduction of MMP synthesis by tenidap was also shown in vivo in the synovium and cartilage of the OA experimental dog model (Fernandes et al, 1995). However, using tenidap (100 μg/ml) on RA synoviocytes repressed the level of MMP-1 but not TIMP-1 gene expression at higher concentration (10 fold higher than the concentration used for OA cells). Interestingly, in RA synoviocytes, using tenidap (1-10 μg/ml) increased the level of MMP expression. Since PGE_2 down-regulates the expression of IL-1β induced collagenase expression (DiBattista et al, 1994), it may be possible that, this action of tenidap is mediated by suppressing endogenous PGE_2 synthesis, as this drug is a potent inhibitor of cyclooxygenase. However, PGE_2 also down-regulates the IL-1β induced TIMP-1 mRNA expression (DiBattista et al, 1995), thus it is unlikely that this would be the sole explanation.

In this study, methotrexate did not alter collagenase or TIMP-1 mRNA levels in IL-1 stimulated RA synoviocytes. In another study on synovial biopsies by quantitative in situ hybridization using computer-assisted image analysis (Firestein et al, 1994), oral methotrexate therapy decreased collagenase, but not TIMP-1 or stromelysin gene expression in the synovium. However in the same study, methotrexate had no effect on collagenase and TIMP-1 mRNA expression in control or IL-1 treated synovial fibroblasts. These data indicate that methotrexate does not directly decrease collagenase or TIMP-1 gene expression whereas probably it has an indirect effect due to an alteration in the synovial cytokine milieu. Several animal models as well as in vitro observations have indicated the direct interference of methotrexate with the activity of pro-inflammatory cytokines such as IL-1 (Segal et al, 1989; 1990). Furthermore, other biological effects of

methotrexate, including the inhibition of synovial fibroblast proliferation and the chemotactic activity of neutrophils, may be indirect consequences of the suppression of IL-1 activity by methotrexate (Meyer et al, 1993).

Inhibiting synovial inflammation and tissue degradation by blocking the synthesis of MMPs, may have therapeutic value in several forms of arthritis. However the mechanism by which these therapeutic agents might regulate synoviocyte function to express the genes for MMPs and TIMP-1 is still not completely understood.

In addition to the regulatory effects of these widely used anti-rheumatic agents, the synthesis of the metalloproteinases and their inhibitors can also be regulated by polypeptide growth factors and cytokines. Therefore our next study is aimed to determine whether TGF-β is capable of modulating the secretion of MMPs and TIMP-1, as this could have potential therapeutic implications for RA and furthermore suggest new avenues of therapeutic application.

Chapter 5

Transforming growth factor-β (TGF-β) modulates the pro-inflammatory effects of IL-1β and TNF-α in human synovial fibroblasts

5.1. Abstract

In connective tissue, extracellular matrix degradation by IL-1 and TNF-α is mediated by a variety of mediators of inflammation and tissue destruction such as, prostaglandins (PGs) and MMPs (Mauviel et al, 1994). In cultured synoviocytes, IL-1β and TNF-α have been shown to stimulate the synthesis of collagenase and prostaglandin E_2 (PGE_2), whereas TGF-β is known to repress the synthesis of metalloproteinases and increase TIMP-1 production. In this study, TGF-β alone slightly suppressed collagenase mRNA expression and significantly increased TIMP-1 mRNA expression by rheumatoid synovial fibroblasts. Incubation of the cells with the combination of TGF-β plus pro-inflammatory cytokines, suppressed the IL-1-mediated increased level of collagenase mRNA expression although synergistically up-regulated the level of TIMP-1 mRNA expression. Similarly, TGF-β inhibited the stimulatory effects of IL-1β and TNF-α on PGE_2 production.

Our observations suggest that TGF-β is capable of protecting the extracellular matrix of human synovial fibroblasts by modulating the expression of collagenase and TIMP-1 genes as well as the production of inflammatory mediator, PGE_2.

5.2. Introduction

The MMP/TIMP ratio has an important role in modulating connective tissue turnover. This enzyme:inhibitor system has been the subject of recent reviews (Lennars and Strittmatter 1991; Matrisian 1992; Emonard and Grimaud 1990).

The expression of MMP-1 and TIMP-1 is regulated by a number of cytokines and hormones, acting on the cells via surface receptors (Dayer and Burger 1994). IL-1 and TNF-α activate chondrocytes and synoviocytes to produce metalloproteinases such as stromelysin and collagenase (Duff, 1994; Bodden et al, 1994). Specific immunoassays for IL-1 have shown, raised levels in synovial fluid from RA joints (Rooney et al, 1990) and in the serum of patients with active RA (Eastgate et al, 1991). Similarly TNF-α has many biological activities in common with IL-1. TNF-α has profound effects on cells in inflamed area, especially the stimulation of collagenase and PGE_2 by synovial cells (Dayer and Burger 1994). In addition, a connection between the induction of IL-1 mRNA with the increased level of collagenase and PGE_2 stimulating activity was observed in rheumatoid synovial cells (Dayer et al, 1984). Several studies using target cells such as dermal fibroblasts, articular chondrocytes, and almost all mesenchymal cells have shown that IL-1 has similar stimulatory effects on the production of collagenase and prostaglandins (for a review see Dayer and Burger 1994).

In contrast, the fibrogenic growth factors, e.g. transforming growth factor β (TGF-β) act to increase the synthesis of matrix components such as collagen, proteoglycan, and fibronectin and to prevent their breakdown. Thus TGF-β is thought to play a key role in fibrosis and wound healing (Wahl 1992). TGF-β, is a 26 KDa homodimeric growth factor which is present at high concentration within platelets (Assoian et al, 1983) and is secreted at the site of tissue repair by inflammatory cells (Assoian et al, 1987) including, activated lymphocytes, macrophages, neutrophils and synovial fibroblasts (Wahl et al, 1990; Brennan et al, 1990; Arend and Dayer 1990). In a study using human synoviocytes it has been shown that, in RA, TGF-β mRNA and peptide

synthesis is substantial (Wahl et al, 1990). TGF-β contributes to matrix regeneration in the joint by inducing the production of collagenase inhibitors and suppression of proteolytic enzymes (Overall et al, 1989) as well as blocking the induction of interstitial collagenase by other cytokines (Matrisian et al, 1992). It also induces transcription of several cytokines including itself (Whal et al, 1987). Being both a potent stimulator of TIMP-1 and procollagen in vitro, implies that this growth factor has a potent ability to accumulate the matrix proteins in tissues (Whal, 1992)

Prostaglandins (PGs) are synthesized in response to cell injury, and their appearance in tissues is a sign of cell damage. PGs play an important role in the pathogenesis of the inflammation such as inflammatory joint diseases. The PGs are 20 carbon fatty acid derivatives characterized by a five membered ring and they are generated by the oxidation of polyunsaturated fatty acids, in particular arachidonic acid. The arachidonic acid is released from cell membranes. Following this initial release, arachidonic acid is oxygenated by cyclooxygenase (the first enzyme in the prostaglandin cascade) to produce two unstable endoperoxide intermediates, PGG_2 and PGH_2. PGH_2 is further isomerized to form two main products, PGE_2 and $PGF_{2\alpha}$. It appears that *de novo* synthesis of PGs must precede their release and biological action since there is little evidence to suggest that PGs are stored intracellularly (Kunze and Vogt 1971). PGs are generally labile in most tissues, and PGE_2 is rapidly (<30 seconds) hydrolysed to inactive metabolites in the circulation (Samuelsson et al 1978).

Several lines of evidence suggest that the PGs, primarily PGE_1 and PGE_2 may act as mediators of inflammation (Vane 1976). Firstly , PGs cause inflammation when injected subcutaneously, they are vasodilators and they potentiate the formation of edema induced by other agents, for example they also sensitize tissue to pain stimulation. Secondly PGE_1 and PGE_2 have been found in inflammatory exudates (Higgs and Salmon 1979) and thirdly, their biosynthesis is strongly inhibited by anti-inflammatory drugs. Thus, PGs are important inflammatory mediators in arthritis and their synthesis is stimulated in synoviocytes by cytokines (Chin and Lin 1988; Gilman 1987; Meyer et al 1990; Dayer et al 1986; Kumkumian et al 1989). In particular, PGE_2

is an important stimulus for the production of intracellular proteases leading to tissue destruction and bone resorption (Rodemann and Goldberg, 1982). Indomethacin, is a non-steroid anti-inflammatory drug, which inhibits prostaglandin synthesis by inhibiting cyclo-oxygenase (Roth et al 1975; Roth and Majerus 1975; Rome and Lands 1975).

In RA, synovium is infiltrated by mononuclear cells and is hyperplastic. These mononuclear cells secrete inflammatory mediators, including IL-1 and TNF. TGF-β is also present in synovium (Wahl et al, 1985) and is the most well studied inhibitor of the degradative factors in synovial cells. Thus, the net efficiency of TGF-β to rescue the synovium from the catabolic effects of the inflammatory cytokines depends on the relative response of cells to cytokines and growth factors in terms of production of proteolytic enzymes and their inhibitors. It was therefore important to analyse, the levels of the collagenase, TIMP-1 genes and production of the PGE_2, to determine the relative responses of human synovial fibroblast to IL-1β, TNF-α as well as the efficiency of TGF-β to reverse this response. In this report, we have examined the effects of TGF-β on cytokine stimulated rheumatoid synovial fibroblasts. We have examined whether, this growth factor modulates the expression of MMP-1 and TIMP-1 as well as the production of PGE_2.

5.3. Materials and Methods

5.3.1. Reagents

Human recombinant TGF-β1, was purchased from the Sigma Chemical Company, St. Louis, Mo 63178 USA. Rabbit anti-PGE_2 polyclonal antibody was purchased from Sigma (St. Louis, Mo, USA). Radiolabelled [5,6,8,11,12,14,15 (n)-^{3}H]-PGE_2 (5.2-6.3 TBq/mmol) was purchased from Amersham International (Amersham UK). PGE_2 standard was purchased from Caymen Chemicals (Ann Arbor, Michigan, USA). Dextran T70 was from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used in the PGE_2 assay were of analytical grade and purchased from either Sigma or Ajax Chemicals (Sydney, Australia).

5.3.2. Fibroblast cultures

Cultured synovial fibroblasts were prepared according to the method of Tessier et al (Tessier et al, 1993), as explained in Chapter 3. In general, before experimentation, confluent cells were exposed to serum free media, containing 0.2% BSA, for 24 hours prior to each experiment. BSA in culture media prevents protein deprivation, since FCS contains a variety of growth factors and cytokines that may induce collagenase and TIMP-1 synthesis by fibroblasts. Following overnight starvation, cells were stimulated for 24 hours by cytokines. In experiments with TGF-β and cytokines, cells were pre-treated with TGF-β for 24 hours, followed by another 24 hours incubation in the presence of the pro-inflammatory cytokine, IL-1.

5.3.3. Northern blot analysis

Northern blot analysis was performed in order to determine the specificity of each probe to its complementary mRNA. This analysis is explained in the methods section of Chapter 3.

5.3.4. Slot-blot analysis

Slot blot analysis was performed according to the method explained in Chapter 3.

5.3.5. RNA, Protein, and data analysis

The results expressed as the RI of MMP-1 or TIMP-1 mRNA normalised to the level of GAPDH mRNA. Percent control of the RI was calculated as the ratio of the RI of collagenase or TIMP-1 band to the RI of the GAPDH band. Values were expressed as mean ± SEM with "n" refers to the number of cell lines (i.e., patients) analysed. Where SEM is not shown, the size of the error bar is the same size or smaller than symbols. Student's t-test was used to test for significant differences between means.

5.3.6. PGE_2 assay

Standard curves were established for each assay between PGE_2 concentrations of 10 pg-10 ng. Standard PGE_2 was diluted in 1 mM Na_2CO_3, pH 10. RA human synoviocytes were seeded in 24 well plates at 2 x 10^4 cells/well. Cells were cultured in serum containing medium at 37°C for 4 days to become confluent. After the incubation time, supernatant was removed and the cells were gently rinsed with PBS. 300 µl of the serum free medium was added to each well. For control, cells were incubated, untreated in serum free media, for 24 hours. For cytokine treatment, cells were treated for 24 hours with each cytokine IL-1β (10 pM) or TNF-α (10 ng/ml). For indomethacin treatment, indomethacin (15 µM) was added to the cells at least 5 minutes before the cytokine. For TGF-β treatment, cells were pretreated with TGF-β (10 ng/ml) for 24 hours, followed by another 24 hours treatment with IL-1β or TNF-α. After the incubation time, the supernatant was removed and used in a PGE_2 assay. Prostaglandins present in synoviocyte supernatants were assayed using a modification of the radioimmunoassay (RIA) described by Jarre and Behrman (1974). Aliquots (100 µl) of sample were added to the RIA assay buffer which consisted of 0.1% gelatin, 0.9% NaCl, 0.01 M Tris base and 0.05% NaN_3, pH 7.3. To this mixture 100 µl of ^3H-

PGE_2 suspended in 1mM Na_2CO_3 was added (stock solution contained 20µl ^3H-PGE_2 (7.07 TBq/mmol) in 10 ml of 1 mM Na_2CO_3). Anti-PGE_2 antibody (100 µl) was added to the reaction mixture (stock solution contained 1 vial anti-PGE_2 reconstituted with 10 ml RIA buffer). This mixture was then incubated at 37°C for 2 hours. After incubation, samples were cooled to 4°C for 1 hour. To separate bound from unbound PGE_2, 500 µl of a solution containing 1% activated charcoal, 1% Dextran T70, 0.05% NaN_3 was added to each sample and mixed. Samples were then centrifuged at 4°C in a pre-cooled centrifuge for 20 minutes at 2,000 g. Aliquots (500 µl) of supernatant were carefully removed and radioactivity was determined with a Beckman LS-6000 LL scintilation counter. Standard PGE_2 was assayed under the same conditions. Standard curves were plotted on a logarithmic scale and quantities of PGE_2 in samples were determined from the standard curve.

5.4. Results

5.4.1. Regulation of collagenase and TIMP-1 mRNA levels by TGF-β

We examined the effects of the anti-inflammatory cytokine TGF-β on the expression of MMP-1 and TIMP-1 mRNA. Cultured human synoviocytes were treated with TGF-β (0.1-10 ng/ml) in a serum free medium at 37°C for 24 hours. After the incubation time, the cells were washed and total mRNA was extracted as described in methods. The levels of mRNA for collagenase and TIMP-1 were assayed using slot-blotting analysis. According to the results in Figure 5.1, TGF-β alone enhanced the expression of TIMP-1 mRNA while suppressed the level of collagenase mRNA. 100% control was the level of mRNA expression for untreated cells. The % control collagenase mRNA expression was decreased by 45% when the cells were treated with 10 ng/ml TGF-β. Whereas the % control TIMP-1 mRNA expression was increased up to 50% with 10 ng/ml TGF-β.

5.4.2. Collagenase and TIMP-1 mRNA levels are time and dose dependently increased by IL-1β or TNF-α

IL-1β or TNF-α stimulation of cultured human RA synoviocytes resulted in up-regulation of collagenase and TIMP-1 mRNA. Figure 5.2 shows that, cytokine stimulation of collagenase mRNA was time dependent. RA synoviocytes were treated with either IL-1β (10 pM) or TNF-α (10 ng/ml) in a serum free media at 37°C for the indicated time points. The level of the collagenase mRNA expression reached a maximum after the cells were treated for 24 hours with either IL-1β or TNF-α.

Figure 5.1.

Differential expression of collagenase and TIMP-1 mRNA in (RA) synovium upon stimulation with TGF-β.

Cultured human synovial fibroblasts were exposed to TGF-β in serum free medium for 24 hours. Results of slot blot analysis of in vitro collagenase and TIMP-1 mRNA levels are shown; values are the mean and SEM levels of collagenase and TIMP-1 mRNA. Each experiment was performed in triplicate. ($n = 3$). Significant differences are indicated ($*$, $p < 0.05$).

Figure 5.1

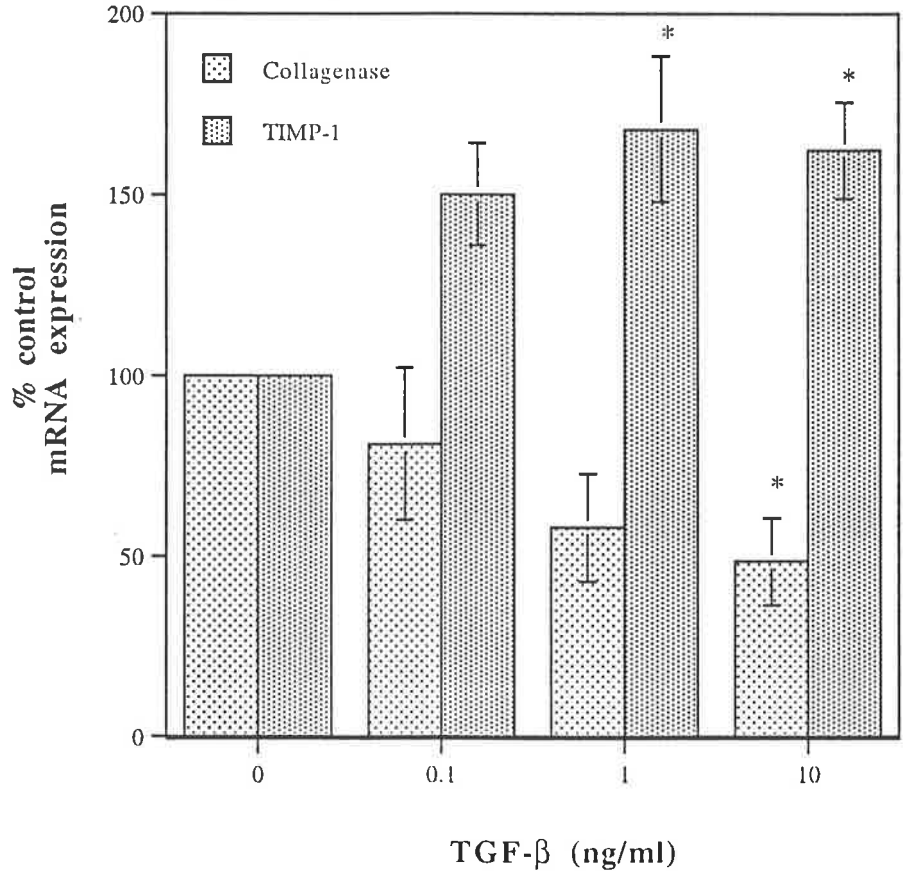
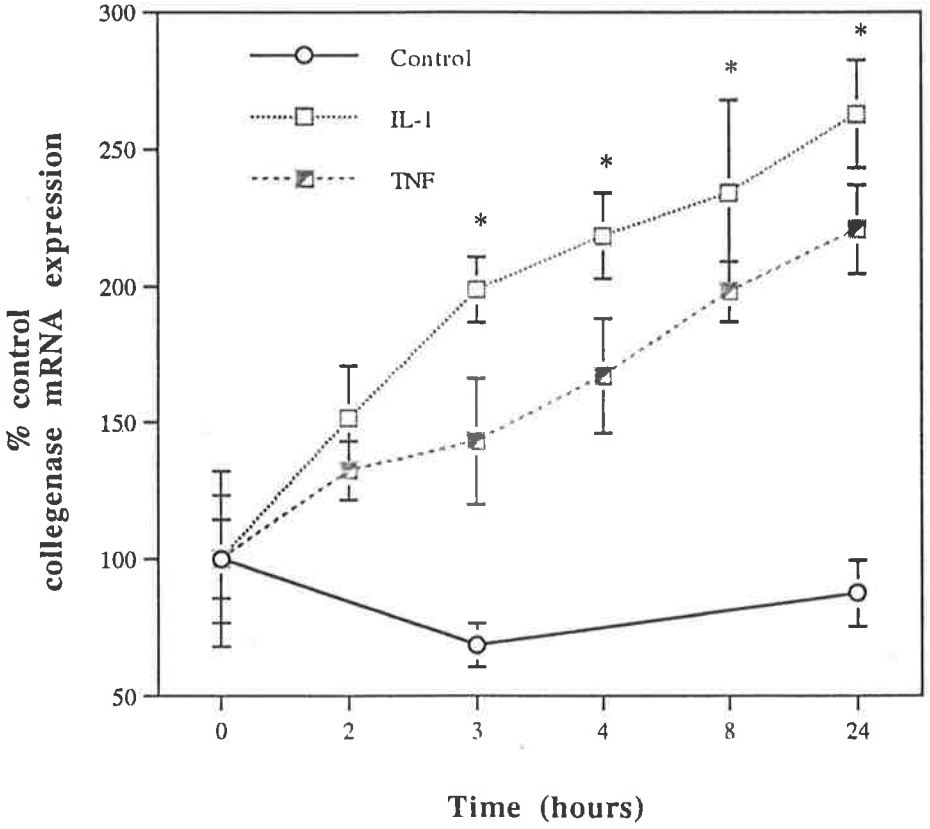

200
150
100
50
0
Collagenase
TIMP-1
*
*
*
0
0.1
1
10
% control
mRNA expression
TGF-β (ng/ml)

Figure 5.2.

Time course of IL-1β and TNF-α stimulation in RA synoviocytes, induced by IL-1β or TNF-α.

Confluent, human synoviocytes were cultured with IL-1β (10 pM) or TNF-α (10 ng/ml) for the lengths of time indicated. At each time point, cells were washed and the mRNA was extracted as described in methods. The level of mRNA for collagenase was assayed, using the slot-blot protocol. Vertical axis, % control of the relative intensity for collagenase mRNA expression. Each experiment was performed in triplicate. ($n = 3$). Significant differences are indicated (*, $p < 0.05$).

Figure 5.2


300
250
200
150
100
50
% control
collegenase mRNA expression
Control
IL-1
TNF
*
*
*
*
0
2
3
4
8
24
Time (hours)

The pro inflammatory-cytokine stimulation of collagenase and TIMP-1 genes occurred in a dose dependent manner by IL-1β or TNF-α. As shown in Figure 5.3 A, IL-1β (0.2-100 pM), TNF-α (0.2-100 ng/ml) and PMA (0.2-100 nM) stimulated the expression of collagenase mRNA, in cultured human synoviocytes, for 24 hours. 100% control is the level of the mRNA expression when the cells were not treated with any stimulant. Using IL-1β (100 pM) increased the level of collagenase gene expression up to 10 fold. When the cells were treated with TNF-α (0.2-10 ng/ml), the % control collagenase gene expression increased in a dose dependent manner reaching to a maximum of 1200% at 10 ng/ml, whereas using higher concentration of this cytokine (i.e., 100 ng/ml) reduced this level down to 800%. PMA did not induce any change in the level of collagenase mRNA expression.

Figure 5.3 B shows the level of TIMP-1 mRNA expression under the same conditions. There was a dose dependent increase in the level of TIMP-1 mRNA expression when the cells were treated with IL-1β or TNF-α at the indicated concentrations. PMA (0.2-10 nM) did not induce any significant change in TIMP-1 mRNA expression, whereas using higher concentrations (i.e., 100 nM) slightly enhanced the level of TIMP-1 mRNA expression.

5.4.3. Modulation of collagenase and TIMP-1 mRNA by IL-1β and TNF-α in the presence of TGF-β

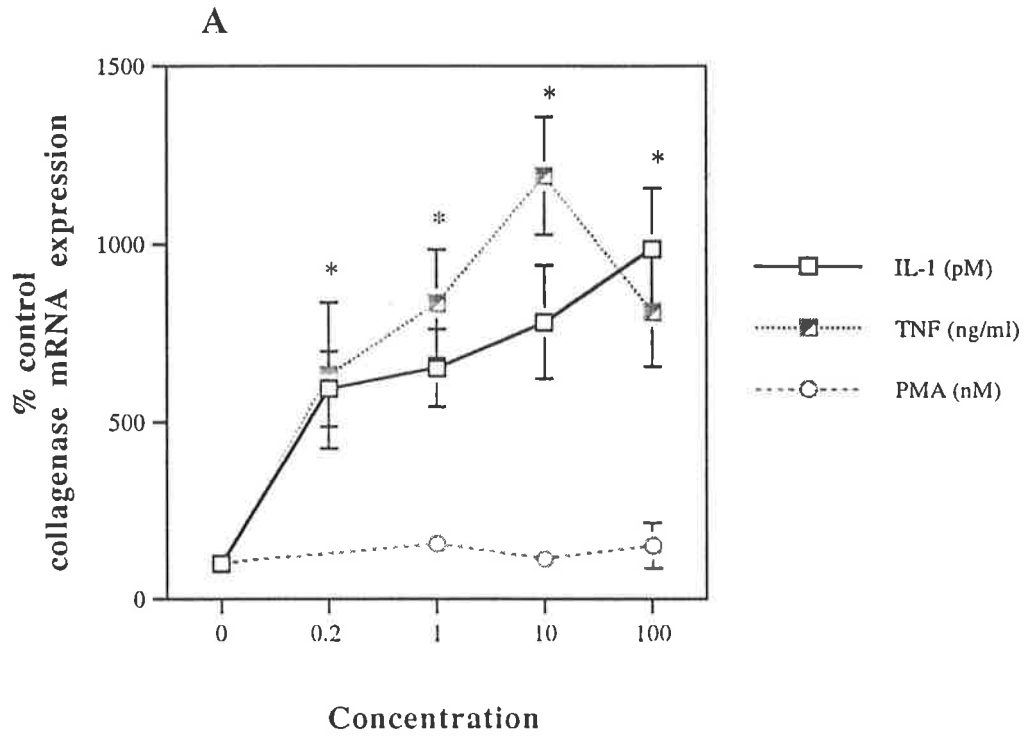
Next we investigated the effects of TGF-β on cytokine stimulated RA synoviocytes.

RA synoviocytes were pre-treated with TGF-β (0.1-10 ng/ml) at 37°C for 24 hours in serum-free media, followed by 24 hours stimulation with IL-1β (10 pM) or TNF-α (10 ng/ml). As shown in Figure 5.4 A, IL-1β or TNF-α-stimulated up-regulation of collagenase gene expression, was significantly reduced in the presence of TGF-β. Results were expressed as, % control of the collagenase mRNA expression. 100% control was determined as the maximum level of the collagenase gene expression,

Figure 5.3.

Dose-response experiment, examining the effects of IL-1β, TNF-α and PMA on collagenase and TIMP-1 mRNA expression.

The % control of collagenase mRNA expression in RA synoviocytes treated with IL-1β, TNF-α, or PMA is determined using the slot-blot protocols (Figure 5.3 A). Human synoviocytes were cultured with various concentrations of stimulants, i.e. IL-1β (0.2-100 pM), TNF-α (0.2-100 ng/ml) , or PMA (0.2-100 nM), for 24 hours. After each incubation, the level of the collagenase mRNA expression was assayed by the slot-blotting technique. 100% control is indicated for the cells which have not been treated with any cytokine. Figure 5.3 B shows the % control of TIMP-1 mRNA expression in RA synoviocytes treated with various concentrations of stimulants, i.e. IL-1β (0.2-100 pM), TNF-α (0.2-100 ng/ml) , or PMA (0.2-100 nM), for 24 hours. Each experiment was performed in triplicate. ($n = 3$). Significant differences are indicated ($*$, $p < 0.05$).


A
1500
1000
500
0
% control
collagenase mRNA expression
*
*
*
*
0
0.2
1
10
100
Concentration
IL-1 (pM)
TNF (ng/ml)
PMA (nM)

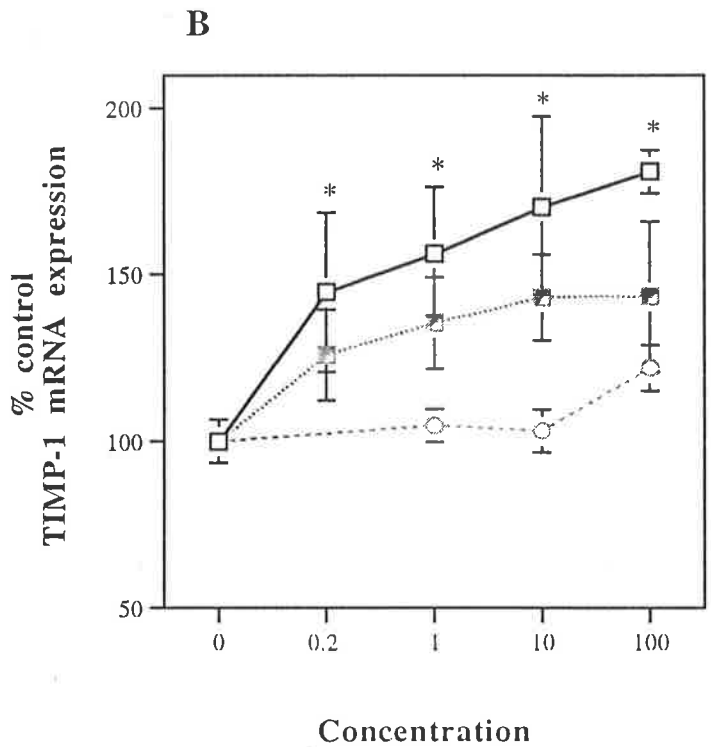
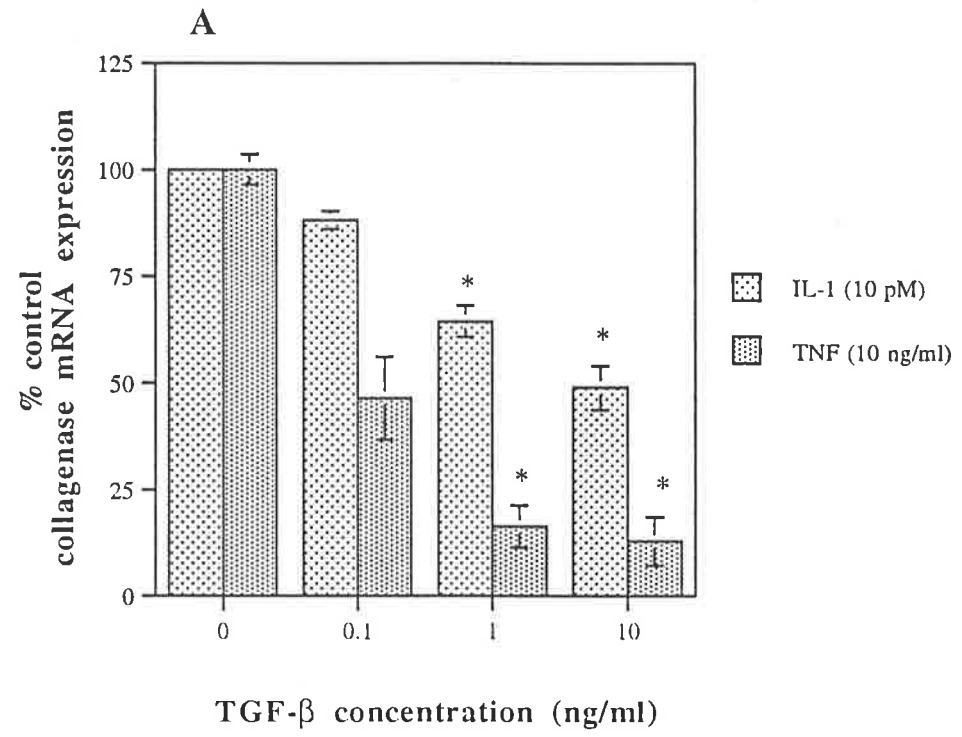

B
200
150
100
50
% control
TIMP-1 mRNA expression
*
*
*
*
0
0.2
1
10
100
Concentration

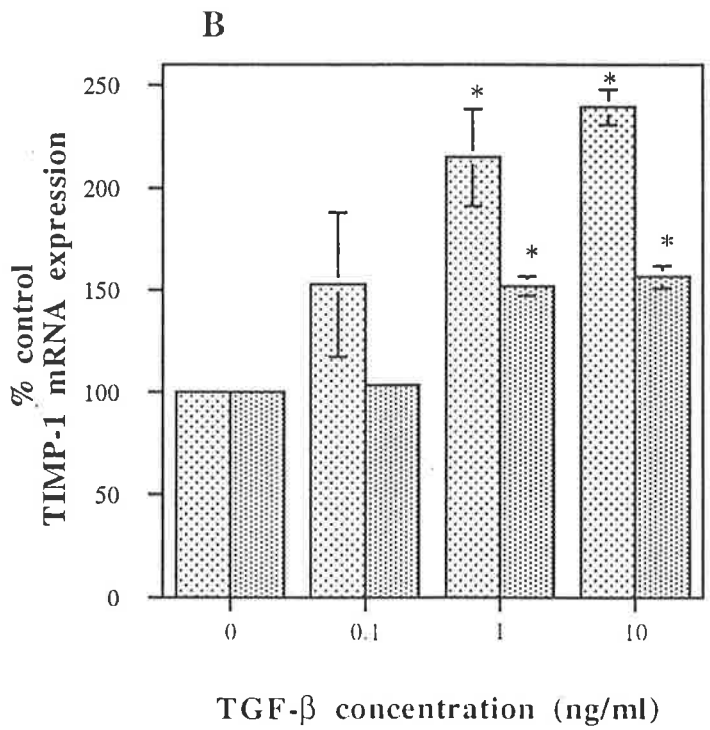
Figure 5.4.

Modulation of collagenase and TIMP-1 mRNA expression by IL-1β and TNF-α in the presence of TGF-β.

Figure 5.4 A shows the % control of collagenase mRNA expression, when RA synoviocytes were treated with IL-1β, or TNF-α in combination with TGF-β. Human synoviocytes were treated with TGF-β (0.1-10 ng/ml) for 24 hours in serum free media, followed by stimulation with IL-1β (10 pM), or TNF-α (10 ng/ml), for another 24 hours in the same media. After the incubation time (48 hours), the level of the collagenase mRNA expression was assayed by the slot-blotting technique.

Figure 5.4 B shows the % control of TIMP-1 mRNA expression, when RA synoviocytes were treated with IL-1β, or TNF-α in combination with TGF-β. Human synoviocytes were treated with TGF-β (0.1-10 ng/ml) for 24 hours in serum free media, followed by stimulation with IL-1β (10 pM), or TNF-α (10 ng/ml), for another 24 hours in the same media. After the incubation time (48 hours), the level of the TIMP-1 mRNA expression was assayed by the slot-blotting technique. Each experiment was performed in triplicate. ($n = 3$). Significant differences are indicated (*, $p < 0.05$).


A
125
100
75
50
25
0
% control
collagenase mRNA expression
*
*
*
*
0
0.1
1
10
TGF-β concentration (ng/ml)
IL-1 (10 pM)
TNF (10 ng/ml)


B
250
200
150
100
50
0
% control
TIMP-1 mRNA expression
*
*
*
*
0
0.1
1
10
TGF-β concentration (ng/ml)

when the cells were treated with IL-1β or TNF-α. Note that the level of collagenase mRNA expression was very low in untreated cells. In cytokine stimulated cultured synoviocytes, TGF-β (0.1-10 ng/ml) decreased the level of collagenase gene expression in a dose dependent manner.

According to Figure 5.4 B, when the cells were exposed to IL-1β or TNF-α in combination with TGF-β, TIMP-1 expression was increased significantly. 100% control, was the level of TIMP-1 gene expression, when the cells were treated with IL-1β (10 pM) or TNF-α (10 ng/ml) alone. Treatment of the cells with either cytokine in combination with TGF-β induced a higher level of TIMP-1 gene expression compared to sum of the data achieved with either cytokine or TGF-β alone. Therefore, here we have demonstrated that TGF-β in combination with IL-1β or TNF-α, interact in a synergistic manner to increase TIMP-1 gene expression. Furthermore according to these data TGF-β significantly reduced the increased level of collagenase mRNA expression with IL-1β or TNF-α.

The paradoxical effects of TGF-β to up-regulate the TIMP-1 gene expression and down-regulate collagenase gene expression in the IL-1β induced cultured cells is illustrated in Table 1.

Table 1. Relative expression of human collagenase and TIMP-1 mRNA, normalized to GAPDH, in human synovial fibroblasts treated with recombinant human IL-1β in the presence or absence of TGF-β. (n = 3)

	mRNA normalized to GAPDH			
No treatment	IL-1β	TGF-β	IL-1 + TGF-β	% Inhibition collagenase gene
Collagenase				
100	319	162	276	13.5
100	390	141	316	20
100	2195	1145	1216	44.5

No treatment	IL-1β	TGF-β	IL-1 + TGF-β	% Enhancement TIMP-1 gene
TIMP-1				
100	94.93	111	159	43
100	136	127	223	75.5
100	93	126	168	33.5
100	233	312	462	48

5.4.4. Comparison of cellular mRNA for collagenase and TIMP-1 using slot-blot protocols

Figure 5.5 shows the autoradiograms of mRNA extracted from cultured human synoviocytes exposed to IL-1β and TGF-β separately or in combination. IL-1β stimulates both collagenase and TIMP-1 mRNA expression. TGF-β exerts a reciprocal effect, where TIMP-1 mRNA levels are increased and collagenase levels are reduced. The combination of TGF-β and IL-1β resulted in suppression of collagenase expression and induction of TIMP-1 expression. Expression of the GAPDH was unaffected by exposure to these cytokines.

Figure 5.5.

Comparison of cellular mRNA obtained from cultured human synovial fibroblasts in serum free media in treatment conditions using the slot blot protocols.

Panel **A,** Standard mRNA extraction dissolved in 20 x SSC (cells with no treatment) **B,** Cells exposed to IL-1β (10 pM) **C,** Cells exposed to TGF-β (10 ng/ml) and **D,** IL-1β (10 pM)+TGF-β (10 ng/ml).

The mRNA samples were applied to a nylon membrane and probed with ^{32}P-labelled complementary DNA (cDNA) for **a.** MMP-1, **b.** TIMP-1 and **c.** GAPDH and exposed for 2 days.

5.4.5. TGF-β modulates the stimulation of PGE_2 production by IL-1β or TNF–α

IL-1 and TNF-α are potent stimulators of PGE_2 expression in rheumatoid synovial fibroblasts (Unemori et al, 1994). Synovial fibroblasts were treated with IL-1β or TNF-α alone or in combination with TGF-β (10 ng/ml). Cells were also treated with either cytokines in combination with indomethacin (15 mM) for 24 hours. Conditioned media from the treated and untreated cells, were collected and assayed for the release of PGE_2 by synoviocytes, as described in methods. TNF-α (10 ng/ml) or IL-1β (10 pM) alone caused large increase in PGE_2 secretion. Indomethacin (15 mM), significantly reduced the stimulation of PGE_2 production by these proinflammatory cytokines. TGF-β (10 ng/ml) alone caused no significant change in PGE_2 secretion, whereas, pretreatment of these cells with 10 ng/ml TGF-β for 24 hours, followed by exposure to IL-1β (10 pM) or TNF-α (10 ng/ml) for a further 24 hours, significantly decreased the cytokine induced production of PGE_2. (Figure 5.6)

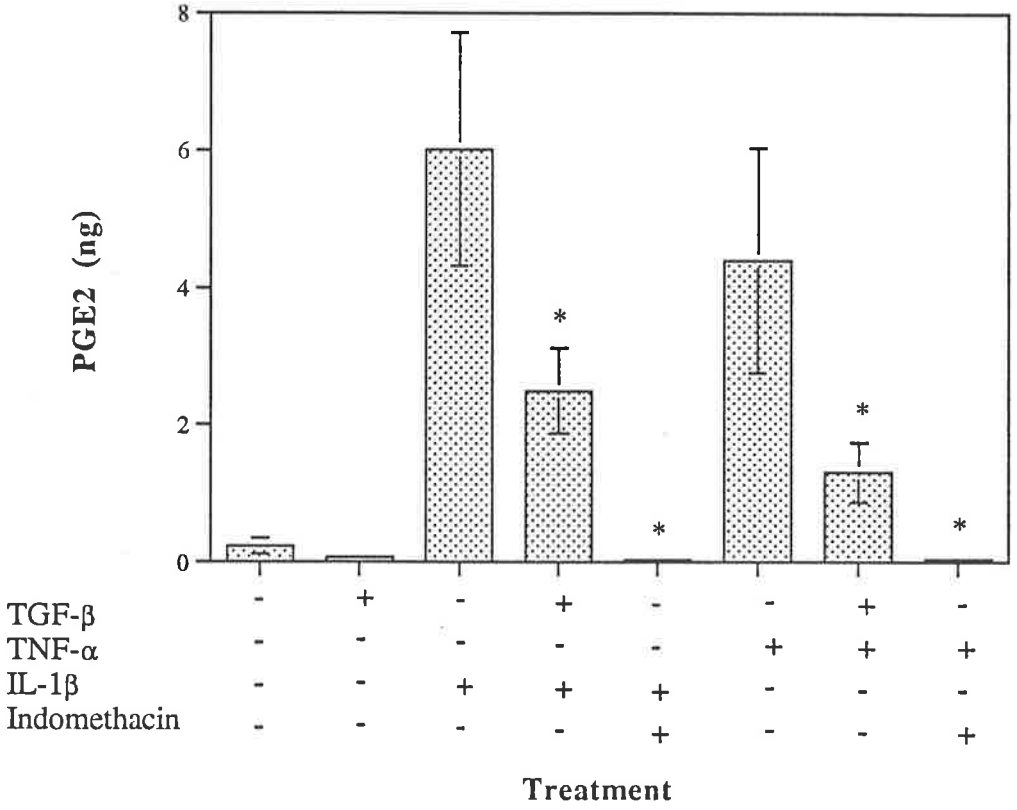
Since TGF-β reduced the enhanced level of PGE_2 by IL-1β or TNF-α, these results indicate the possible involvement of TGF-β in modulating the synthesis of PGE_2 in stimulated human synoviocytes.

Figure 5.6.

Production of PGE_2 in response to cytokines alone and in combination with TGF-β.

Confluent, human synoviocytes in serum free medium were exposed to IL-1β (10 pM), TNF-α (10 ng/ml) alone or in combination with TGF-β (10 ng/ml) or indomethacin (15 μM). Following these incubations, cell supernatants were collected and assayed for the PGE_2 production. Vertical axis is the concentration of PGE_2 (ng). Values represent the mean ± SEM of three separate experiments (in quadruplicate). Significant differences are indicated (*, $p < 0.05$).

Figure 5.6



5.5. Discussion

Extracellular matrix dynamics are regulated by the balance between the deposition of structural components such as collagen, and their degradation by extracellular secreted proteases e.g. collagenase and stromelysin. The activity of these metalloproteinases is regulated partly by the activity of their specific inhibitor TIMP. Therefore the control of metalloproteinase and TIMP expression is an important regulatory mechanism in extracellular matrix turnover (Edwards et al, 1987).

Our results demonstrate another important mechanism in extracellular matrix turnover; selective modulation of the transcriptional induction of MMP-1 and TIMP-1 by TGF-β alone and in combination with pro-inflammatory cytokines including IL-1β and TNF-α. TGF-β is capable of reducing the collagenase mRNA level whereas stimulating the expression of TIMP-1 mRNA by human synovial fibroblasts in serum free media. Furthermore, TGF-β synergistically induces the TIMP-1 mRNA expression when the cells are simultaneously exposed to IL-1β or TNF-α. There is also evidence that TGF-β is able to reduce the collagenase mRNA secretion in conjunction with IL-1β or TNF-α. These opposing effects of TGF-β on the expression of the enzyme and inhibitor, effectively leads to inhibition of extracellular matrix breakdown.

In a study by Wright, using a diffuse fibril assay, TGF-β and IL-1β in combination, significantly increased the TIMP secretion in cultured media of the human synoviocytes (Wright et al, 1991). Howéver our results make it clear that TGF-β regulates the expression of MMP-1 and TIMP-1 gene at transcriptional level and possibly the effects of this growth factor are not post-transcriptional. Another study on the human articular chondrocytes, have shown the ability of TGF-β to modulate the catabolic effects of IL-1β (Lum et al, 1996). Our results in this study showing an inhibition of collagenase and induction of TIMP-1 production in response to TGF-β are in agreement with their findings. Other mediators of inflammation, PGs, can cause joint pain and swelling as seen in RA (Mehindate et al, 1995). Elevation of PGE_2 is

found in synovial effusions of patients with inflammatory joint disease (Sepalla et al, 1985). PGE_2 expression is stimulated by pro-inflammatory cytokines such as IL-1 and TNF, and is believed to be an important stimulus for production of intracellular proteases (Rodemann and Goldberg 1982). In this study, we show that, the enhanced secretion of PGE_2 by IL-1β or TNF-α stimulated synovial cells, is significantly inhibited by TGF-β. There is a similarity in the effects of IL-1β and TNF-α in combination with TGF-β, on PGE_2 production and collagenase synthesis. In both cases, TGF-β is capable of inhibiting the stimulatory effects of IL-1β and TNF-α. This suggest that a particular step(s) in the biochemical pathway of synthesis is possibly induced by TGF-β. On the other hand, these beneficial effects of TGF-β might occur in two separate pathways in parallel. Our results indicate that the human synovial fibroblast response to cytokines and growth factors is complex and is dependent on local cytokine concentrations, as well as their antagonising interactions. Although local cytokine concentrations in synovium and their levels during the disease process are not known, measurements of these substances in rheumatoid synovial fluid indicates that there is marked variation between samples (Duff et al, 1988; Wahl et al, 1990). Further studies are needed to clarify the role of these cytokines in human synovial fibroblasts.

In conclusion, TGF-β is known to promote wound healing and matrix synthesis, thus it would prevent matrix breakdown and initiate repair of connective tissues. The net balance between the levels of activated enzyme and the amount of TIMP-1 results in the overall enzyme activity in the extracellular milieu. If TGF-β both inhibits MMP-1 whilst stimulating TIMP-1 secretion by synoviocytes, then it has a powerful means of shifting the MMP/TIMP-1 ratio away from matrix degradation. Our results confirm and extend observations of above studies. These effects of TGF-β are seen at the mRNA levels, suggesting the involvement of pretranslational mechanisms in these effects. The capacity of TGF-β to antagonise the immunoregulatory effects of several pro inflammatory cytokines suggest that this growth factor may be valuable, in developing new therapeutic agents, for the treatment of inflammatory diseases. Further

studies are clearly needed to resolve the possible use of this growth factor in the therapy of joint disease such as RA and OA.

Chapter 6

Inhibition of IL-8 mRNA synthesis and secretion in rheumatoid synoviocytes as a mechanism of action for glucocorticoids

6.1. Abstract

Rheumatoid inflammation is characterized by the presence of neutrophils in the synovial fluid and synovium. Interleukin-8 (IL-8) is a novel neutrophil chemotactic factor released from cells at the site of infection or tissue injury in response to inflammatory stimuli. We investigated whether glucocorticoids, suppress the production of this cytokine by cultured RA synoviocytes stimulated with IL-1β or TNF-α . The level of IL-8 production in culture supernatants of RA synoviocytes, was measured by specific enzyme-linked immunosorbent assay (ELISA). The gene expression of IL-8 was determined using slot-blot analysis. Dexamethasone exerted a potent suppressive effect on the production of IL-8 from cultured synoviocytes IC_{50} =1 nM. This suppressive effect was also observed at the level of mRNA IC_{50} = 0.5-1 nM. These results suggest that, suppression of IL-8 synthesis and secretion might be another mechanism for the usefulness of glucocorticoids by which glucocorticoids suppress synovial inflammation in the treatment of RA .

6.2. Introduction

An early event in the inflammatory process is migration of neutrophils into the synovial joint of patients with RA. Neutrophils are inflammatory cells which contain a wide range of proteinases such as MMPs. Upon stimulation, these tissue degrading enzymes are released from neutrophils along with oxygen metabolites. In this way activated neutrophils contribute to tissue damage at sites of inflammation. Therefore, one mechanism for controlling inflammatory reactions is to regulate the migration and activation of neutrophils (Welbourn et al, 1991).

In RA a number of mediators secreted by endothelial and /or mononuclear cells such as platelet-activating factor, leukotriene B4 (Ford et al. 1980, Lee et al, 1985), IL-1, and TNF-α (Le J and Vilcek J 1987, Shalaby et al, 1985) may influence the migration of neutrophils into the synovial membrane. Interleukin-8 (IL-8), has been described as a novel leukocyte-derived chemotactic cytokine (Matsushima et al, 1989). IL-8 is a member of the family of chemokines, which are factors responsible for signalling during both acute and chronic inflammation (Oppenhiem et al, 1991). There is considerable evidence that IL-8 is elevated at sites of inflammation and can induce inflammatory states. For example, in RA, the presence of IL-8 is increased in joint fluid and cells of the pannus (Houssiau et al, 1988). There is also evidence that, intra-articular injection of IL-8 into the knee joint space, induces a rapid infiltration of a large number of neutrophils into the joint space and synovial tissues (Endo et al, 1991). Furthermore, intradermal injection of IL-8 also results in massive neutrophil infiltration (Colditz et al, 1989). IL-8 is known to exert these effects through its specific receptors which are located on the plasma membrane of neutrophils. Therefore migration of neutrophils is directed in response to very low concentrations of IL-8 (Oppenhiem et al, 1991). The mature form of IL-8 consists of 72-amino acids with the molecular mass of 8 kDa. Upon exposure to IL-1 or TNF, endothelial cells (Schroeder et al, 1989), human fibroblasts (Strieter et al, 1989), and human synovial cells (Watson et al, 1988), express IL-8. Since, various cell types can only express the IL-8 gene and IL-8 protein in the

presence of stimulants, but not constitutively, it appears that IL-8 gene expression is tightly controlled consistent with its primary role in regulating neutrophil migration (Mokaida et al 1992). Therefore, repression of IL-8 gene activation may be a novel way to suppress inflammation by reducing the leukocyte infiltration. Preliminary evidence for this is suggested in a study by Seitz, where some evidence for modulation of IL-8 expression by NSAIDs, glucocorticoids, and IFN-gamma is provided (Seitz et al, 1991).

Synovial cells are capable of producing IL-8 in response to inflammatory cytokines, although the amount of secreted IL-8 varies from cell to cell (Hirota et al, 1992). The amounts of IL-8 detected in synovial fluid of patients with active RA are increased compared to that of patients with OA (Endo et al 1991). In addition, IL-1 and TNF-α (mRNA and protein) are produced at high levels by cells isolated from the synovial membrane of individuals with RA (Buchan et al, 1988; Hirota et al, 1992). Glucocorticoids are also used as anti-inflammatory agents in various types of cells including, peripheral blood mononuclear cells, fibrosarcoma cells, and glioblastoma cells, to suppress the IL-8 mRNA expression and protein production induced by IL-1, TNF, or LPS. (Mokaida et al 1994).

In summary, since the influx of neutrophils is a critical step in the pathogenesis of RA, determining the regulation of the factors recruiting these cells, is of major importance. IL-8 is believed to be an important factor in attracting neutrophils to the site of inflammation. As the activities of IL-8 are relevant to the pathogenesis of RA, we conducted this study to determine the level of IL-8 gene expression and protein secretion in cytokine stimulated human synoviocytes from patients with RA. We also examined the inhibitory effects of the synthetic glucocorticoid, dexamethasone, on cytokine induced IL-8 mRNA expression as well as protein production in rheumatoid human synoviocytes.

6.3. Materials and methods

6.3.1. Reagents

Recombinant human IL-8 was from Amgen, Amgen Centre, 1840, Deavilland Drive, Thousand Oakes, CA 91320-1789 U.S.A. Anti-human IL-8 neutralizing antibody was from R&D Systems, 614 Mckinley Place N.E Minneapolis, MN 55413 U.S.A. Streptavidin, horseradish peroxidase conjugated was from Pierce, Rockford, Illinios 61105 U.S.A. Biotinylated rabbit anti-goat immunoglobulins was from DAKO Corporation, Carpinteria, CA, U.S.A. Collagenase, clostridiopeptidase A (EC 3.4.24.3) and O-phenylene diamine-HCl tablets were from Sigma Chemicals, St. Louis, MO. Nunc Immuno Plate Maxisorb flat-bottom 96-well ELISA trays were purchased from Nunc, Denmark. Giga-prime DNA labelling kit, (α^{32}P)dCTP and (γ^{32}P)ATP were from Bresatec (Thebarton, South Australia).

6.3.2. Fibroblast cultures

Synovial tissue was obtained from informed patients with RA. Cultured synovial fibroblasts were prepared according to the protocol, described in Chapter 3. Fibroblasts from the 3th-10th passage were inoculated onto 60 x 15 mm sterile polystyrene tissue culture dishes at a density of 5 x 10^5 cells/well, and grown to semi-confluency. In general, before experimentation, cells were exposed to serum free media containing 0.2% BSA for 24 hours. The following day, the cells were treated as described in the Figure legends. After each incubation, cultured supernatants were collected and stored at -20°C until assayed for IL-8 protein production, and the cellular RNA was obtained from the remaining cells.

6.3.3. Isolation of messenger RNAs

Following each experiment, total cellular RNA was isolated according to the method outlined in Chapter 3.

6.3.4. Radiolabeling of cDNA probes

The IL-8 probe used in this study was the 244-base pair PstI/EcoRI cDNA fragment representing the coding region of the IL-8 cDNA from nucleotides 49 to 293. The IL-8 cDNA was then radiolabelled with (α-^{32}P)dCTP, using a random primer DNA labelling system (Giga-prime DNA labelling kit, Bresatec). A γ^{32}P-labelled synthetic oligonucleotide (5'-GTTGGTTTCTTTTCCTC-3'), that binds to the 28 S ribosomal RNA was used to monitor mRNA binding to the membrane. This oligonucleotide was constructed from the known consensus sequences for 28 S RNA (kindly provided by Dr. Shaun McColl, Dept. of Immunology, University of Adelaide), and radiolabelled with (γ^{32}P)ATP using a 5' DNA terminus labelling system.

6.3.5. Northern analysis

Northern analysis (of RNA prepared by the standard protocol) was carried out essentially as described in previous Chapters (Sambrook et al, 1989).

6.3.6. Slot-blot analysis and hybridization

This technique was adopted specifically to prevent any denaturing of the RNA during the loading process and reduce RNA degradation. The membrane was then hybridized (according to the same protocol in Chapter 3) with the denatured IL-8 probe in 25 ml for 18-24 hours (Maniatis et al, 1982). To reprobe each membrane, the first probe was removed by using the solution of boiled 0.5% SDS for 15 mins at room temperature.

Each membrane was then reprobed with the oligonucleotide probe for 28 S RNA as a control for mRNA loading, as it is constitutively expressed.

The results are expressed as the relative intensity (RI) of IL-8 mRNA normalised to the level of 28 S mRNA. Percent control of the RI was calculated as the ratio of the RI of the IL-8 band to the RI of the 28 S band. Values were expressed as mean ± SEM with "n" refers to the number of cell lines (i.e., patients) analysed. Where SEM is not shown, the size of the error bar is the same size or smaller than symbols. Student's t-test was used to test for significant differences between means.

6.3.7. IL-8 enzyme linked immunosorbent assay (ELISA)

The assay described here is a competitive ELISA, based on the method used for quantification of Substance P (Scicchitano et al, 1995). Intra- and inter-assay variance is 6% and 15%.

All steps were performed at room temperature unless otherwise stated. The layout for the plates is shown in figure 6.1. Trays were kept covered to prevent evaporation.

1. Nunc Immuno Plate Maxisorb flat-bottom 96-well ELISA trays were sensitized with 100 µl/well of IL-8 (15 ng/ml), for 4 hours.

2. Trays were washed three times with washing buffer (0.01M PBS pH = 7.4 containing 0.05% (v/v) Tween 20) by the flicking technique.

3. Nonspecific binding sites were blocked by adding 100 µl/well of blocking buffer (1% (v/v) horse serum in 0.01 M PBS pH = 7.4 containing 0.05% (w/v) sodium azide) for a minimum of 30 min.

4. Trays were washed as in step 2.

5. A total of 50 µl of either standard IL-8 (wells A3, A4) or unknown samples (cultured supernatant) (wells A5-A12) were added and then serially titrated in 1:2

dilutions down the tray in primary antibody diluent (0.1 M PBS/Tween containing 1% horse serum and 0.05% sodium azide). For example, 50 µl of standard IL-8 was added to A3, A4 with 50 µl diluent, mixed then, 50 µl transferred to wells B3, B4.

6. Quality controls (50 µl) were added to wells E1, E2 --> H1, H2 without further dilution. Quality control samples for the assay are, concentrations of IL-8 at (5 ng/ml) and (10 ng/ml) in 0.1 M PBS/Tween containing 0.75% BSA (w/v) which were stored at -70°C in 500 µl aliquots in microtubes. We detected no loss in concentration after storage for 6 months.

Negative control wells include:

A1, A2 are antigen-free and contain 100 µl of 0.01M PBS.

B1, B2 are primary antibody-free which contain 100 µl primary antibody diluent and act as a control for nonspecific binding of secondary antibody and subsequent steps.

C1, C2, D1, D2 are positive controls which contain primary antibody but no soluble IL-8, to compete with bound IL-8 on plate and yield the maximum OD_{490}. This maximum OD_{490} was 1.2.

7. A total of 50 µl of a 1:2000 primary antibody, goat anti-human IL-8 was added to the wells giving a final dilution of 1:4000.

8. The trays were covered and left overnight at 4°C.

9. Next day, trays were washed as in step 2.

10. A total of 100 µl of the secondary antibody (biotinylated rabbit anti-goat) was used at 1:40,000 final dilution for 1 hour at 37°C.

11. Trays were washed as in step 2.

12. A total of 100 µl of streptavidin, horseradish peroxidase conjugated was used at a final dilution of 1:2000 for 1 hour at 37°C.

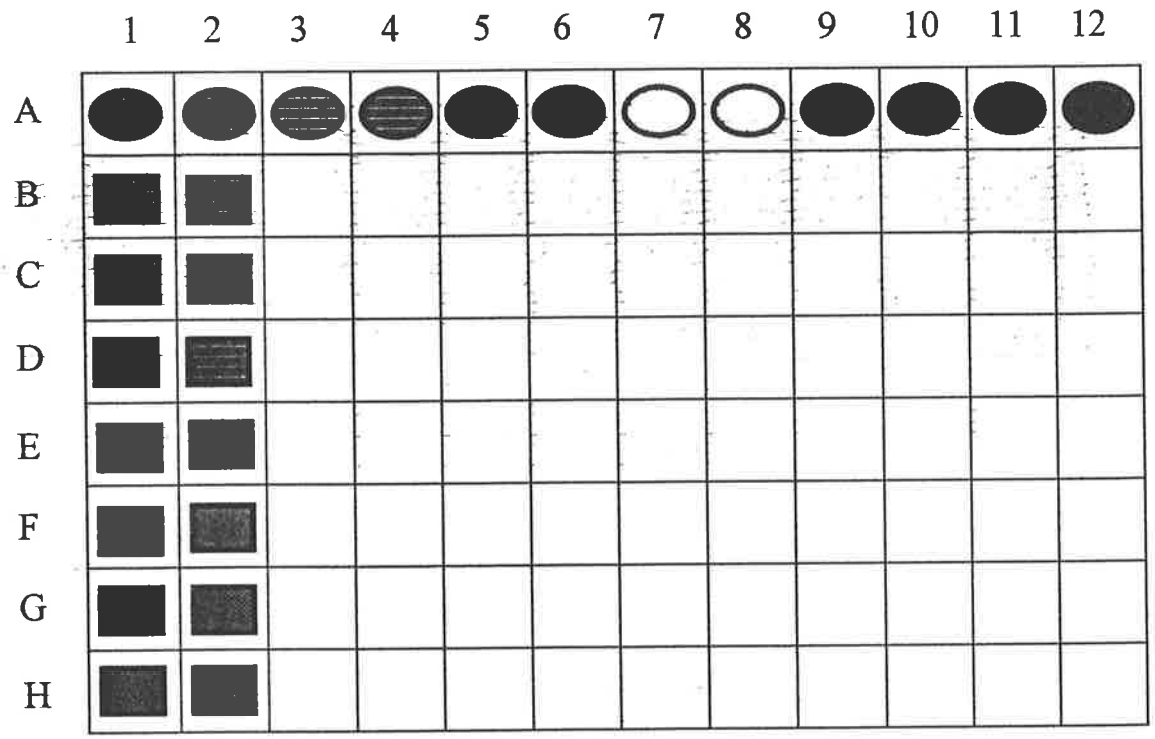
13. Trays were washed as in step 2.

14. For detection, O-phenylene diamine-HCl tablets were used in substrate buffer (40 mM citrate/2.7 mM phosphate pH 5). Five 10 mg tablets and 35 ml of 30% H_2O_2 was added to 100 ml substrate buffer, then 100 µl was added to each well. Trays were incubated for 30 min in the dark.

15. The reaction was then inhibited by the addition of 100 µl of 2.5 M sulfuric acid to each well.

Optical density (OD) was read at 490 nm, blanked on air, with reference at 630 nm using an MR 7000 EIA-CALC Dynateck ELISA reader (Dynateck, USA). A curve was constructed by plotting OD_{490} vs standard concentration. The sigmoid program used for curve fitting was based on the Rodbarbs Four Parameter equation, according to the method of Folkesson (Folkesson et al, 1985) as described in the operating manual for the ELISA reader. This fitted curve routinely generates an r value of ≥0.99. OD reading for samples were compared to those of the standards for quantitation, provided that the OD values obtained for samples fell within the linear range of the curve fit. The sensitivity of the ELISA was 19 ng/well.

Figure 6.1. Layout of the ELISA tray.



Antigen-free

Standard soluble IL-8

Sample 1

Sample 2

Sample 3

Sample 4

No primary antibody

No soluble IL-8 (max OD)

Quality control 1

Quality control 2

6.4. Results

6.4.1. Specificity of IL-8 probe to its complementary mRNA

We initially investigated the expression of the IL-8 mRNA in our system by cultured human synoviocytes upon stimulation with IL-1β (10 pM) in serum free medium. We used a complementary DNA probe corresponding to IL-8. Figure 6.2 shows the autoradiograph of Northern blot analysis using mRNA isolated from confluent cultured human synoviocytes in serum free medium for 24 hours. RNA from these cells showed a detectable level of hybridization to the band corresponding to IL-8 ^{32}P-labelled cDNA, indicating the specificity of the IL-8 cDNA probe to its complementary mRNA.

6.4.2. Detection of antigenic IL-8 by ELISA

A standard curve representing a compilation of 15 separate assays is shown in figure 6.3. The lower limit of detection of IL-8 was about 19 ng/well. To assess selectivity, negative and positive controls for nonspecific bindings were tested in each ELISA tray. For a negative control for nonspecific binding of antibodies to each tray, the OD_{490} in wells which were not sensitised with IL-8 protein was about 0.02. For a negative control for nonspecific binding of secondary antibody to IL-8 bound to tray, the OD_{490} for the (primary antibody-free) wells was about 0.02. For a positive control, all reagents were added with no soluble IL-8 to compete with the bound IL-8 on each plate. The OD_{490} for the positive controls were about 1.2, which represents the maximum level.

Figure 6.2.

Expression of IL-8 mRNA in cultured human synovial fibroblasts.

Autoradiograph of Northern blot analysis using mRNA isolated from human synovial fibroblasts in serum-free medium for 24 hours. The Northern blot was hybridised, using α ^{32}P radiolabelled IL-8 cDNA.

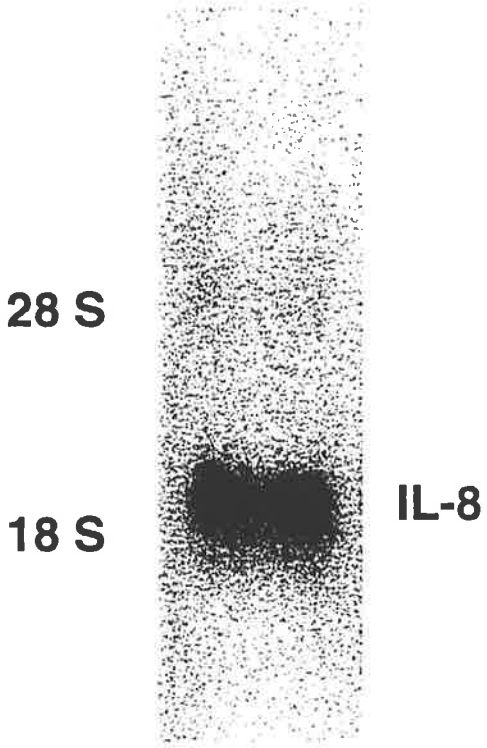
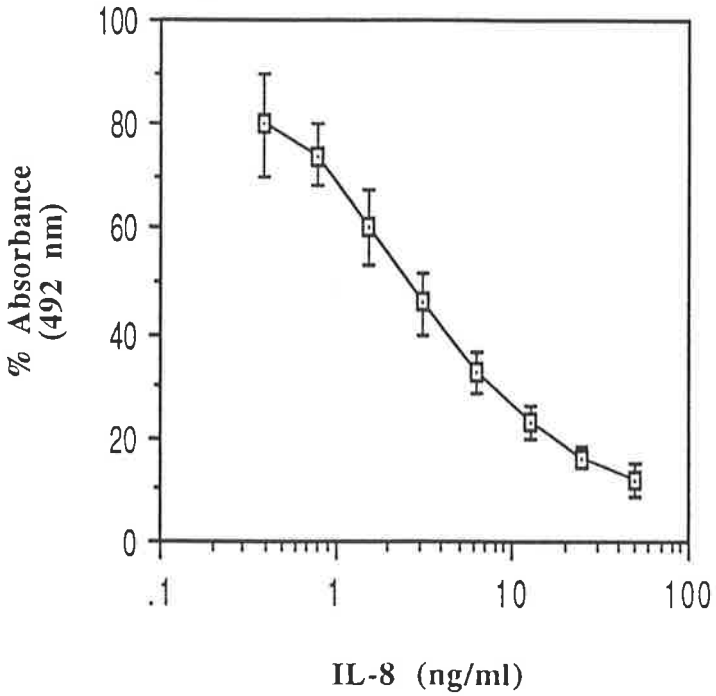

28 S
18 S
IL-8

Figure 6.3.

Standard curve of the interleukin-8 (IL-8) ELISA.

Each point represents the mean of 15 separate determinations carried out in duplicate and vertical bars indicate standard deviation. The lower limit of detection was 19 ng/well.

Figure 6.3



6.4.3. Time dependent induction of IL-8 synthesis and production in response to IL-1β and TNF-α

RA human synoviocytes were cultured with either IL-1β or TNF-α to induce IL-8 secretion and production. IL-1β (10 pM) or TNF-α (10 ng/ml) were applied to the cells in a serum free media for the indicated times. Cells were collected at each time point and the level of mRNA expression for IL-8 was determined using the standard protocols for slot-blot analysis. Figure 6.4 A shows that IL-1β and TNF-α increase the level of IL-8 mRNA expression in a time dependent manner. 100% RI of IL-8 mRNA expression is the level of IL-8 gene expression when the cells were not treated with any cytokine. Whereas exposure of the cells to either IL-1β or TNF-α rapidly and dramatically increased IL-8 mRNA expression. This increase was observed within 2 hours after stimulation with IL-1 or TNF and progressively increased thereafter. The maximal level of expression was achieved at 24 hours after stimulation. The dose of IL-1β and TNF-α used in these experiments was sufficient for maximal stimulation of IL-8 mRNA accumulation. We observed that, the level of IL-8 gene expression induced by IL-1β and TNF-α was variable from donor to donor although the kinetics of induction was the same.

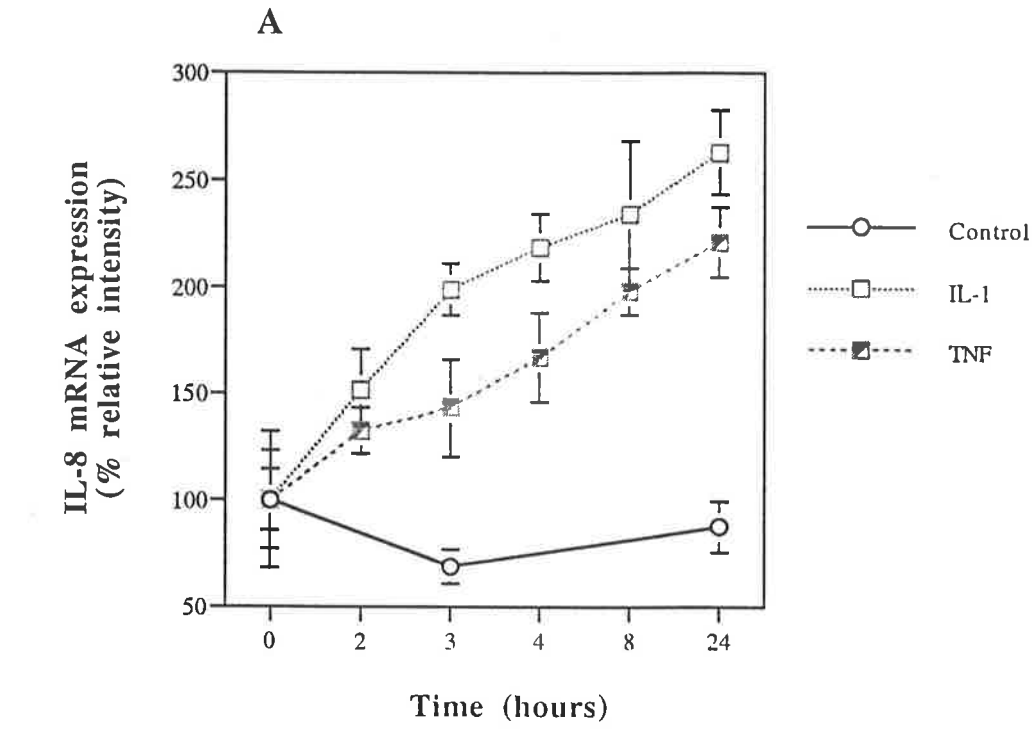
Figure 6.4 B shows the level of IL-8 protein production in cultured supernatant of the cells treated with IL-1β (10 pM) or TNF-α (10 ng/ml) at each indicated time point. The time points on the horizontal axis indicate the period of incubation time for the treatment of the cells with either cytokine. The results of the competitive ELISA illustrates that the induction of IL-8 protein was increased between 2 to 24 hours in cultured supernatants of the cytokine stimulated synoviocytes The level of IL-8 protein achieved statistical significance ($P < 0.05$) at 24 hours of cytokine treatment. The maximal level of IL-8 secretion was achieved after 5 days for cells treated with IL-1β and 7 days for cells treated with TNF-α.

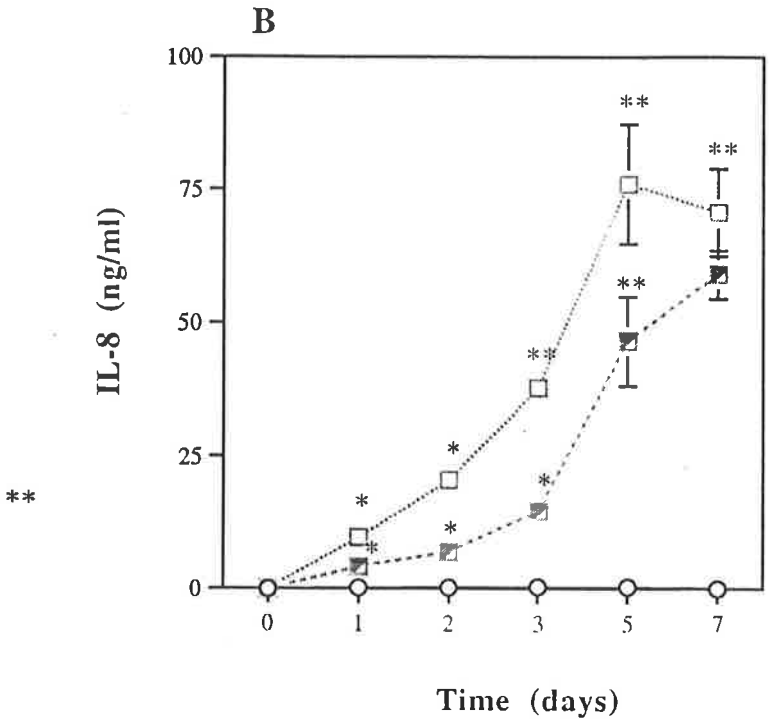
Figure 6.4.

Time course of IL-8 mRNA expression and production in RA synoviocytes treated with IL-1β (10 pM) and TNF-α (10 ng/ml).

A. RA synoviocytes in culture were treated with IL-1β, 10 pM or TNF-α, 10 ng/ml for the times indicated. RNA was isolated and analysed by slot-blot using a single-stranded RNA probe for IL-8 mRNA.

B. IL-8 production was also determined in the culture supernatants of the cultured RA synoviocytes using a competitive ELISA assay. Each data point represents the mean values ± SEM for cells from three RA patients. Significant differences are indicated (*, $p < 0.05$; **, $p < 0.005$).


A
IL-8 mRNA expression
(% relative intensity)
300
250
200
150
100
50
0
2
3
4
8
24
Time (hours)
Control
IL-1
TNF


B
IL-8 (ng/ml)
100
75
50
25
0
0
1
2
3
5
7
Time (days)

**

6.4.4. Dose dependent induction of IL-8 synthesis and production in response to IL-1β and TNF-α

The cytokine induction of IL-8 secretion was also increased in a dose dependent manner between IL-1β (0.2-100 pM) and TNF-α (0.2-100 ng/ml). RA human synovial fibroblasts were treated with IL-1β (0.2-100 pM) or TNF-α (0.2-100 ng/ml) in serum-free media at 37°C, for 24 hours. After the incubation time, total mRNA was isolated from the remaining cells, and the level of IL-8 mRNA expression was determined using the standard protocols for slot-blot analysis. Figure 6.5 A shows IL-1β and TNF-α increased the level of IL-8 mRNA expression in a dose dependent manner. Specific IL-8 mRNA expression was calculated as the % RI of IL-8 to 28S mRNA expression and IL-8 expression in stimulated cells was expressed as % RI to the expression in unstimulated cells. IL-1β (100 pM) increased the RI of IL-8 mRNA expression to a maximum of 750% compared to the control (100%). Using TNF-α (100 ng/ml) the RI of IL-8 mRNA expression reached a maximum of 600% compared to the control.

Cultured supernatants of the above treated cells, were assayed for the production of IL-8 protein. The level of IL-8 protein secretion was increased by IL-1β (0.2-100 pM) and TNF-α (0.2-100 ng/ml) in a dose dependent manner over these ranges (Figure 6.5 B). The maximal level of IL-8 production was achieved using IL-1β (100 pM) and TNF-α (100 ng/ml). However the use of IL-1β (10 pM) or TNF-α (10 ng/ml) produced substantial amount of IL-8 protein in cultured supernatants of the rheumatoid synoviocytes.

6.4.5. Effects of dexamethasone on IL-1β induced IL-8 mRNA expression and synthesis

We examined the inhibitory effects of a synthetic glucocorticoid, dexamethasone on the level of IL-8 mRNA expression as well as IL-8 production in IL-1β induced human synovial fibroblasts. Figure 6.6 A shows the addition of dexamethasone (1-100 μM) significantly decreased the level of IL-8 mRNA expression in IL-1β stimulated synoviocytes. 100% control IL-8 mRNA expression indicates the level of IL-8 expression in cells stimulated with IL-1β (10 pM) for 24 hours at 37°C. Dexamethasone (100 μM) induced a 70% inhibition in IL-8 secretion.

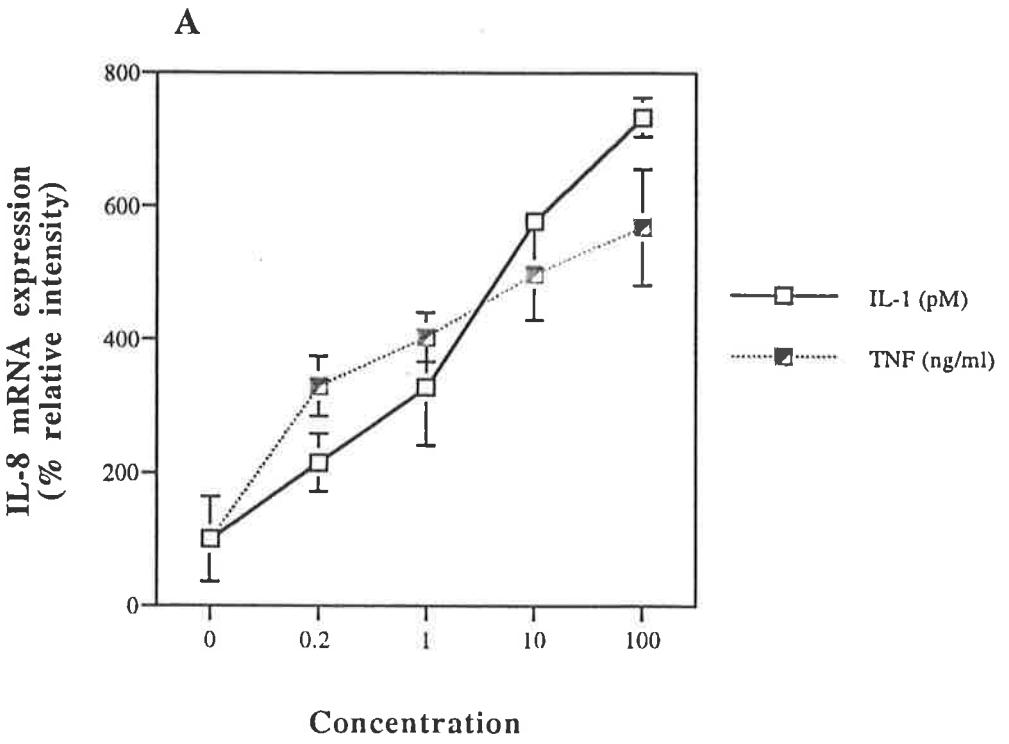
Cultured supernatants of the above treated cells, were collected and assayed for the production of IL-8 protein synthesis (Figure 6.6 B). 100% IL-8 release indicates the maximum level of the IL-8 protein secretion in IL-1β (10 pM) treated rheumatoid synoviocytes for 24 hours at 37°C. The maximum level of IL-8 protein production (100% IL-8 release) was between 13-127 ng/ml. Using dexamethasone in as low as 0.1μM induced a significant reduction in the level of IL-8 protein secretion in these cells. 10μM dexamethasone decreased the level of IL-8 protein secretion down to 25%. These results are likely to indicate that the inhibitory effect of dexamethasone is mediated by direct effect on gene expression, although IL-8 protein secretion was also reduced with this drug.

Figure 6.5.

Effects of various concentrations of IL-1β, TNF-α and PMA on IL-8 mRNA expression and production in RA synoviocytes.

A. Cells were grown to confluence and stimulated with increasing concentrations of IL-1β (0.1-100 pM), TNF-α (0.1-100 ng/ml), and PMA (1-100 nM) for 24 hours. The cells were harvested, RNA was prepared and IL-8 mRNA was then detected as described under " Materials and methods". The filters were then stripped and reprobed for 28S ribosomal RNA. The data presented are the mean values ± SEM for cells from three RA patients.

B. The supernatants of the above cells were collected, and IL-8 protein was determined by competitive ELISA as described under "Materials and methods".


A
IL-8 mRNA expression
(% relative intensity)
800
600
400
200
0
0
0.2
1
10
100
Concentration
IL-1 (pM)
TNF (ng/ml)

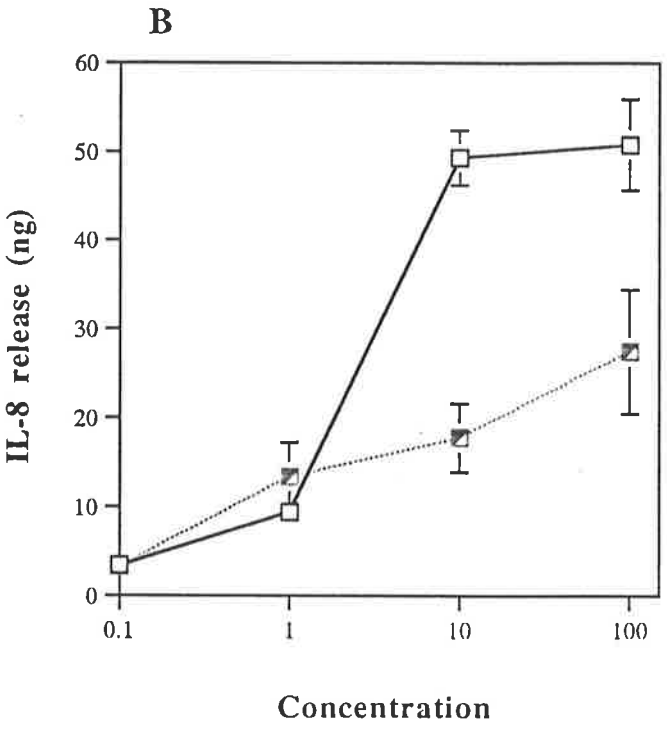

B
IL-8 release (ng)
60
50
40
30
20
10
0
0.1
1
10
100
Concentration

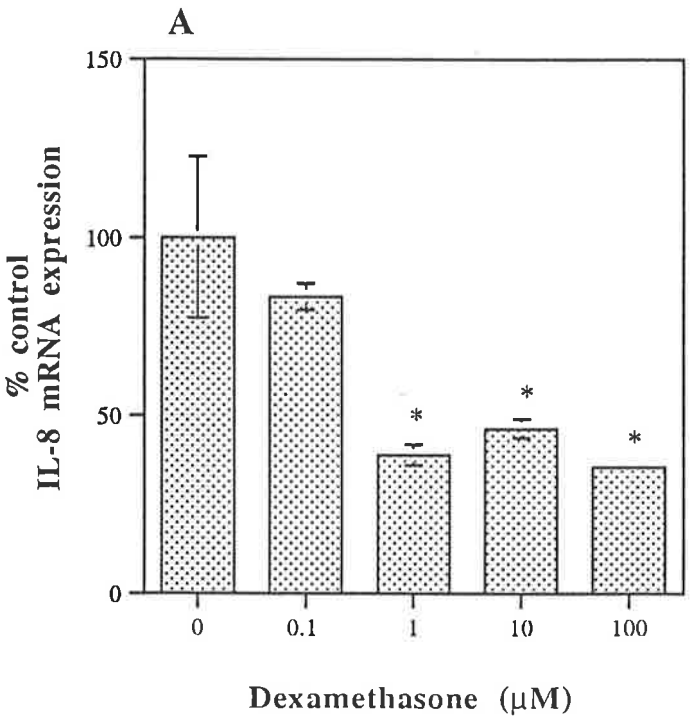
Figure 6.6.

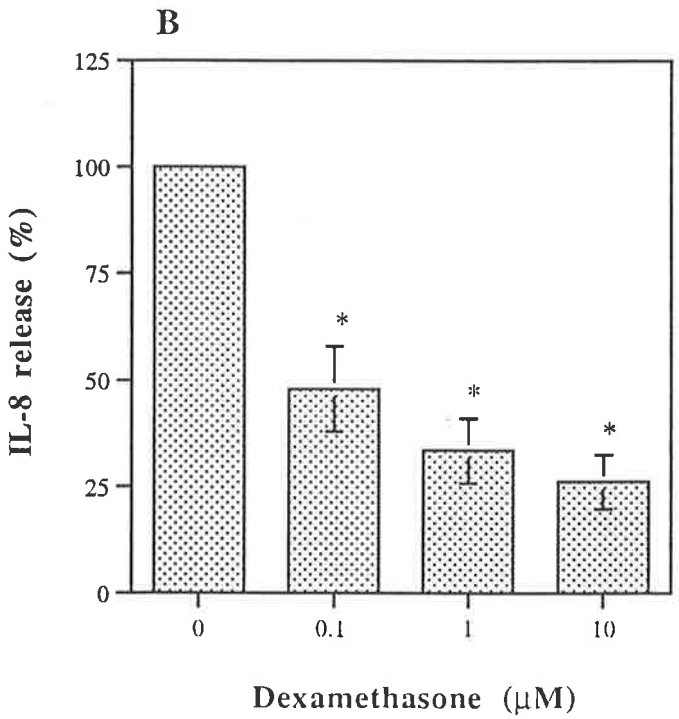
The effect of dexamethasone on cytokine induced up-regulation of IL-8 gene expression and production in RA synoviocytes.

Confluent cells were preincubated with increasing concentrations of dexamethasone for 24 hours. The cells were then incubated with 10 pM IL-1β for another 24 hours.

A. After incubation time, the cells were harvested and analysed for IL-8 mRNA and 28S ribosomal RNA, using slot blot analysis, as described under "Materials and methods".

B. The cultured supernatants were collected, and IL-8 protein was detected by ELISA as described in "Materials and methods". % control with respect to control (no dexamethasone) is shown. Control values (100%) were 12.95 to 127 ng/ml for IL-8 protein released.


A
150
100
50
0
% control
IL-8 mRNA expression
*
*
*
0
0.1
1
10
100
Dexamethasone (μM)


B
125
100
75
50
25
0
IL-8 release (%)
*
*
*
0
0.1
1
10
Dexamethasone (μM)

6.5. Discussion

There is a body of evidence documenting the accumulation of neutrophils within the synovial joint of patients with RA. The production of chemotactic factors within the joint is believed to have a major role in migration of neutrophils into the inflamed area. IL-8 is one of the major mediators of inflammation and joint destruction, and is released by activated synoviocytes in the proliferating synovium. This implies an important role for this cytokine in the pathogenesis of RA. We investigated this by examining the expression and secretion of IL-8 in cultured human synoviocytes from patients with RA. Our results confirm the IL-8 expression and production in cytokine stimulated RA synoviocytes. We have also shown that dexamethasone, regulates the expression and production of IL-8 by these cells.

The level of IL-8 expression and production was not detectable in cultured synoviocytes incubated in the absence of IL-1β or TNF-α. However exposure of these cells to exogenous pro-inflammatory cytokines, IL-1β or TNF-α resulted in enhanced level of IL-8 mRNA and protein secretion. The mechanism of IL-8 gene expression by IL-1, TNF or PMA is not completely understood. Although different factors are believed to play a role in IL-8 gene regulation by various stimulants. For example, in a TNF-α stimulated human melanoma cell line, the activation of IL-8 gene transcription was attributable to enhanced NF_kB binding (Harrant et al, 1996). Whereas in HL-60 cells, elevation of IL-8 gene expression with LPS and PMA was attributed to the increased stability of IL-8 mRNA (Kowalski and Denhardt 1989). However in our model, it is not determined whether either, enhancing binding sites for transcriptional factors or increasing the stability of IL-8 mRNA could increase IL-8 gene expression in cytokine stimulated human synoviocytes.

Furthermore, pre-treatment of human synoviocytes with dexamethasone followed by stimulation with cytokines, resulted in decreased level of IL-8 gene expression. The inhibitory effect of dexamethasone was also evident in IL-8 protein secretion.

The effect of glucocorticoids on IL-8 expression and production is well documented. We observed significant regulatory activity of dexamethasone on the production and expression of IL-8 by RA synoviocytes. Glucocorticoids have recently been shown to inhibit the induction of IL-8 by IL-1 or TNF in human fibrosarcoma cells. This was controlled mainly at the activation step of the transcription factors bound to the NF_kB-site (Mukaida et al, 1992b). Similarly, dexamethasone inhibited the IL-1β induced IL-8 secretion in human retinal pigment epithelial cells (Kurtz et al, 1997), human bone marrow stromal cells (Chaudhary and Avioli 1996) and the LPS-stimulated monocytes (Canadra and Bucala 1997). The 5'-flanking region of the IL-8 gene contains a potential binding site for glucocorticoid receptor (Oppenhiem et al, 1991) It is also suggested that glucocorticoids modulate IL-8 expression by a combination of genomic mechanisms (Brattsand and Linden 1996). Several mechanisms for the regulation of IL-8 gene expression by glucocorticoids have been suggested including inactivation of transcription factors (e.g, AP-1, NF_kB) and /or mRNA destabilization (Brattsand and Linden 1996). Further studies are needed to determine the mechanism of action for glucocorticoids in synoviocytes.

Taken together, these results demonstrate that IL-1 and TNF are important stimulators of IL-8 induction from human synovial fibroblasts. They confirm and extend studies on the effect of cytokines on IL-8 protein and mRNA regulation. In the previous studies we determined that dexamethasone was capable of reducing the level of MMPs but not TIMP-1 genes. Here we demonstrated another modulatory effect of this agent to inhibit the pro-inflammatory process by reducing the level of IL-8 secretion and production. Results in Chapter 4 showed that dexamethasone was a significant inhibitor of MMP mRNA expression. These observations suggest that the regulation of IL-8 gene expression resemble the regulation of MMP genes. Furthermore, our data

suggest that a decrease in the synthesis of IL-8 in inflamed synovium may be another mechanism by which glucocorticoids exert their anti-inflammatory actions in diseases such as RA. Dexamethasone is an important anti-inflammatory agent in the treatment of arthritis, as it is able to minimize IL-8 and MMPs secretion without affecting TIMP expression from the same population of RA synoviocytes.

Chapter 7

General discussion

The aims of the experiments described in this thesis were to determine the effects of anti-rheumatic agents on TIMP-1 oxidative inactivation and to a wider extent, on the expression of MMPs, TIMP-1 and IL-8 mRNA in the connective tissue cells of the joint, i.e. synovial cells. The findings of this study have supported and demonstrated the role of anti-rheumatic agents as well as anti-inflammatory cytokines in the joint.

The first major observation was the role of the neutrophil derived oxidant, HOCl in inactivation of TIMP-1. This suggests that neutrophils, which infiltrate inflammatory sites, may contribute to TIMP-1 inactivation by releasing their major product of oxidative burst, HOCl. HOCl released from neutrophils reacts rapidly with readily available amines and is thus unlikely to diffuse away from the immediate pericellular environment of the neutrophil. The most abundant amine in the pericellular environment of the neutrophil is taurine, thus most HOCl is converted to NCT (Weiss et al, 1989). This study has shown that NCT does not inactivate hrTIMP-1. Thus, oxidative inactivation of hrTIMP-1 is likely to occur only in the pericellular environment of the neutrophil, or in a cellular micro-environment, where HOCl is found. The differential reactivity of these oxidants may be an effective way in which the non specific effects of neutrophil derived oxidants may be contained in a biological milieu. The inactivation of protease inhibitors by oxidants leading to enhanced proteolytic activity has been suggested as a model for neutrophil mediated matrix degradation. This study enhances this model by demonstrating that the neutrophil mediated matrix degradation may also apply to TIMP-1 oxidative inactivation. To determine whether neutrophils can or cannot attack extracellular tissues by oxidative inactivation of TIMP-1 in a physiologic system, an in vitro model of an inflammatory

site needs to be constructed. In this model neutrophils can become triggered in vicinity of an insoluble matrix of connective tissue proteins in the presence of purified TIMP-1. In this condition the ability of neutrophils to generate and use HOCl to oxidize the TIMP-1 needs to be clearly determined. Further experimental attempts to elucidate these events may not only provide insights into tissue damage but also lead to the identification of important new targets of pharmacologic agents.

This study has also shown that a spectrum of anti-rheumatic agents have the ability, at sufficient concentrations, to prevent the oxidative inactivation of TIMP-1. For D-penicillamine it is evident at concentrations approaching those reported in patients taking this agent (i.e. about 100 μM). With the other agents studied however, suppression of HOCl induced inactivation of hrTIMP-1, occurs at concentrations significantly higher than plasma concentrations in patients. However, this in itself does not dismiss a potential therapeutic effect since selective cellular uptake of drugs may result in intracellular concentrations higher than plasma levels. This strategy, in common with many previous investigations into the effects of anti-rheumatic drugs, has been to take currently used agents, albeit of inconsistent efficacy, and explore their effects in a simplified model of the biological situation. An alternative strategy is to look purely at mechanisms and devise agents that are derived from a knowledge of the inflammatory process-the most notable recent example being trials with anti-TNF antibodies. Both strategies have their place and it is clear from the studies presented here and elsewhere that the currently used agents still have much to tell us about the process(es) of joint inflammation.

The second observation was the determination of a biochemical basis for the effects of a wide range of the pharmacological agents used in RA. Because the imbalance between the MMPs and TIMP is likely to play a critical role in the pathogenesis of this disease, these studies were designed to examine the effects of these therapeutic agents on the expression of MMPs and TIMP-1 on pro-inflammatory cytokine stimulated human synovial fibroblasts. To construct an in vitro situation of an inflammatory

system, human synovial cells were cultured with IL-1β. High levels of MMPs and TIMP-1 mRNA expression were observed in these cells. The use of gold containing anti-rheumatic agents showed that, GSTM but not its TM component, down-regulated collagenase gene expression in human rheumatoid synovial fibroblasts. TIMP-1 expression was not affected by GSTM or its TM salt. The ineffectiveness of TM in this system suggests that the relative inhibition of MMP-1 gene expression is mediated by gold rather than its thiomalate ligand. It has been claimed that the organic ligands were beneficial parts of the drug. However our data implies that the gold component is necessary to inhibit collagenase mRNA in an in vitro study. Organic ligands form a suitable delivery system and follow very different metabolic pathways from that of the gold. They reach the blood stream very efficiently and quickly and once there, the gold-sulphur link cleave rapidly. The thiolate parts of the drug are then almost completely eliminated via kidneys and urine and thus would appear unlikely to play a part in long term effects of the drugs. Whether gold compounds act by inhibiting transcription or by promoting transcript degradation remains to be determined. Recent studies have suggested that anti-rheumatic gold compounds may have direct inhibitory actions on protein kinase C in several types of cells (Hashimoto et al, 1992; Froscio et al, 1989). Because AP-1 is considered to be one of the nuclear signals for protein kinase C (Karin 1991), it is likely that GSTM suppresses collagenase gene expression by reducing the AP-1 activity. Further studies are clearly needed to clarify whether GSTM-sensitive protein kinase C is involved in AP-1 activation. Following these experiments therefore, it would be necessary to clarify the effects of GSTM, on AP-1 activity and to a wider extent on c-fos and c-jun gene transcription.

We have also demonstrated that GSTM partially liberated collagenase gene expression from glucocorticoid dependent repression. This is in agreement with the similar work by Makino, indicating that GSTM counteract the repressive effects of glucocorticoids on collagenase gene expression using phorbol-ester-treated human skin fibroblasts. This antiglucocorticoid action of GSTM is attributed to the interaction between GSTM, or Au(I), and GR. In any case, additional studies are needed to elucidate the molecular

nature of gold action in patients with RA.

Indomethacin, as a representative NSAID, significantly enhanced the level of MMP-1 gene expression in RA fibroblast-like synoviocytes. Whereas TIMP-1 mRNA expression was not changed. It was shown that, prostaglandins inhibit IL-1β induced MMP expression and synthesis in human synovial fibroblasts (DiBattista et al, 1994). Indomethacin is a potent inhibitor of prostaglandin production. These two facts gave an explanation to support the observation of enhancement of collagenase gene expression by indomethacin in our system. These results also demonstrate that CyA inhibits IL-1β induced MMP-1, but not TIMP-1, mRNA. However, the precise mechanism by which CyA inhibits collagenase mRNA expression needs to be resolved. Since there have also been reports of CyA interference or competition with the binding of IL-1 (Bendtzen and Dinarello 1984), therefore suppression of collagenase gene expression in our system may simply be explained by CyA inactivation of IL-1 as a result of the IL-1 receptor blockage or IL-1 receptor gene suppression on the RA synoviocytes. CyA collagenase gene repression might not be necessarily within the same DNA sequences that participate in the induction of transcription. Further studies are required to determine whether using the specific mutations within the collagenase promoter that abolish transcription activation, may or may not abolish repression by CyA. Another possibility would be, that collagenase mRNA half-life may be affected by CyA, resulting to the decreased level of collagenase mRNA in our system. The synthetic glucocorticoid, dexamethasone suppressed the expression of MMP-1, and MMP-3 significantly, but did not have any effect on TIMP-1 gene expression. Glucocorticoid hormones repress transcription of the collagenase gene by the interaction of glucocorticoid receptors with the AP-1 proteins, fos and jun (Vincenti et al, 1996). The fact that TIMP-1 gene expression was not coordinately regulated by dexamethasone in our study would suggest the presence of other promoter elements in each of these genes that may explain the differences between regulation of MMPs and TIMP-1 genes by glucocorticoids. The other reason would simply be the lack of a glucocorticoid response element (GRE) at the AP-1 site of the TIMP-1 promoter, which does not allow

the glucocorticoid receptor-hormone complex (GRh) to interact with the AP-1 and repress the transcription. The effects of tenidap were also examined on OA and RA synoviocytes. These results determined that tenidap effectively repressed the expression of MMP-1, MMP-3, and TIMP-1 genes in OA synoviocytes. This was in agreement with the study by Pelletier and his colleagues (Pelletier et al, 1996), who showed the suppressive effect of tenidap on IL-1 induced collagenase synthesis in synoviocytes. These authors suggested that this indirect effect of tenidap on MMP expression occurs via a decreased level of the IL-1 receptor. However, tenidap (100 μg/ml) suppressed MMP-1 production by RA synoviocytes but not TIMP-1 gene expression at higher concentration (10 fold higher than the concentration used for OA cells). Since PGE_2 down-regulates the expression of IL-1β induced collagenase expression (DiBattista et al, 1994), it may be possible that, this action of tenidap is mediated by the suppression of endogenous PGE_2 synthesis, as this drug is a potent inhibitor of cyclooxygenase. However, PGE_2 also down-regulates the IL-1β induced TIMP-1 mRNA expression (DiBattista et al, 1995), thus it is unlikely that this would be the sole explanation.

Methotrexate did not alter collagenase or TIMP-1 mRNA levels in IL-1 stimulated RA synoviocytes, whereas probably it has an indirect effect due to an alteration in the synovial cytokine milieu. This indicates that methotrexate does not directly decrease collagenase or TIMP-1 production. Methotrexate therapy was shown to decrease the activity of proteolytic enzymes in articular tissue. This effect may be related to the inhibition of IL-1 activity and the decreased migration of polymorphonuclear cells to inflamed joints. Although the positive effect of methotrexate on cartilage lesions in animal models may support this mode of action, it is interesting that no clinical study has demonstrated that methotrexate inhibits the development of joint erosions (Bannwarth et al, 1994). Several animal models as well as in vitro observations have indicated the direct interference of methotrexate with the activity of pro-inflammatory cytokines such as IL-1 (Segal et al, 1989; 1990). Clearly further studies are needed to elucidate the mechanism(s) by which these agents act in RA. In addition to the

regulatory effects of these widely used anti-rheumatic agents, the synthesis of the metalloproteinases and their inhibitors can also be regulated by polypeptide growth factors and cytokines.

The third observation was the role of TGF-β in extracellular matrix turnover by selective modulation of the expression and secretion of MMP-1, TIMP-1 and PGE_2. TGF-β was capable of reducing the collagenase mRNA level at the same time stimulating the expression of TIMP-1 mRNA in cultured human synovial fibroblasts. Furthermore, TGF-β induced a significant increase in the TIMP-1 mRNA expression when the cells were simultaneously exposed to IL-1β or TNF-α in combination with TGF-β. There was also evidence that TGF-β was able to reduce the collagenase mRNA secretion in conjunction with IL-1β or TNF-α. These opposing effects of TGF-β on the expression of the enzyme and inhibitor, effectively leads to inhibition of extracellular matrix breakdown. Furthermore, TGF-β significantly inhibited the enhanced secretion of PGE_2 by cytokine stimulated synovial cells. These results showed a similarity in the effects of TGF-β in combination with IL-1β and TNF-α, on PGE_2 production and collagenase synthesis. In both cases, TGF-β was capable of inhibiting the stimulatory effects of IL-1β and TNF-α. This suggest that a particular step(s) in the biochemical pathway of synthesis is induced by TGF-β. Although, these beneficial effects of TGF-β might occur in two separate pathways in parallel. Our results indicate that the human synovial fibroblast response to cytokines and growth factors is complex and is dependent on local cytokine concentrations, as well as their antagonising interactions. Since TGF-β both inhibits MMP-1 whilst stimulating TIMP-1 secretion by synoviocytes, it is a powerful means of shifting the MMP/TIMP-1 ratio away from matrix degradation. These effects of TGF-β are present at the level of mRNA expression, thus suggesting the involvement of pretranslational mechanisms in these effects. The capacity of TGF-β to antagonise the immunoregulatory effects of several pro-inflammatory cytokines suggests that this growth factor may be valuable in developing new therapeutic agents, for the treatment of inflammatory diseases. Further studies are also needed to resolve the possible use of this growth factor in the therapy of

joint disease such as RA and OA, where tissue destruction events present a great problem.

Studies of IL-8 production by human synovial cells are also consistent with an important role of this cytokine in the pathogenesis of RA. The production of chemotactic factors within the joint by cells that are native to the joint (i.e. synovial cells) underlines the importance of these cells in the early phase of the inflammatory response within the joint. IL-8 is one of the major mediators of inflammation and joint destruction, released by activated synoviocytes in the proliferating synovium. We examined the expression and secretion of IL-8 in cultured human synoviocytes from patients with RA. These results support the presence and role of IL-8 in response to pro-inflammatory cytokines in joint cells. The role of the commonly used glucocorticoid, dexamethasone, in regulating the expression and production of IL-8 by these cells was also studied.

The mechanism of regulation of IL-8 gene expression by IL-1 or TNF is not completely understood. Different factors are believed to play a role in IL-8 gene regulation by various stimulants. For example, in a TNF-α stimulated human melanoma cell line, the activation of IL-8 gene transcription was attributed to the enhanced NF_kB binding (Harrant et al, 1996). Whereas in HL-60 cells, elevation of IL-8 gene expression with LPS and PMA was attributed to the increased stability of IL-8 mRNA (Kowalski and Denhardt 1989). However in our synoviocyte culture system, further studies are needed to determine whether either, enhancing binding sites for transcriptional factors or increasing the stability of IL-8 mRNA could increase IL-8 gene expression in cytokine stimulated human synoviocytes.

Furthermore, pre-treatment of human synoviocytes with dexamethasone followed by stimulation with cytokines, resulted in a decreased level of IL-8 gene expression and protein secretion. The effect of glucocorticoids on IL-8 expression and production is well documented. We observed significant regulatory activity of dexamethasone on the production and expression of IL-8 by RA synoviocytes. Glucocorticoids have recently

been shown to inhibit the induction of IL-8 by IL-1 or TNF in human fibrosarcoma cells. This was controlled mainly at the activation step of the transcription factors bound to the NF$_k$B-site (Mukaida et al, 1992b). It is suggested that, glucocorticoids modulate IL-8 expression by a combination of genomic mechanisms (Brattsand and Linden 1996). Several mechanisms for the regulation of IL-8 gene expression by glucocorticoids have been suggested including inactivation of transcription factors (e.g, AP-1, NF$_k$B) and /or mRNA destabilization (Brattsand and Linden 1996). Further studies are needed to determine the mechanism of action for glucocorticoids in synoviocytes.

Taken together, our results confirm and extend studies on the effect of cytokines on IL-8 regulation. In the previous studies we determined that dexamethasone was capable of reducing the level of MMPs but not TIMP-1 genes. Here we demonstrated another modulatory effect of this agent to inhibit the pro-inflammatory process by reducing the level of IL-8 secretion and production. These observations suggest that the regulation of IL-8 gene expression is likely to resemble the regulation of MMP genes. This might be simply attributed to the similar binding site regions for the transcription factors (e.g. AP-1, NF-kB) on the promoter region of these genes. Furthermore, our data suggest that the decrease in IL-8 expression and secretion by dexamethasone may reduce the recruitment of leukocytes to rheumatoid synovium and thus may contribute to the anti-inflammatory effects of this compound. Dexamethasone is likely to be an important anti-inflammatory agent in the pathology of arthritis, as it is able to minimize IL-8 and MMPs secretion without affecting TIMP from the same population of RA synoviocytes.

In summary MMPs, TIMP and IL-8 have a premier role in the inflammation and irreversible degradation of the ECM. Therefore considerable attention has been paid for developing strategies to reduce their levels in diseased joints. Several studies directed attention on synthesis of these factors. These preclinical studies have been carried out in cell-free and/or cell culture systems and in animal models. However there have been no significant successes in the clinical area. The reasons for this are

several. The complicated structure of collagen along with the biochemistry of inflamed synovial tissue are only a part of these difficulties. Some of the technical difficulties involve include, designing inhibitors of these factors and delivering them to the affected joints without reducing their specificity and efficacy. The lack of fundamental knowledge about the biochemistry and molecular biology of these factors are more important problems. Understanding of mechanisms controlling the regulation of both MMP and TIMP genes as well as IL-8 genes gives us the ability to know how to turn these genes on and off, and hopefully, to moderate disease progression. Indeed, although these studies are still not at clinical level these possible approaches may become a reality in the future. By increasing the knowledge of the basic mechanisms underlying the therapeutic pathways, we hope further studies will help to open the areas to formulate agents that are targeted at specific molecular mechanisms and become effective in vivo. Successful inhibition of MMPs and IL-8 which is the focus of our studies, are surely among them.

Appendix

Publication

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The Oxidative Inactivation of Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) by Hypochlorous Acid (HOCl) is Suppressed by Anti-Rheumatic Drugs

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Tissue inhibitors of metalloproteinases (TIMPs) prevent uncontrolled connective tissue destruction by limiting the activity of matrix metalloproteinases (MMPs). That TIMPs should be susceptible to oxidative inactivation is suggested by their complex tertiary structure which is dependent upon 6 disulphide bonds. We examined the oxidative inactivation of human recombinant TIMP-1 (hr TIMP-1) by HOCl and the inhibition of this process by anti-rheumatic agents.

TIMP-1 was exposed to HOCl in the presence of a variety of disease modifying anti-rheumatic drugs. TIMP-1 activity was measured by its ability to inhibit BC1 collagenase activity as measured by a fluorimetric assay using the synthetic peptide substrate (DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg), best cleaved by MMP-1.

The neutrophil derived oxidant HOCl, but not the derived oxidant N-chlorotaurine, can inactivate TIMP-1 at concentrations achieved at sites of inflammation. Anti-rheumatic drugs have the ability to protect hrTIMP-1 from inactivation by HOCl. For D-penicillamine, this effect occurs at plasma levels achieved with patients taking the drug but for other anti-rheumatic drugs tested this occurs at relatively high concentrations that are unlikely to be achieved in vivo, except possibly in a microenvironment. These results are in keeping with the concept that biologically

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derived oxidants can potentiate tissue damage by inactivating key but susceptible protein inhibitors such as TIMP-1 which form the major local defence against MMP induced tissue breakdown.

Keywords: Tissue inhibitor of metalloproteinases (TIMP), oxidants, anti-rheumatic drugs and hypochlorous acid

INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of proteolytic enzymes that are capable of degrading the macromolecules of the extracellular matrix. They represent an important mechanism by which the turnover of structural proteins such as collagen is regulated.[1] MMP-1 can cleave native collagen molecules and allow the resultant denatured collagen to be digested by a range of other enzymes including other MMPs. In this way MMP-1 regulates fibrillar collagen turnover.[2,3]

Tissue inhibitors of metalloproteinases (TIMPs) form a family of proteins and are the major local inhibitors of MMPs with activity against all known MMPs.[4] TIMPs regulate the activity of MMPs by tightly binding to the active site and forming inactive complexes with a 1:1 stoichiometry.[5] The balance between the activities of MMPs and TIMPs is a crucial factor in regulating extracellular matrix breakdown in vivo.[6] Thus far, four members of the TIMP family have been identified, namly TIMP-1, TIMP-2, TIMP-3 & TIMP-4. TIMP-1 is a 30 KDa glycoprotein found in most body fluids. TIMP-2 is an unglycosylated 21 KDa protein. These two proteins have 40% amino acid sequence homology. Average concentrations of MMPs and TIMP fragments are significantly elevated in the joint fluid of patients with osteoarthritis (OA) as compared with volunteers with healthy knees.[7] Structural studies of TIMP-1 suggest a highly conserved secondary structure. There are 12 cysteine residues which form six conserved disulfide bonds, giving a protein structure of six loops and two domains. TIMP activity is dependent upon this elaborate tertiary struc-

ture.[8] The high density of disulfide bonds and relatively complicated tertiary structure, which is a functional requirement, would suggest that this proteinase inhibitor is susceptible to oxidants.[6]

HOCl is a major product of the oxidative burst of neutrophils.[9] There is accumulation of polymorphonuclear leukocytes (PMN) in the synovial fluid of patients with rheumatoid arthritis (RA) which, after stimulation, may release inflammatory mediators.[10] The oxidative burst of neutrophils generates superoxide anion ($O_2^{\cdot-}$), which is rapidly dismutated to form hydrogen peroxide (H_2O_2). The neutrophil enzyme, myeloperoxidase, catalyzes the reaction of H_2O_2 with chloride ions to form the highly reactive and cytotoxic agent hypochlorous acid (HOCl).[11] In aqueous solution HOCl exists in essentially equal concentration as the conjugate base hypochlorite (OCl^-) and together form a powerful biologically relevant oxidizing agent capable of causing damage to proteins.[12]

HOCl will react with amino acids such as taurine to generate a derivative group of oxidants known as the chloramines.[13] Generally, chloramines are less powerful oxidants than HOCl itself.[9] Taurine, whose biological role remains unclear, is found at concentrations approaching 20 mM in neutrophils and macrophages.[14] N-Chlorotaurine has been reported to be essentially unreactive with major cellular components. It has been suggested that taurine may act as a "sink" for the oxidizing potential of HOCl/OCl^-, generating a chlorinated species of lower reactivity and thereby minimizing indiscriminate damage.[14] Since taurine is the most abundant endogenous amine in the pericellular environment of the neutrophil,[14] we have used N-chlorotaurine as an example of chloramines in our model. In many cases oxidation appears to result in a partial unfolding or rearrangement of target proteins.[15,16]

Previous reports have shown that latent neutrophil collagenase (MMP-8) can be activated by neutrophil derived oxidants, principally HOCl.[9,11,12] This has highlighted the way that

oxidant generation and enzymatic mechanisms can interact to lead to tissue breakdown. Direct oxidative inactivation of enzyme inhibitors is a more direct mechanism of interaction between oxidant generation and the equilibrium between proteases and their inhibitors. The most well known instance, where this occurs, is the oxidative inactivation of plasma proteinase inhibitors, such as α_1-proteinase inhibitor (α_1-PI), where oxidation of the methionine at the active site of α_1-PI results in loss of affinity of this inhibitor for its target enzyme.[17] In another study by Frears *et al.* the inactivation of TIMP-1 by peroxynitrite ($ONOO^-$) has been demonstrated.[18] We have reasoned that since hrTIMP-1 has an elaborate 6 loop structure, each loop maintained by interchain disulfide bonds, that oxidative cleavage of these disulfide bonds would result in major structural disintegration and consequent loss of affinity for matrix metalloproteinases.

Thiol-containing antirheumatic drugs, such as D-penicillamine, sodium aurothiomalate, and aurothioglucose, are widely used in treatment of RA.[19] Some studies have investigated the effects of antirheumatic drugs, including thiol-containing drugs, on the oxidative activity of myeloperoxidase.[20,21] It has been shown that D-penicillamine effectively scavenges HOCl formed by myeloperoxidase and inhibits the enzyme itself.[17]

In this study, we have examined the possibility that neutrophil derived oxidants may inactivate hrTIMP-1 and thus allow MMPs to be unfettered in areas of HOCl generation, as for example the pericellular environment of the activated neutrophil. We have also examined the hypothesis that anti-rheumatic drugs, particularly those shown to act as anti-oxidants, may protect hrTIMP-1 from oxidative inactivation.[20]

MATERIALS AND METHODS

Materials

D-Penicillamine, gold sodium thioglucose (aurothioglucose), chloroquine, taurine and Tris (hydroxymethyl aminomethane) [Tris], were purchased from the Sigma Chemical Company, St. Louis, MO., USA. Gold sodium thiomalate (aurothiomalate) was from Aldrich, U.K. Reagent grade chemicals were purchased from BDH Chemicals Australia Pty. Ltd., Kilsyth, Victoria, Australia. Media were from Cytosystems Pty Ltd, Castle Hill, NSW, Australia. Auranofin was from Smith, Klein and French (Pty Ltd). Methotrexate was from David Bull Laboratories, Melbourne, Australia. Aurothiomalate and aurothioglucose were stored desiccated as powder at –20°C. D-penicillamine, methotrexate, chloroquine and auranofin were stored desiccated at 4°C.

Human recombinant TIMP-1 (hrTIMP-1) was a gift from Dr. A. Docherty (Celltech Research Slough, Berkshire, U.K). The rat mammary carcinoma cell line BC-1 was a gift from Dr. J. O'Grady, University of Technology, Sydney, Australia.[23]

N-chlorotaurine was made by the addition of a solution of NaOCl to taurine buffer (50 mM Taurine, 10 mM $CaCl_2$, 100 mM NaCl, pH 7.5) according to the method of Weiss *et al.*[12]

BC-1 Collagenase

Serum-free medium (HAMS F-12.10.65g/2L, DMEM 18.42g/2L, $NaHCO_3$ 2g/2L, Pen/Strep 5U/ml, insulin 5µg/ml, transferrin 1mg/ml, L-glutamine 0.292mg/ml, BSA 5mg/ml), was conditioned by BC-1 cells to produce MMP-1 (serum-free-BC-1). These cells have the ability to produce large quantities of matrix metalloproteinases, particularly MMP-1. The enzyme, BC-1 collagenase was purified by passing the conditioned media through Heparin-Sepharose affinity column, and was eluted from the column using an eluting buffer consisting of Tris-Cl 50 mM, NaCl 800 mM, $CaCl_2$ 10 mM, 0.2% NaN_3.[23] Column eluates were assayed for protein by using serial concentrations of the bovine serum albumin (BSA) as standard curve and measuring the absorbance at 280 nm.

Activation of BC-1 Collagenase

Enzyme BC-1 collagenase was incubated at 35°C for 15 mins with trypsin at a final concentration of 2.5 μg/ml in the assay buffer (Tris 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM, NaN_3 0.02%, pH 7.5). The reaction was terminated using 12.5 μg/ml SBTI. A time course for this assay of collagenase activity showed that the maximum activity was achieved between 5–15 mins incubation of the collagenase with trypsin.

Fluorimetric Assay of Collagenase Activity

This assay was performed according to the method of Netzel-Arnett *et al.*[24] The assay relies on cleavage of the substrate, DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg at the Ala-Leu bond, thus releasing Trp fluorescence from quenching by the DNP group at the N-terminal region of the heptapeptide substrate. The substrate used, is best cleaved by MMP-1. The heptapeptide substrate was synthesized using the solid phase method with Nα-Fmoc-aminoacids. The synthetic peptide was coupled with dinitrofluorobenzene prior to cleavage from the resin. It was purified by HPLC using a Waters C18 Novapak column and subsequently recovered by lyophilization. The heptapeptide was solubilized in 4% DMSO.

Samples, after dialysis against the appropriate buffer, were activated and then equilibrated at 35°C for 30 seconds in a 5 mm path length quartz cuvette (Starna type 3–5) and the assay started with the addition of the heptapeptide substrate to give a final concentration of 12 μM. The reaction was monitored over time at 35°C in a Perkin-Elmer LS-5 fluorimeter (excitation l = 280 nm, slit width: 10 nm; emission l = 360 nm, slit width: 10 nm). Collagenolytic activity was calculated from the initial linear slope of the curve being expressed as the rate of increase in fluorescence at 360 nm per second.

Collagenase Inhibition Assay

The inhibitory capacity of hrTIMP-1 was determined using the same spectrophotometric assay as above. In this assay, trypsin activated enzyme was used as the indicator of the hrTIMP-1 inhibitory activity. To establish the dose curve with hrTIMP-1, different concentrations of the hrTIMP-1 between 1 to 50 nM was applied to the active enzyme to make the inactive complex in 30 seconds. Synthetic peptide, 12 μM, was then added to show the % inhibition of the enzyme with the inhibitor. Initially concentrations of HOCl between 0.1 and 200 μM were incubated with hrTIMP-1 at 35°C for 1 hour. A time course for this inhibitory assay showed that maximum inhibition was achieved after 30–60 mins pre-incubation of TIMP-1 with HOCl. Subsequently reactants were added to the cuvette in the following sequence: assay buffer, oxidant-exposed hrTIMP-1, enzyme (BC1 collagenase), substrate (synthetic peptide).

Results were expressed as change in fluorescent intensity per unit time. For experimental samples this was expressed as a percent of control, which was the reaction of activated BC1 collagenase and substrate alone.

Drugs

For experiments involving anti-rheumatic agents, hrTIMP-1 was pre-treated with a range of anti-rheumatic drugs prior to the addition of HOCl. The sequence of addition was as follows: assay buffer, drug, hrTIMP-1, oxidant, BC1 collagenase, substrate. Fresh solutions of drugs were prepared on a daily basis.

Statistical Analysis

Each experiment was repeated three times. All results were expressed as the mean ± standard error of the mean (SEM).

RESULTS

Inhibition of BC-1 Collagenase by Human Recombinant TIMP-1

BC-1 collagenase was activated by trypsin and the reaction was stopped using SBTI. This activated enzyme was used as the control in the following fluorimetric assays. As shown in Figure 1, hrTIMP-1 was able to inactivate BC-1 collagenase that had been activated by pre-exposure to trypsin. The inhibition was investigated for a maximum hrTIMP-1 concentration of 35 nM, at which the BC1 collagenase activity was reduced to 10% of the control. There was a sigmoidal relationship between the concentration of inhibitor and enzyme activity.

Oxidative Inactivation of TIMP-1

We examined the loss of activity of hrTIMP-1 to inhibit BC1 collagenase, after pre-exposure to HOCl. TIMP-1 was diluted in PBS to give the final concentration of 1.1 μM. Figure 2A shows the inactivation of TIMP-1 after pre-exposure to HOCl between concentrations of 100–200 μM. 100% control TIMP-1 activity is when no HOCl was applied. 50% inactivation was achieved using 175 μM HOCl, while 200 μM HOCl caused complete inactivation of the inhibitor. This represents an oxidant to inhibitor molar ratio of 200:1. In order to show that this was not due to a direct effect of HOCl on BC1 collagenase, HOCl was exposed to BC1 collagenase at the final concentration achieved in this experiment (ie. 200 μM) and no change in collagenase activity was observed (data not shown). When N-chlorotaurine (NCT) was used as an oxidant in concentrations of NCT between (0.1–10 mM) no inactivation of hrTIMP-1 was seen.

A time course of TIMP-1 oxidative inactivation is shown in Figure 2B. TIMP-1 was incubated with HOCl (200 μM) at 37°C for the indicated time points. TIMP-1 oxidative inactivation started after 15 minutes incubation with HOCl reaching to a maximum after 60 minutes.

Effects of Anti-rheumatic Drugs on BC1 Collagenase Activity

Figure 3 shows the direct effect of several anti-rheumatic agents on BC1 collagenase activity. Auranofin has no inhibitory effect up to a concentration of 500 μM, whereas chloroquine, aurothiomalate and aurothioglucose give moderate inhibition at 500 μM but not 100 μM. Methotrexate and D-penicillamine have significant effects at 100 μM. Subsequent experiments to examine oxidative inactivation of TIMP-1 were therefore designed such that BC1 collagenase used in the collagenase inhibition assay was not exposed to concentrations of each drug greater than 30 μM, where there is no significant effect of the drug on BC1 collagenase activity.

Effects of Anti-rheumatic Drugs on the Oxidative Inactivition of the TIMP-1

Figure 4 shows the results of experiments where hrTIMP-1 is pre-incubated with a variety of anti-rheumatic drugs prior to HOCl treatment. At 0% TIMP-1 loses its ability to inhibit collagenase when treated with HOCl (200 μM). At 100% TIMP-1 activity, no oxidative inactivation of TIMP-1 is evident as the result of TIMP-1 pre-treatment with some drugs. The first stage of this experiment involves exposure of TIMP-1 to oxidant in the presence of drug to determine whether the drug can protect TIMP-1 from oxidative inactivation. In the second stage of the experiment, TIMP-1, in combination with the drug and HOCl, is exposed to BC1 collagenase to determine TIMP-1 activity. At this stage, because the samples are diluted, BC1 collagenase is not exposured to drug concentrations over 30 μM.

D-penicillamine shows the greatest ability to protect hrTIMP-1 from oxidative inactivation by HOCl, this effect being evident at a concentration of 10 μM. Of the other drugs tested, auranofin, chloroquine and aurothiomalate had the ability to protect TIMP-1 from oxidative inactivation at a concentration of 100 μM. All drugs were partially

effective at the 500 μM concentration, although this is about 20 fold higher than the serum levels acheived during treatment with these agents.

DISCUSSION

In the present study we have shown that HOCl can directly inactivate TIMP-1 in vitro. TIMP-1 was shown to inhibit the cleavage of a synthetic peptide by an active mammalian MMP(BC1 collagenase). TIMP-1 loses this ability after being pretreated by HOCl. Since HOCl is a product of activated neutrophils, this suggests that neutrophils, which infiltrate inflammatory sites, may contribute to TIMP-1 inactivation. Quantitative analyses have demonstrated that 10^6 maximally triggered neutrophils produce approximately 2×10^{-7} mol of HOCl during a two-hour incubation.[25] The quantity of oxidants generated by the neutrophil are impressive.

The dependence of the inhibitory activity of TIMP-1 on this 6-loop structure is underlined by its inactivation by reducing and alkylating agents such as dithiothreitol and indoacetamide.[26] Another agent, known to modify the stability of the inhibitor by altering its tertiary structure is guanidine hydrochloride (GdnHCl), resulting in structural disintegration of TIMP-1.[27] The structure of TIMP-1 has also been modified using diethylpyrocarbonate (DEPC), which is a potent inactivator of human TIMP-1. Exposure to DEPC specifically modifies histidine residues, resulting in loss of human TIMP-1 activity.[28]

We demonstrated that pre-exposure of hrTIMP-1 to HOCl resulted in loss of its capacity to inhibit activated collagenase. Our results are in agreement with the work already has been done by Stricklin and Hoidal, indicating TIMP degradation with hypochlorite ion.[29] HOCl released from neutrophils rapidly reacts with readily available amines and is thus unable to diffuse away from the immediate pericellular environment of the neutrophil. The most abundant amine in the pericellular environment of the neu-

trophil is taurine, thus most HOCl is converted to NCT.[9,11–13] We have shown that NCT does not inactivate hrTIMP-1. Thus, oxidative inactivation of hrTIMP-1 is likely to occur only in the pericellular environment of the neutrophil, or in a cellular micro environment, where HOCl is found.

We have also shown that a spectrum of anti-rheumatic agents has the ability, at sufficient concentrations, to prevent the oxidative inactivation of TIMP-1. In several this occurs at concentrations above serum concentration, however this effect may be relevant in an extracellular microenvironment. For D-penicillamine it is evident at concentrations approaching those reported in patients taking this agent 20 μg/ml (100 μM).[30,31] With the other agents studied however, suppression of HOCl induced inactivation of hrTIMP-1 occurs at concentrations significantly higher than plasma concentrations in patients. However, this in itself does not dismiss a potential therapeutic effect since selective cellular uptake of drugs may result in intracellular concentrations higher than plasma levels. For example, in vivo uptake of gold occurs over days or weeks,[32,33] and the final distribution of gold in cells may be very different from simple serum levels. In a study by Wasil and his group, where HOCl inactivated alpha 1-antiprotease, most anti-inflammatory drugs were capable of reacting with HOCl. They have also suggested that, the anti-inflammatory effects of D-penicillamine and gold sodium thiomalate might be the result of rapid scavenging of HOCl by these drugs.[34]

The inactivation of protease inhibitors by oxidants leading to enhanced proteolytic activity has been suggested as a model for neutrophil mediated matrix degradation.[9] Our study enhances this model by demonstrating that it may also apply to TIMP-1. Furthermore we showed that therapeutic agents may interfere with this balance between oxidants and enzyme inhibitors.

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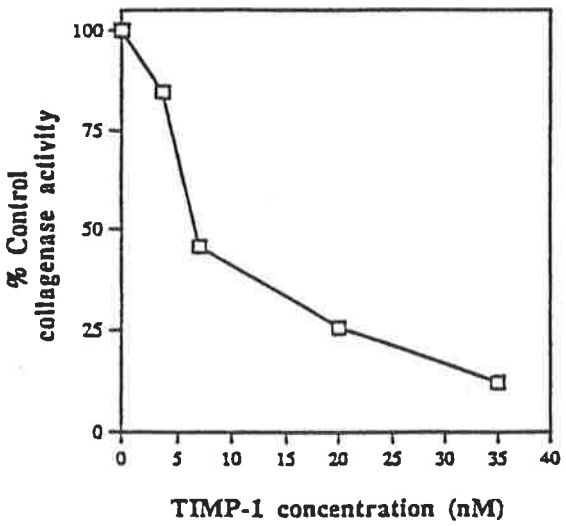

FIGURE 1 Inhibition of trypsin activated BC1 collagenase by TIMP-1. Serial concentrations of TIMP-1 applied to the active collagenase at 37°C. A synthetic peptide was used as the substrate to determine the level of the BC1-collagenase activity. 100% control collagenase activity was the level of BC1-collagenase activity when this enzyme was not treated with any concentration of TIMP-1. 50% inhibition of the BC1-collagenase activity was achieved using 7 nM TIMP-1. Values represent mean ± SEM for three separate experiments expressed as % control collagenase activity. Where SEM is not shown, it is less than or equal to the size of the markers.

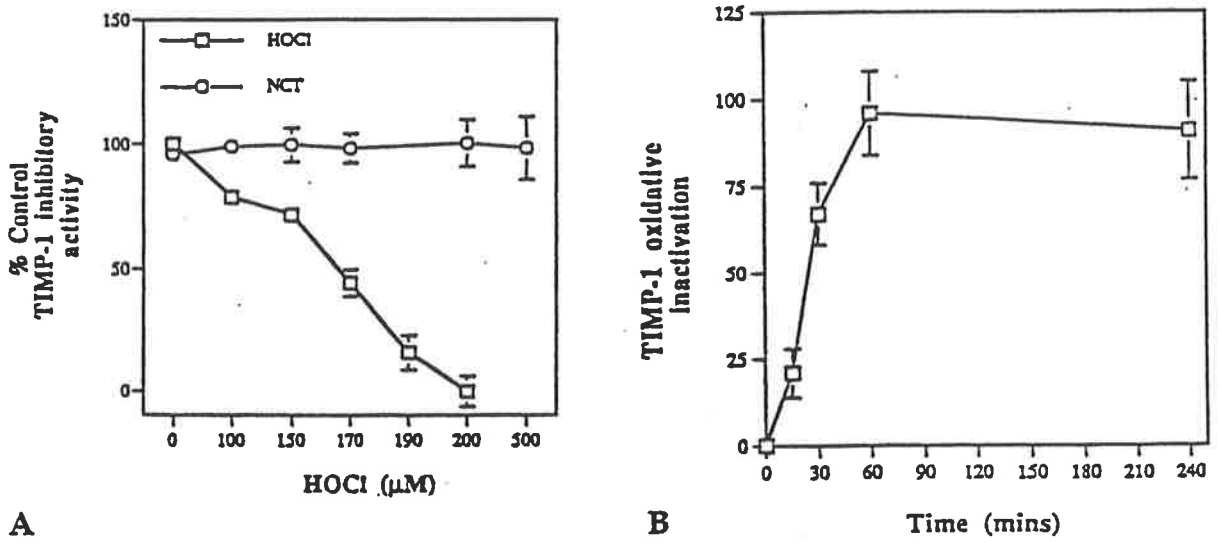

FIGURE 2 Oxidative inactivation of human recombinant TIMP-1 by HOCl. Figure 2A shows, TIMP-1 inactivation with HOCl but not NCT is regulated in a dose dependent manner. TIMP-1 was incubated with increasing concentrations of HOCl (100–200 μM) or NCT (100–500 μM) at 37°C for 1 hour. 100% control TIMP-1 inhibitory activity was the level of TIMP-1 activity when this inhibitor was not incubated with any oxidant. 175 μM HOCl caused 50% inactivation of TIMP-1, whereas no inactivation of TIMP-1 was achieved using NCT up to 500 μM. Using HOCl 200 μM completely abolished the inhibitory activity of TIMP-1. Values represent means ± SEM for three separate experiments expressed as % control TIMP-1 inhibitory activity. TIMP-1 oxidative inactivation was also time dependently regulated. TIMP-1 was incubated with HOCl (200 μM) at 37°C for the indicated time points. According to figure 2B, the oxidative inactivation of TIMP-1 was started after 15 minutes. Maximum activation was acheived after 60 minutes incubation. Values represent means ± SEM for three separate experiments expressed as % control TIMP-1 oxidative inactivation.

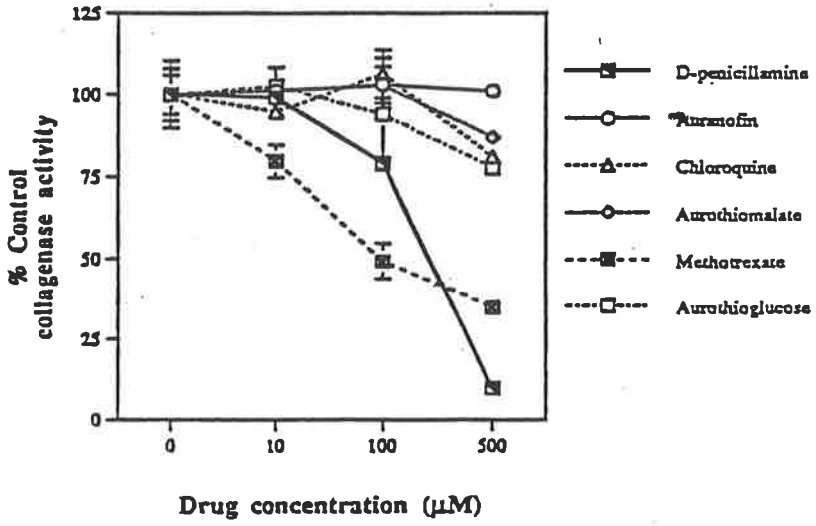

FIGURE 3 Direct effect of several anti-rheumatic agents on BC1-collagenase activity. Trypsin-activated BC1-collagenase was incubated with serial concentrations of different anti-rheumatic drugs including Aurothioglucose (10–500 μM), Aurothiomalate (10–500 μM), Auranofin (10–500 μM), Chloroquine (10–500 μM), D-penicillamine (10–500 μM), and Methotrexate (10–500 μM) for 1 hour at 35°C. 100% control collagenase activity indicates the level of BC1-collagenase activity, when this enzyme was not treated with any drug. Values represent means ± SEM for three separate experiments expressed as % control collagenase activity.

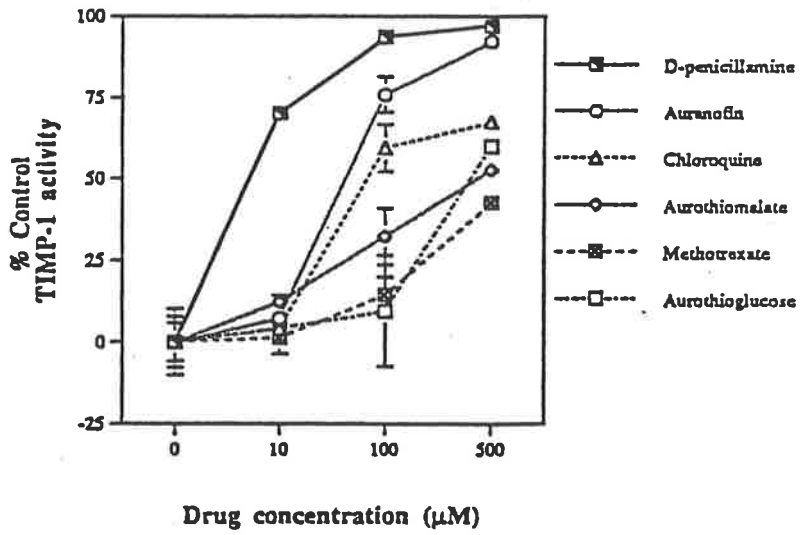

FIGURE 4 Activity of hrTIMP-1 in the prescence of drugs and oxidant. Human recombinant TIMP-1 was pretreated with a variety of anti-rheumatic drugs followed by exposing to HOCl for 1 hour at 35°C. At 0% control TIMP-1 activity, where this inhibitor was not pretreated with drugs, the hrTIMP-1 inhibitory effect against collagenase was completely suppressed by HOCl. Whereas, at 100% control TIMP-1 activity, the oxidative inactivation of hrTIMP-1 was completely abolished by anti-rheumatic drugs. Values represent means ± SEM for three separate experiments expressed as % control TIMP-1 activity.

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