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EXPRESSION AND REGULATION OF MMP-1 AND MMP-3 IN HUMAN GINGIVAL FIBROBLASTS

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To my mother Kristina and to
the memory of my father Per

ABSTRACT

Matrix metalloproteinases (MMPs) are a group of proteolytic enzymes collectively responsible for the metabolism of extra cellular matrix components. Disturbance of the physiological balance between MMPs and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), is implicated in several inflammatory diseases characterized by tissue degradation. Enhanced expression of MMPs including MMP-1 and MMP-3 is demonstrated in gingival connective tissue of individuals with periodontal diseases, but the underlying mechanisms are not well understood.

The general aim of this thesis was to investigate the expression and regulation of MMP-1 and MMP-3 as well as their inhibitor TIMP-1 in human gingival fibroblasts in response to cell interactions with monocytes or to inflammatory mediators.

The effect of cell interactions between gingival fibroblasts and monocytes on the expression of MMP-1 and TIMP-1 was studied using a co-culture model, where the cells were cultured either in direct cell-to-cell contact or separated by a membrane to prevent cell contact. Cell interactions of fibroblasts and monocytes stimulated the expression of MMP-1 and TIMP-1 in the fibroblasts as well as in the co-culture medium. The stimulatory effect of cell interactions of fibroblasts and monocytes on the expression of MMP-1 was partly dependent on cell-to-cell contact as well as on the number of fibroblast and monocytes in the co-cultures. The increased expression of MMP-1 in the fibroblasts was mediated partly by the adhesion molecule inter-cellular adhesion molecule-1 (ICAM-1) and the signal pathway p38 mitogen activated protein kinase (p38 MAPK). Furthermore, the expression of MMP-1 was reduced in the presence of dexamethasone and doxycycline.

The effect of cytokines on the expression of MMP-1 and MMP-3 was investigated by culturing the gingival fibroblasts in the absence or presence of interleukin-1β (IL-1β), tumor necrosis factor α (TNFα) or epidermal growth factor (EGF). These cytokines stimulated the expression of MMP-1 as well as MMP-3 in a time-dependent manner. Furthermore, the stimulatory effect of IL-1β and TNFα on MMP-1 and MMP-3 expression was mediated partly by the signal transduction pathways tyrosine kinase and p38 MAPK.

The expression of MMP-1 and TIMP-1 in gingival fibroblasts was also stimulated by the protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA). The PMA stimulated MMP-1 expression was synergistically upregulated by IL-1β as well as by the Ca^{2+}-ionophore A23187. However, the expression of TIMP-1 was not upregulated by the cytokine IL-1β and reduced by the Ca^{2+}-ionophore.

In summary, this thesis demonstrates that cell interactions between gingival fibroblasts and monocytes result in enhanced expression of MMP-1 and TIMP-1 in human gingival fibroblasts and that ICAM-1 and p38 MAPK are involved in the stimulated MMP-1 expression. Expression of MMP-1 and MMP-3 in gingival fibroblasts is also stimulated by the cytokines IL-1β, TNFα and EGF and partly regulated by p38 MAPK, tyrosine kinase and PKC. The stimulatory effect of monocytes and inflammatory mediators on the expression of MMP-1 and MMP-3 in gingival fibroblasts may contribute to tissue degradation in periodontal diseases.

Keywords: matrix metalloproteinases, tissue inhibitor of metalloproteinases, gingival fibroblasts, monocytes, periodontal diseases

LIST OF PUBLICATIONS

This thesis is based on the following articles, which are referred to in the text with their roman numbers (I-IV).

I. Helena Domeij, Tülay Yucel-Lindberg, Thomas Modéer. Cell interactions between human gingival fibroblasts and monocytes stimulate the production of matrix metalloproteinase-1 in gingival fibroblasts. *J Periodontal Res*. In press.

II. Helena Domeij, Thomas Modéer, Hernán Concha Quezada, Tülay Yucel-Lindberg. Cell expression of MMP-1 and TIMP-1 in co-cultures of human gingival fibroblasts and monocytes; the involvement of ICAM-1.
Biochem Biophys Res Commun. In press.

III. Helena Domeij, Tülay Yucel-Lindberg, Thomas Modéer. Signal pathways involved in the production of MMP-1 and MMP-3 in human gingival fibroblasts. *Eur J Oral Sci* 2002; 110: 302-306.

IV. Helena Domeij, Thomas Modéer, Tülay Yucel-Lindberg. Matrix metalloproteinase-1 and tissue inhibitors of metalloproteinase-1 production in human gingival fibroblasts: the role of protein kinase C.
J Periodontal Res 2004; 39: 308-314.

CONTENTS

ABBREVIATIONS

BIS	Bisindolylmaleimide
BME	Eagle´s basal medium
COX-2	Cyclooxygenase-2
DAB	Diaminobenzidine
DMEM	Dulbecco´s modified Eagle´s medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbant assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
HRP	Horse radish peroxidase
HSS-HRP	High sensitivity streptavidine HRP
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin-1β
MAPK	Mitogen activated protein kinase
MFI	Mean fluorescence intensity
MMP-1	Matrix metalloproteinase-1
MMP-3	Matrix metalloproteinase-3
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PGE_2	Prostaglandin E_2
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PTK	Protein tyrosine kinase
RNA	Ribonucleic acid
mRNA	Messenger RNA
RT-PCR	Reverse transcription polymerase chain reaction
TIMP-1	Tissue inhibitor of metalloproteinase-1
TNFα	Tumor necrosis factor α

INTRODUCTION

Maintenance of the extracellular matrix (ECM) is critical for the normal development and function of the tissues. The integrity of the ECM requires a balance between the amount and activity of matrix-degrading enzymes and their associated tissue inhibitors. In this context, the matrix metalloproteinases (MMPs) are of major importance. The MMPs are a group of zinc- and calcium-dependent endopeptidases capable of degrading almost all constituents of the extracellular matrix and the basement membrane (Egeblad and Werb, 2002). In addition, MMPs have the ability to cleave other molecules including cytokines, growth factor precursors, cell adhesion molecules as well as MMPs themselves (McCawley and Matrisian, 2001). Because of this broad substrate specificity, MMPs may be regarded as a group of multifunctional enzymes involved in physiological processes such as embryogenesis, normal tissue remodelling, wound healing and angiogenesis (Birkedal-Hansen, 1993a; Kähäri and Saarialho-Kere, 1997; Nagase, 1997; Rundhaug, 2005). However, MMPs are also involved in pathological conditions, such as rheumatoid arthritis, atherosclerosis, cancer and periodontal diseases (Nagase, 1997; Lijnen, 2002; Egeblad and Werb, 2002; Vincenti and Brinckerhoff, 2002; Ejeil et al., 2003). With respect to oral diseases, correlations between tissue degradation and MMPs have been investigated most intensively in periodontal diseases, but MMPs have also been detected in dentinal caries, inflamed dental pulps, periapical lesions, lichen planus lesions and oral squamous cell carcinomas (Sutinen et al., 1998; Tjäderhane et al., 1998; Shin et al., 2002; Sorsa et al., 2004).

PERIODONTAL DISEASES

Periodontal diseases, ranging in severity from gingivitis to periodontitis, are inflammatory conditions of the periodontium, which comprises four principal components: gingiva, periodontal ligament, alveolar bone and cementum. The primary sequence of events in periodontal diseases is the initiation of an inflammatory-immune response in the gingiva to the bacterial biofilm at the tooth surface. This highly-regulated response involves neutrophils, macrophages, epithelial cells, endothelial cells, fibroblasts and the complement system (Genco and Slots, 1984; Bartold et al., 2000).

Bacterial compounds in the bacterial biofilm, including lipopolysaccharides (LPS) and lipoteichoic acids, interact with toll-like receptors on epithelial cells,

leukocytes and fibroblasts and stimulate the production of cytokines such as interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), interleukin-6 (IL-6), IL-8, macrophage inflammatory protein-1a, monocyte chemotactic protein-1, -5, and prostaglandin E_2 (PGE_2) (Page, 1991; Madianos et al., 2005). These mediators, as well as bradykinin and kallidin, stimulate the extravasation of neutrophils and monocytes from the blood vessels by increasing the vascular permeability as well as the expression of adhesion molecules such as intercellular adhesion molecule -1 (ICAM-1) and P-selectin on endothelial cells (Gemmel et al., 1994; Nedbal et al., 2002; Madianos et al., 2005). In order to accommodate the infiltrate of leukocytes, fibroblasts stimulated by IL-1β and TNFα secrete MMPs that degrade ECM molecules, including collagen (Madianos et al., 2005).

Although the above-described immuno-inflammatory response is intended to eliminate the infection, it may also result in tissue destruction. The increased expression of inflammatory mediators, including IL-1β and TNFα, as well as bacterial products, including LPS, stimulates the expression of MMPs in fibroblasts, epithelial cells, keratinocytes, endothelial cells, mast cells, macrophages, neutrophils and lymphocytes (Makela et al., 1994; Tervahartiala et al., 2000; Dahan et al., 2001; Naesse et al., 2003). Several MMPs, including MMP-1, -2, -3, -7 -8, -9, -13, have been detected in gingival tissue and in gingival exudates, the gingival crevicular fluid (GCF) (Aiba et al., 1996; Kubota et al., 1996; Nomura et al., 1998; Tervahartiala et al., 2000; Alpagot et al., 2001; Séguier et al., 2001; Ejeil et al., 2003).

The activity of MMPs is tightly controlled by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs), especially TIMP-1 and TIMP-2, produced by fibroblasts, endothelial cells, keratinocytes, mast cells and macrophages (Kubota et al., 1997; Naesse et al., 2003). In periodontal diseases, there is an imbalance between MMPs and TIMPs, primarily due to increased MMPs, but also as a result of decreased expression of TIMPs. Several reports have demonstrated an imbalance of MMP-1 and MMP-3 with their inhibitor TIMP-1 in periodontal diseases (Soell et al., 2002; Tüter et al., 2002; Tüter et al., 2005). Furthermore, the increased expression of MMP-1 and MMP-3 detected in gingival tissue correlates to increased collagen degradation in the gingival tissue (Séguier et al., 2001; Ejeil et al., 2003). Gingival fibroblasts, the predominant cell type in gingival connective tissue, in response to inflammatory response, are believed to contribute to the enhanced levels of MMP-1 and MMP-3 detected in periodontal disease.

MATRIX METALLOPROTEINASES (MMPs)

Functional and structural properties of MMPs

The first MMPs were discovered in 1962, on a frog tail undergoing resorption (Gross and Lapière, 1962). Since then a number of additional MMPs have been identified. At present, the MMP family comprises 23 members, numbered in order of discovery, beginning with MMP-1. The MMPs are structurally related membrane or soluble endopeptidases. Despite overlapping substrate specificity, the MMPs are classified into five main subgroups, according to substrate specificity: collagenases, gelatinases, stromelysins, membrane-type MMPs and other MMPs (Table 1) (Kelly and Jarjour, 2003). The collagenases are primarily responsible for degradation of collagen fibers. The gelatinases are capable of cleaving gelatin, as well as some collagen molecules, especially type IV collagen. The stromelysins have a broad substrate spectrum, including collagen, elastin, laminin, and proteoglycans. The membrane-type MMPs cleave proteoglycans, fibronectin, laminin and collagen (Brinckerhoff et al., 2000; McCawley and Matrisian, 2001; Kelly and Jarjour, 2003; Chakraborti et al., 2003).

Most cell types have the ability to produce one or several MMPs, as demonstrated in Table 1. The MMPs are synthesized as inactive proenzymes (proMMP) and most are activated extracellularly, with the exception of MMP-11, MMP-23, MMP-28 and the membrane-type MMPs that can be activated intracellularly. The inactive proMMPs have a characteristic multidomain structure common for most MMPs, including MMP-1 and MMP-3. Starting from the N-terminus, MMPs consist of (i) the signal peptide (prepeptide), (ii) the propeptide, (iii) the catalytic domain, (iv) the hinge region and (v) the hemopexin-like domain in the C-terminus (Fig. 1). The signal peptide guides the MMP in the cell and is cut off during transit from the cell. The propeptide domain maintains the latency of the proMMP through a cysteine residue that ligates a zinc atom in the active site of the catalytic domain. During activation of proMMP, which is a stepwise process involving several proteinases, the linkage between the cysteine residue in the prodomain and the catalytic zinc in the catalytic domain is disrupted and the prodomain is eventually cut off. The catalytic domain contains the zinc-containing active site as well as additional zinc and calcium ions, which contribute to the maintenance of the three-dimensional structure of MMP. The hinge region that links the catalytic domain to the hemopexin-like domain mediates interactions with its endogenous inhibitors TIMPs. The hemopexin-like domain plays a key role in

substrate specificity as well as in the maintenance of the structure of MMP (Lijnen, 2002; Egeblad and Werb, 2002).

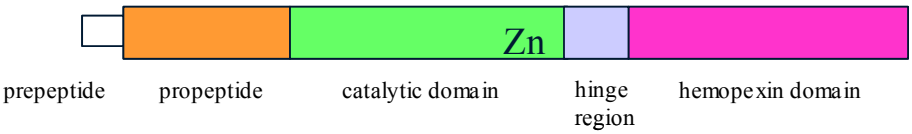


Fig.1. Schematic arrangement of the domain structures of MMP-1 and MMP-3. Modified from Woessner and Nagase 2000.

MMP-1 and MMP-3

The first human collagenase to be purified was collagenase-1 (MMP-1), also referred to as interstitial collagenase or fibroblast collagenase (Brinkerhoff and Matrisian, 2002; Pardo and Selman, 2005). This enzyme was designated MMP-1 and has served as a prototype for the collagenases. Like many MMPs, it is undetectable in normal resting tissue, although *in vitro* it is produced by a variety of cells such as fibroblasts, keratinocytes, endothelial cells, epithelial cells and monocytes (Table 1) (Pardo and Selman, 2005). MMP-1 is situated on chromosome 11 and the protein comprises an unglycosylated form of ~52 kDa as well as a glycosylated form of 61 kDa. After activation extracellularly, which involves proteinases including plasmin, chymase, kallikrein and other MMPs such as MMP-3, the size is reduced to ~43 kDa (Murphy et al., 1987; Woessner and Nagase, 2000; Kerkelä and Saarialho-Kere, 2003).

Active MMP-1 initiates the degradation of fibrillar collagen (Brinckerhoff and Matrisian, 2002). Additionally, other matrix molecules are substrates for MMP-1, including aggrecan, versican, perlecan and gelatin. MMP-1 can also cleave cell surface molecules and other non-matrix molecules such as IL-1β, proTNFα and insulin growth factor binding proteins (McCawley and Matrisian, 2001; Chakraborti et al., 2003).

Stromelysin-1 (MMP-3), previously also known as collagenase-activating protein, is produced by a variety of cells, *e.g.* fibroblasts, monocytes, epithelial cells, endothelial cells, keratinocytes, vascular smooth muscle cells and chondrocytes (Table 1). MMP-3 and MMP-1 have many characteristics in common. For example MMP-3 is also located on chromosome 11 and comprises an unglycosylated form of 52 kDa and a glycosylated form of 64 kDa. After activation extracellularly by

Table 1. The subgroups of the MMP family members and their source

Substrate specificity	MMP-number	Source
Collagenases	MMP-1	fibroblasts, endothelial cells, epithelial cells, hepatocytes, keratinocytes, monocytes, osteoblasts,
	MMP-8	fibroblasts, endothelial cells, monocytes, neutrophils, B-cells, T-cells
	MMP-13	fibroblasts, bone cells
Gelatinases	MMP-2	fibroblasts, endothelial cells, macrophages, neutrophils, T-cells
	MMP-9	fibroblasts, dentritic cells, endothelial cells, eosinophils, epithelial cells, keratinocytes, macrophages, neutrophils, osteoblasts, T-cells
Stromelysins	MMP-3	fibroblasts, endothelial cells, epithelial cells, monocytes, vascular smooth muscle cells, keratinocytes, chondrocytes
	MMP-10	fibroblasts, epithelial cells, keratinocytes, T-cells, monocytes
	MMP-11	fibroblasts, epithelial cells, B-cells
Membrane-type MMPs	MMP-14	fibroblasts, epithelial cells, macrophages, vascular smooth muscle cells, osteoblasts
	MMP-15	fibroblasts, macrophages, T-cells
	MMP-16	vascular smooth muscle cells, brain, placenta, T-cells
	MMP-17	monocytes, B-cells, brain, reproductive tissues
	MMP-24	T-cells, brain
	MMP-25	neutrophils, monocytes
Other MMPs	MMP-7	epithelial cells, monocytes, T-cells, B-cells, mesangial cells
	MMP-12	macrophages
	MMP-19	monocytes, T-cells
	MMP-20	dental enamel
	MMP-21	epithelial cells, keratinocytes, monocytes, B-cells, T-cells
	MMP-23	monocytes, B-cells, T-cells, ovarium
	MMP-26	B-cells, keratinocytes
	MMP-27	B-cells
	MMP-28	T-cells, cartilage

Ahokas et al., 2003; Kelly and Jarjour, 2003; Bar-Or et al., 2003; Momohara, 2004; Ahokas et al., 2005; Pardo and Selman, 2005

proteinases such as plasmin, tryptase, kallikrein, chymase and MMP-3 itself (autoactivation), MMP-3 is reduced in size to 43 kDa (Woessner and Nagase, 2000; Kerkelä and Saarialho-Kere, 2003; Chakraborti et al., 2003). Active MMP-3 has a wide variety of substrates, including ECM molecules such as collagen III-V and IX, gelatin, elastin, proteoglycans and osteonectin. MMP-3 also cleaves non-matrix molecules such as proIL-1β, proTNFα, MMP-1, MMP-9 and MMP-13 (McCawley and Matrisian, 2001; Chakraborti et al., 2003; Kelly and Jarjour, 2003).

REGULATION OF MMP-1 AND MMP-3

Regulation of MMP-1 and MMP-3 expression is dependent on both the cell type producing the MMP and the location of the cell (Chakraborti et al., 2003). In general MMPs are regulated at three levels: (i) gene transcription, (ii) activation of the latent proform and (iii) inhibition by the endogenous inhibitors TIMPs (Kelly and Jarjour, 2003). The promoters of MMP-1 and MMP-3 have many similarities (Wenk et al., 1999), containing sites for the transcription factors ETS and activator protein-1 (AP-1) (Westermarck and Kähäri, 1999; Chakraborti et al., 2003; Pardo and Selman, 2005). At transcriptional level, both MMP-1 and MMP-3 are regulated by cell-to-cell interactions, cell-matrix interaction and by cytokines such as IL-1β, TNFα, epidermal growth factor (EGF), interferon γ (IFNγ), IL-6, IL-4, prostaglandins including PGE_2, and vitamin A derivatives (Ravanti et al., 1999; Westermarck and Kähäri, 1999; Wassenaar et al., 1999, Martelli-Junior et al., 2003; Jenkins et al., 2004; Ruwanpura et al., 2004; Sakaki et al., 2004; Maldonado et al., 2004). These extracellular signals transduce their signals in the cells through different intracellular signal transduction pathways that activate or repress the expression of MMP-1 and MMP-3 (Fig 2).

Cell-to-cell interactions

Several *in vitro* studies have demonstrated that the expression of MMPs can be stimulated by cell-to-cell interactions between structural cells including fibroblasts and endothelial cells and leukocytes including monocytes/macrophages and lymphocytes (Huybrecht-Godin et al., 1979; Burger et al., 1998; Hojo et al., 2000; Zhu et al., 2001a). In periodontal disease fibroblasts are in close contact with monocytes/macrophages that are heavily distributed in the gingival tissue (Lo et al., 1999). Interaction between fibroblasts and monocytes may involve several adhesion molecules such as the integrin family and the family of cellular adhesion molecules

(CAM). The expression of ICAM-1 on gingival fibroblasts is upregulated in inflamed gingival tissue and may facilitate interactions between fibroblasts and leukocytes (Hayashi et al., 1994). ICAM-1 is a member of the immunoglobulin superfamily, expressed on the cell surface of fibroblasts as well as on endothelial cells and leukocytes (Anderson and Siahaan, 2003). ICAM-1 interacts with leukocyte function-associated antigen-1, a member of the β2-integrin family, which is usually expressed on leukocytes. The intracellular effect of the interaction of ICAM-1 with its ligand is not well documented (Anderson and Siahaan, 2003; Witkowska and Borawska, 2004), although the expression of ICAM-1 is known to be stimulated by cytokines such as IFNγ, IL-1 and TNFα (Hayashi et al., 1994).

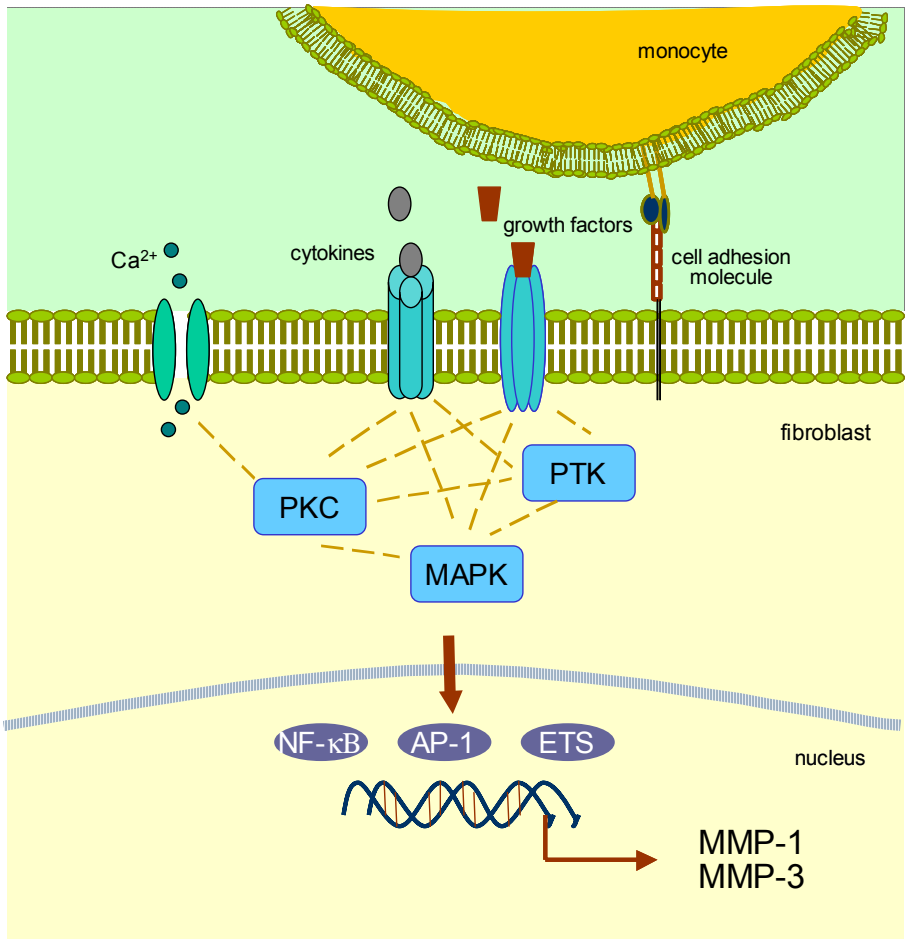


Fig 2. Schematic drawing of signal pathways involved in the regulation of the expression of MMP-1 and MMP-3. Protein kinase C (PKC), mitogen activated protein kinase (MAPK), protein tyrosine kinase (PTK).

Cytokines

Cytokines are soluble proteins that mediate cellular effects through interaction with their receptors on the cell membrane. Cytokines are denoted as pro- or anti-inflammatory mediators depending on their role in the inflammatory response. The effects of the cytokines depend on the local environment in which they are expressed, the presence of competing or co-operating elements, and the expression of their receptors on the target cell, as well as the status of the intracellular signal pathways processing the cytokine signal in the target cell. In this study the cytokines IL-1β, TNFα and EGF were used to simulate the inflammatory response.

IL-1β is a pro-inflammatory cytokine that together with IL-1α and IL-1Ra constitutes the IL-1 gene family. It is produced primarily by monocytes, synthesized as inactive proIL-1β that is converted to active IL-1β in the presence of interleukin-1-converting enzyme and specific cellular proteases, including MMPs (Dinarello, 1996). IL-1β is a potent inflammatory mediator that *inter alia* has the capability to induce the production of cytokines, prostaglandins and MMPs (Dinarello, 1997, Wassenaar et al., 1999). Through its two membrane-bound and soluble receptors, IL-1RI and IL-1RII, IL-1β transduces intracellular signals, partly by mitogen activated protein kinase (MAPK), protein kinase C (PKC) and tyrosine kinase and activates or induce transcription factors including nuclear factor-κB (NF-κB), AP-1 and ETS (Schütze et al., 1994; Matthews et al., 1999; Vincenti and Brinckerhoff, 2002; Siwik and Colucci, 2004; Maldonado et al., 2004; Kida et al., 2005).

TNFα is a pleiotropic cytokine with strong pro-inflammatory and immunomodulary properties. TNFα induces production of cytokines, prostaglandins and MMPs (Papadakis and Targan, 2000). While monocytes/macrophages are the major source of TNFα, other cells, such as lymphocytes, keratinocytes and fibroblasts also produce TNFα (Baud and Karin, 2001). TNFα-induced responses are mediated through either of its two receptors, TNFR1 and TNFR2, which transduce intracellular signals partly through MAPK and tyrosine kinase promoting transcription or activation of transcription factors such as AP-1, NF-κB and ETS (Baud and Karin, 2001, Liacini et al., 2003; Siwik and Colucci, 2004).

Epidermal growth factor (EGF) is a polypeptide involved in inflammatory conditions (Chang et al., 1996). In consistens with IL-1β and TNFα, EGF also stimulates the production of cytokines and prostaglandins as well as MMPs (Hiraoka et al., 1992; Modéer et al., 1993; Yucel-Lindberg et al., 1999a). EGF mediates its

intracellular signals through its receptors, the EGF receptors, which are members of the family of receptor tyrosine kinase (Wells, 1999). Subsequently signal pathways including MAPK and PKC and transcription factors, including NF-κB, AP-1 or ETS are activated (Wells, 1999; Venook, 2005).

Signal transduction pathways

There are vast numbers of intracellular signaling messengers, which in co-operation transduce extracellular signals, resulting in stimulation or repression of gene transcripts of proteins, including MMPs (Fig. 2). The studies in this thesis included investigations of the involvement of the protein kinases tyrosine kinase, p38 MAPK and PKC on the expression of MMP-1 and MMP-3. Protein kinases modify other proteins by phosphorylation, causing activation of the protein, changes in cellular location of the target protein or association with other proteins (Davies et al., 2000).

Protein tyrosine kinase is a group of kinases that phosphorylate tyrosine residues in a target protein. There are 90 tyrosine kinases in the human genome: 58 are transmembrane receptors and 32 are non-receptor proteins. Src-family is a subgroup of nonreceptor tyrosine kinases that are expressed ubiquitously in vertebrate cells (Roskoski, 2005). Aberrations in tyrosine kinase signaling occur in inflammatory diseases and in cancer (Roskoski, 2005; Venook, 2005). Tyrosine kinase play key roles in cell differentiation, proliferation, mobility and survival and are controlled by growth factors, integrins, cytokines and steroids (Venook 2005; Thomas and Brugge, 1997). The tyrosine kinases transduce their signals through co-operation with other kinases and activate several transcription factors including AP-1 and NF-κB and ETS (Geng et al., 1993; Pankov et al., 1994; Jalali et al., 1998).

MAPK including p38 MAPK are a family of serine and threonine protein kinases that are activated by phosphorylation. Four subgroups of p38 MAPKs are described: α and β which are widely expressed, δ which is expressed in lung and kidney, and γ which is limited to skeletal muscle (Antonescu et al., 2005). All four p38 MAPKs are activated by upstream kinases in response to inflammatory cytokines, growth factors and osmotic stress (Antonescu et al., 2005). The p38 MAPK is involved in transcriptional control of MMPs by promoting transcription or activation of AP-1, ETS and NF-κB (Guha and Mackman, 2001; Chase et al., 2002; Vincenti and Brinckerhoff, 2002; Kida et al., 2005).

PKC represents a family of more than 11 calcium and phospholipid-dependent enzymes, which play a pivotal role in cell signaling systems (Cataisson et al., 2003) The PKC family is divided into three groups: conventional PKC that requires calcium and diacylglycerol (DAG) for activation, novel PKC that requires only DAG for activation and atypic PKC that requires neither calcium nor DAG for activation (Huang, 1989; Mellor and Parker, 1998; Cataisson et al, 2003). Furthermore, PKC is also reported to be activated by phorbol esters as well as cytokines, including IL-1β and TNFα (Schütze et al., 1994). PKC, in co-operation with other kinases such as MAPK and tyrosine kinase, activates the transcription factors AP-1, ETS and NF-κB, resulting in transcription of various proteins, including MMPs (Schütze et al., 1994; Mellor and Parker, 1998; Naito et al., 2002; Ding et al., 2005).

Inhibitors of MMPs

Tissue inhibitors of metalloproteinases (TIMPs)

The activity of MMPs is controlled unspecifically by α2macroglobulins and specifically by their endogenous inhibitors: tissue inhibitors of metalloproteinases (TIMPs) (Hornebeck, 2003; Kelly and Jarjour, 2003; Pardo and Selman, 2005). TIMPs comprises a family of four members (TIMP-1, -2, -3, -4), which firmly controls MMP activity by forming a 1:1 complex with the catalytic zinc at the active site of MMP (Brinckerhoff and Matrisian, 2002). TIMP-1 and TIMP-2 are expressed in periodontal tissue and are capable of inhibiting the activities of most MMPs (Gomez et al., 1997; Brew et al., 2000) although MMP-1 and MMP-3 are preferably regulated by TIMP-1. The inhibitor TIMP-1 is located at chromosome 11 and has a molecular weight of 20.6 or 28.5 kDa, depending on glycosylation (Hornebeck, 2003). Normally, TIMPs including TIMP-1 are found only in tissues undergoing remodelling or breakdown (Woessner, 1991). To balance the action of MMP under physiological conditions, the same cell producing MMP usually produces TIMP-1 upon stimulation. In gingival tissue affected by periodontitis, TIMP-1 is expressed by fibroblasts, endothelial cells, keratinocytes, mast cells and macrophage-like cells and is frequently found in extracellular matrix, often along fibrous structures (Naesse et al., 2003). TIMP-1 expression is stimulated by several factors including tumor growth factor β1, EGF and phorbol esters (Gomez et al., 1997). Despite the firm regulation of MMPs, an imbalance between MMPs, *e.g.* MMP-1 and MMP-3 and their inhibitor

TIMP-1 has been detected in pathological conditions, such as arthritis and periodontitis (Reynolds, 1996; Soell et al., 2002; Tüter et al., 2005).

Synthetic inhibitors

Agents that modulate the expression or activity of MMPs have been proposed for the treatment of various MMP-associated diseases, including periodontal diseases (Peterson, 2004; Salvi and Lang, 2005).

The tetracycline doxycycline has traditionally been recognized for its antibiotic properties. More recently, doxycycline has been shown to have a number of anti-inflammatory effects, reducing the production of several inflammatory cytokines as well as nitric oxide (Peterson, 2004). Furthermore, doxycycline has been shown to decrease the production as well as the activity of MMPs (Solomon et al., 2000; Peterson, 2004).

The glucocorticoids, including dexamethasone, are another group of agents with well-known anti-inflammatory effects. The hydrophobic agent dexamethasone diffuses through the cell membrane and is transported by its glucocorticoid receptor in the cytosol and nucleus, where it interacts and inhibits several transcription factors, including AP-1 and NF-κB involved in the transcription of MMPs as well as in the transcription of inflammatory mediators (De Bosscher et al., 2001).

The non-steroidal anti-inflammatory drugs (NSAIDs) are known to inhibit the synthesis of PGE_2 via cyxlooxygenase (COX-1 and COX-2) isoenzymes. Furthermore, NSAIDS have also been shown to reduce the expression of MMPs in tissue affected by periodontal disease (Lee et al., 2004). The inhibitory effect of NSAIDs on MMP expression may be due to inhibition of the inflammatory induced COX-2, which in cooperation with kinases including tyrosine kinase and PKC (Yucel-Lindberg et al., 1999a; Yucel-Lindberg et al., 1999b) may regulate the transcription of MMPs.

Excess production of MMPs including MMP-1 and MMP-3 is associated with tissue degradation in inflammatory conditions including periodontal diseases (Séguier et al., 2001; Ejeil et al., 2003; Pay et al., 2005). Gingival fibroblasts, the major constitutents in the gingival tissue, are believed to play a major role in the production of MMP-1 and MMP-3 in gingival tissue. However, the expression and regulation of these MMPs in gingival fibroblasts is not well studied. The present study was therefore designed to test the hypothesis that the expression of MMP-1 and MMP-3 in gingival fibroblasts is regulated by monocytes and inflammatory cytokines.

AIMS OF THE THESIS

- To investigate the effect of cell interactions between human gingival fibroblasts and monocytes on the expression of MMP-1 and its inhibitor TIMP-1 in gingival fibroblasts, using a co-culture model.

- To study the expression of MMP-1 and TIMP-1 in human gingival fibroblasts in cell-to-cell contact with monocytes; to investigate the involvement of the adhesion molecule ICAM-1 and the signal transduction pathway p38 MAPK in the expression of MMP-1 and TIMP-1.

- To investigate the effect of the cytokines IL-1β, TNFα or EGF on the expression of MMP-1 and MMP-3 in human gingival fibroblasts; to study the involvement of the signal transduction pathways p38 MAPK and tyrosine kinase in the regulation of MMP-1 and MMP-3.

- To study the effect of the PKC activator PMA, in the absence or presence of the cytokine IL-1β and the calcium-ionophore A23187, on the expression of MMP-1 and TIMP-1 in human gingival fibroblasts.

MATERIALS AND METHODS

The methods are briefly described in this section. The detailed descriptions of the methods are found in each individual paper. The methods used in the analysis of MMP expression are summarized in Figure 5.

CELLS

Fibroblasts

Cultures of gingival fibroblast were established from gingival biopsies obtained from ten healthy individuals between 3 and 18 years of age with no clinical signs of periodontal disease. The plan to take gingival tissue biopsies was approved by the Ethical Committee of Huddinge University Hospital. Minced pieces of the tissue were explanted to 25-cm^2 Nunc tissue cultures containing 5 ml of Eagle´s basal medium (BME). The fibroblasts were obtained by trypsinisation of the primary outgrowth of cells as described previously (Modéer et al., 1982) and grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with foetal calf serum (FCS) (5%). The fibroblasts were incubated at 37^{o}C in a humidified incubator aerated with 5% CO_2 in air and routinely passaged using 0.025% trypsin in PBS containing 0.02% EDTA. Cells used for the experiments proliferated in logarithmic phase between the 7th and 12th passages.

Monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by gradient centrifugation (1000 x *g* for 30 min) on Lymphoprep (density: 1.077 g/ml). The PBMC were washed with DMEM and remaining erythrocytes were lysed with 1.5 M ammonium chloride containing 0.1 M potassium hydrogen carbonate and 0.9 mM EDTA. Monocytes were then purified from the PBMC fraction by gradient centrifugation; The PBMC were resuspended in 60% percoll (density 1.131, diluted 1:9 with 10% PBS) in DMEM. Subsequently 47.5% and 34% percoll in DMEM were layered upon the cell suspension. After centrifugation (1700 x *g* for 40 min), the low-density fraction representing monocytes were collected and washed with DMEM.

CULTURES

Cultures of fibroblasts

Gingival fibroblasts were seeded in wells with DMEM containing 5% FCS. After incubation, the cell layer was rinsed three times with DMEM and the cells were thereafter incubated in DMEM supplemented with 1% FCS with or without various substances including the cytokines IL-1β, TNFα, EGF or the phorbol ester phorbol 12-myristate 13-acetate (PMA) in the absence or presence of the calcium ionophore A23187, the glucocorticoid dexamethasone, as well as inhibitors of signal pathways including the p38 MAPK inhibitor SB203580, the tyrosin kinase inhibitor herbimycin A, the PKC inhibitor bisindolylmaleimide (BIS) or the COX-2 inhibitor NS-398.

Co-cultures of fibroblasts and monocytes

Fibroblasts and monocytes were co-cultured by adding newly purified monocytes, in DMEM supplemented with 5 % FCS, to the cell layer of fibroblasts. The co-culture experiments were carried out in two ways: (1) co-cultures with cell-to-cell contact with monocytes layered onto fibroblasts, (2) co-cultures separated with a microporous membrane (pore size 0.4 μm) separating fibroblasts in the lower compartment from the monocytes in the upper compartment (Fig. 3). In some experiments dexamethasone, doxycycline, the p38 MAPK inhibitor SB203580 or an anti-ICAM-1 antibody was added to the co-cultures.

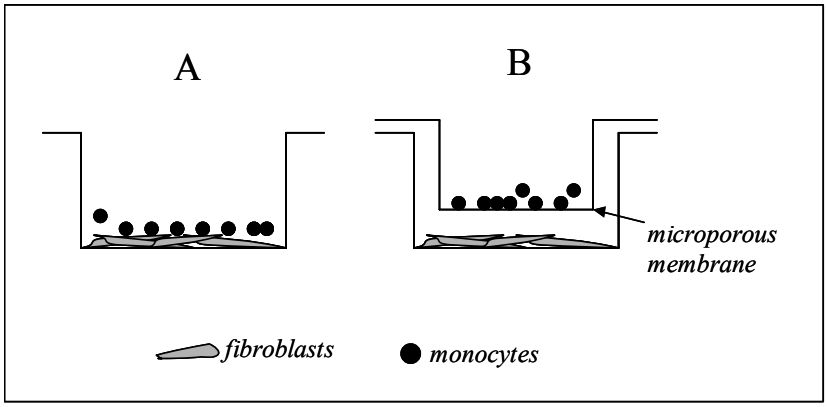


Fig. 3. The co-culture model with fibroblasts and monocytes in direct cell-to-cell contact (A) or separated by a microporous membrane (B).

ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

At the end of incubation, the medium was withdrawn, monocytes present were removed by centrifugation, and the medium was stored at -80°C for analysis.

In the experiments where the intracellular levels of MMP-1 and MMP-3 were to be detected (study III), the cells were washed with ice-cold PBS, detached by 0.25% trypsin-versen, washed with PBS, and resuspended in Tris-HCl. The cells were lysed by repeated freeze thawing and ultrasonication.

The protein levels of MMP-1 and MMP-3 and TIMP-1 in the co-culture medium or in the lysed cell suspension were analysed by commercially available ELISA-kits.

WESTERN BLOTTING

Fibroblasts were cultured with PMA in the absence or presence of BIS, the medium was withdrawn and the protein concentrations were determined according to the Bradford method using bovine serum albumin as the standard. Equivalent amounts of protein were separated by electrophoresis on a 4-15 % gradient SDS PAGE gel and transferred thereafter to a nitrocellulose membrane. The membrane was then blocked in 5% dry milk for 1 h, incubated at 4ºC overnight with polyclonal primary antibody for MMP-1 and thereafter with secondary horseradish rabbit peroxidase-conjugated antibody (1 h). The membranes were developed using enhanced chemiluminescence (ECL) and exposed to hyperfilm-ECL.

IMMUNOHISTOCHEMISTRY

Fibroblasts were cultured in 4-well glass slides with DMEM supplemented with 5% FCS. The monocytes in DMEM containing 5% FCS were added to the fibroblasts and the cells were co-cultured with cell-to-cell contact. The cells adhering to the glass slides were washed twice with PBS and fixed with ice-cold methanol for 10 min, followed by 10 seconds in ice-cold acetone. Thereafter, the cells were stained for immunohistochemistry by the avidin–biotin–peroxidase method, using the HSS-HRP immunostaining kit according to the manufacturer's directions. The cells were incubated at 4°C overnight with primary antibodies for MMP-1 (polyclonal) or TIMP-1 (monoclonal). The peroxidase reaction was disclosed with 0.3% (v/v) diaminobenzidine (DAB) in DAB buffer containing 0.1% (v/v) hydrogen peroxide. Finally, the cells were washed with distilled water, dehydrated through an ethanol series and xylene, mounted, and photographed using a Zeiss light microscope.

FLOW CYTOMETRY

The cell suspensions of fibroblasts and monocytes, cultured alone or co-cultured, were fixed in 2% paraformaldehyde and washed with PBS prior to permeabilization with PBS containing 0.1% Saponin (SAP buffer). Thereafter, the cells were stained for surface and intracellular MMP-1, TIMP-1, CD14, CD90 and ICAM-1 (CD54) in the dark for 30 min at room temperature using phycoerythrin (PE)-labeled monoclonal antibodies for MMP-1, TIMP-1, or CD14 and fluorescein isothiocyanate (FITC)-labeled antibody for CD54 and CD90. After washing with SAP-buffer, the cells were resuspended in PBS and analyzed in a FACSCalibur^{TM} flow cytometer using CellQuest software. To analyze the expression of MMP-1, TIMP-1, ICAM-1, CD14, CD54 and CD90 a gated area (Fig. 4) was selectively determined for the cells using forward vs scatter parameters in order to discriminate the fibroblasts and monocytes.

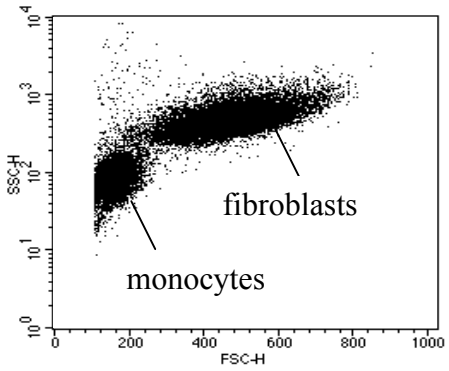


Fig. 4. Cell populations, fibroblasts and monocytes, based on forward (FSC) and side scatter (SSC)

RNA ISOLATION AND RT-PCR

In cultures of fibroblasts alone (study IV), the fibroblasts were washed with ice-cold PBS and frozen immediately in liquid nitrogen and stored at -80^{o}C for isolation of total RNA. Total RNA was isolated from fibroblasts by phenol-chloroform extraction and ethanol precipitation according to the method described by Durnam and Palmiter (1983).

In co-cultures of fibroblasts and monocytes (study I), the monolayer of fibroblasts was washed with ice-cold PBS, frozen immediately in liquid nitrogen and stored at -80^{o}C for isolation of total RNA. The monocytes in the co-cultures were isolated with repeated washing with ice-cold PBS and by a short period of trypsinisation. Thereafter, the monocyte fraction was centrifuged and the pellet with

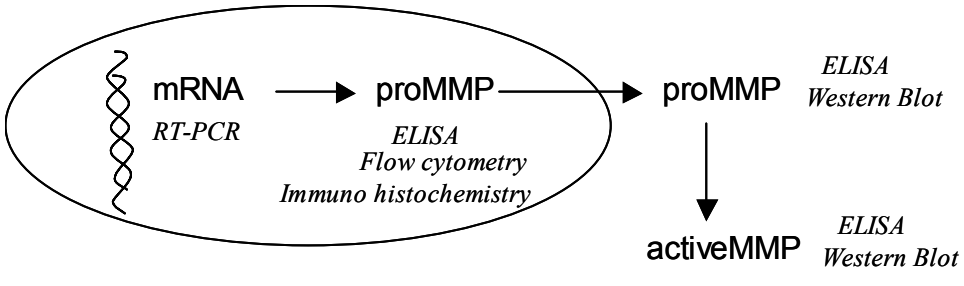
monocytes was frozen immediately in liquid nitrogen and stored at -80°C for isolation of total RNA. Total RNA was obtained using a Qiagen RNA-isolating mini kit.
The amounts of total RNA, obtained by either method, were quantified by spectrophotometry at 260/280 nm (Sambrook et al., 1989).

To obtain first strand cDNA, 1.0 µg of total RNA was reverse transcribed using Superscript II in a total volume of 20 µl. The cDNA (2 µl) obtained was used in a final reaction volume of 50 µl for PCR amplification. The PCR reactions were performed, using specific primers and positive controls for MMP-1, TIMP-1 and GAPDH, according to the manufacturer's recommendation. For each experiment, PCR amplification was also performed without cDNA to check for contamination. 10 µl of each PCR reaction product was run on 2 % agarose gel next to 1kb DNA-ladder standard and visualised with ethidium bromide.

CONFOCAL LASER SCANNING MICROSCOPY

Fibroblasts were cultured in 4-well glass slides with DMEM containing 5% FCS. The monocytes in DMEM containing 5 % FCS were added to the fibroblasts and co-cultured with cell-to-cell contact. After co-culture incubation, the medium was withdrawn and the adhered cells were washed with PBS, fixed with 4% paraformaldehyde and incubated overnight at 4°C with CD90 FITC-labeled mAb, recognized on fibroblasts and CD14 PE-labeled mAb, recognized on monocytes. The cells were thereafter washed with PBS and mounted with fluorescent mounting medium. The cells were viewed in a Leica TCS-NT confocal laser scanning microscope equipped with an Argon-Krypton laser.

Fig 5. Summary of the methods used to detect MMP



RESULTS

Study I. Cell interactions between human gingival fibroblasts and monocytes stimulate the production of MMP-1 in gingival fibroblasts

The effect of cell interactions between human gingival fibroblasts and human monocytes on the production of MMP-1 was studied in a co-culture model. The fibroblasts were cultured in either cell-to-cell contact with monocytes, or in separated co-cultures, using a microporous membrane to prevent cell-to-cell contact. Images of cell-to-cell contact during co-culture were captured by confocal laser scanning microscopy. Co-culturing gingival fibroblasts with monocytes in cell-to-cell contact increased the mRNA expression of MMP-1 in both fibroblasts and monocytes. Furthermore, the protein levels of MMP-1 also increased in the culture media of the co-cultures in a time dependent manner (4-24 h) and correlated to the number of fibroblasts as well as to the number of monocytes. In separated co-cultures the mRNA expression of MMP-1 in fibroblasts as well as the protein levels in the medium increased, although the protein levels of MMP-1 in the separated co-cultures were lower than when the cells were co-cultured with cell-to-cell contact. In addition, MMP-1 production in fibroblasts was also stimulated by conditioned medium from monocytes. Moreover, the levels of the MMP-1 inhibitor TIMP-1 increased in co-cultures with cell-to-cell contact, but not in fibroblasts of separated co-cultures. The glucocorticoid dexamethasone and the tetracycline doxycycline reduced the enhanced level of MMP-1 in the co-cultures with cell-to-cell contact. The results of this study show that interactions of fibroblasts and monocytes, both by cell-to-cell contact and soluble factors stimulate the production of MMP-1, suggesting that interactions of fibroblasts and monocytes contribute to increased MMP-1 expression seen in inflammatory processes.

Study II. Cell expression of MMP-1 and TIMP-1 in co-cultures of human gingival fibroblasts and monocytes; the involvement of ICAM-1

This study is a continuation of study I investigating the intracellular protein expression of MMP-1 and TIMP-1 in co-cultures of fibroblast and monocytes with cell-to-cell contact and the possible mediating role of the adhesion molecule ICAM-1. Co-culturing fibroblasts and monocytes for 16 h increased the levels of both active and total MMP-1 as well as the level of TIMP-1 in the co-culture medium. In co-cultures, the expression of MMP-1 and TIMP-1 markedly increased in gingival

fibroblasts. The monocytes, on the other hand, did not show increased expression of MMP-1 or TIMP-1, although the number of MMP-1$^+$ and TIMP-1$^+$ adhered monocytes increased in the co-cultures. In the presence of an anti-ICAM-1 antibody, the expression of MMP-1 in the co-cultured fibroblasts was reduced. In contrast to MMP-1, the expression of TIMP-1 in the fibroblasts was not affected by anti-ICAM-1 treatment. However, the number of TIMP-1$^+$ adhered monocytes increased in co-cultures treated with anti-ICAM-1 antibody. Moreover, co-culturing fibroblasts with monocytes markedly increased the expression of ICAM-1 in the fibroblasts as well as in the adhered monocytes. Treatment of the co-cultures with the p38 MAPK inhibitor SB203580 reduced the expression of MMP-1 and ICAM-1 in the fibroblasts and the expression of ICAM-1 in the adhered monocytes. These results suggest that the resident cell fibroblast is stimulated by monocytes resulting in enhanced cellular expression of MMP-1, TIMP-1 and ICAM-1. Furthermore, the enhanced MMP-1 expression, in contrast to TIMP-1, is partly mediated by the adhesion molecule ICAM-1 and the p38 MAPK signal pathway.

Study III. Signal pathways involved in the production of MMP-1 and MMP-3 in human gingival fibroblasts

The production and regulation of MMP-1 and MMP-3 in human gingival fibroblasts challenged with the cytokines IL-1β, TNFα or EGF was studied. The results showed that gingival fibroblasts constitutively produce MMP-1 and MMP-3 and that the cytokines IL-1β, TNFα and EGF stimulate the production of MMP-1 as well as MMP-3. Time-course studies showed that the stimulated MMP-1 and MMP-3 production was apparent at 8 h of incubation and increased continuously during 48 h of incubation. The IL-1β and TNFα stimulated production of MMP-1 and MMP-3 was reduced by the tyrosine kinase inhibitor herbimycin A, the p38 MAPK inhibitor SB203580 and the COX-2 inhibitor NS-398. In addition, the production of MMP-1 and MMP-3 stimulated by IL-1β, TNFα or EGF, was markedly inhibited by the presence of the glucocorticoid dexamethasone. These findings demonstrate that the cytokines IL-1β, TNFα and EGF, respectively, enhance both MMP-1 and MMP-3 production in human gingival fibroblasts, and that the signal pathways p38 MAPK and tyrosine kinase are partly involved in the production of MMP-1 and MMP-3.

Study IV. MMP-1 and TIMP-1 production in human gingival fibroblasts: the role of protein kinase C

The production and regulation of MMP-1 and its inhibitor TIMP-1 in gingival fibroblasts were studied with special reference to the enzyme PKC. The results showed that the PKC activator PMA stimulated the production of both MMP-1 and TIMP-1 in 24 h cultures. The PMA stimulated MMP-1 and TIMP-1 production was abolished by the PKC inhibitor BIS. Furthermore, treatment of the cells with IL-1β or the Ca^{2+}-ionophore A23187 synergistically upregulated the stimulatory effect of PMA on the production of MMP-1. This synergistic up-regulation was abolished in the presence of the PKC inhibitor BIS. In contrast to MMP-1, TIMP-1 production was unaffected by IL-1β and reduced by the Ca^{2+}-ionophore A23187.

To further elucidate intracellular signal pathways regulating the PMA stimulated MMP-1 and TIMP-1 production, the involvement of p38 MAPK and tyrosine kinase was also studied. The tyrosine kinase inhibitor herbimycin A reduced the PMA-stimulated MMP-1 production whereas the p38 MAPK inhibitor SB203580 synergistically upregulated the stimulatory effect of PMA on both MMP-1 and TIMP-1 production. Taken together, these findings indicate that MMP-1 and TIMP-1 production is regulated differently by IL-1β and calcium in human gingival fibroblasts and that this difference is markedly amplified in the presence of the PKC activator PMA.

DISCUSSION

MMP-1 EXPRESSION IN FIBROBLASTS IN CELL INTERACTIONS WITH MONOCYTES

The endopeptidases MMP-1 and MMP-3 are key enzymes involved in the pathogenesis of several inflammatory diseases including periodontal diseases (Soell et al., 2002; Vincenti and Brinckerhoff, 2002; Pay et al., 2005). Enhanced expression of MMPs including MMP-1 and MMP-3 is demonstrated in GCF as well as gingival connective tissue of individuals with periodontal diseases and relates to increased degradation of collagen in the gingival tissue (Séguier et al., 2001; Soell et al., 2002; Ejeil et al., 2003). The mechanisms whereby the inflammatory response stimulates the expression of MMPs is not clear, although, leukocytes including monocytes, which infiltrate the gingival tissue, produce pro-inflammatory cytokines that may stimulate the expression of MMP-1 and MMP-3 in gingival fibroblasts (Lo et al., 1999; Dahan et al., 2001). The overall objective of this study was therefore to investigate the *in vitro* expression and regulation of MMP-1 and MMP-3 in human gingival fibroblasts in response to cell interactions with monocytes and to inflammatory mediators.

The effect of cell interactions between gingival fibroblasts and monocytes on the expression of MMP-1 was studied using a co-culture model. The fibroblasts and monocytes were either co-cultured with direct cell-to-cell contact or in transwell co-culture where the cells were separated by a membrane that only allowed soluble factors to pass. Co-culturing fibroblasts and monocytes with direct cell-to-cell contact resulted in enhanced expression of MMP-1 in the fibroblasts, indicating that cellular adhesion between fibroblasts and monocytes stimulates the expression of MMP-1 in fibroblasts. The levels of MMP-1 in the co-culture medium also increased and correlated to the number of both fibroblasts and monocytes in the co-cultures. In this context, it is of interest to note that *in vivo* studies have demonstrated that increased number of monocytes/macrophages in the gingival tissue correlates to degradation of collagen and that gingival fibroblasts at inflammatory sites in the gingival tissue express MMP-1 (Uden et al., 1998; Dahan et al., 2001; Séguier et al., 2001).

The stimulatory effect of monocytes on the expression of MMP-1 in fibroblasts may be mediated by adhesion molecules and/or by soluble factors. Several adhesion molecules, including integrins and their ligands CAMs, are involved in interactions between fibroblasts and monocytes. This present investigations show that the

expression of ICAM-1 is upregulated in co-cultured fibroblasts as well as in co-cultured monocytes adhered to fibroblasts. Furthermore, this study provides evidence that the adhesion molecule ICAM-1 is involved in the cell-to-cell stimulated MMP-1 expression by fibroblasts. The involvement of ICAM-1 in the expression of MMP-1 was verified by treatment of the co-cultures with an anti-ICAM-1 antibody, which reduced the expression of MMP-1 in fibroblasts. The involvement of ICAM-1 in the expression of MMP-1 in gingival fibroblasts is well compatible with *in vivo* studies demonstrating that gingival fibroblasts at inflammatory sites express MMP-1 as well as ICAM-1 (Hayashi et al., 1994; Dahan et al., 2001).

Taking into account that p38 MAPK stimulates ICAM-1 expression (Blaber et al., 2003), the involvement of p38 MAPK in the ICAM-1 mediated MMP-1 expression was investigated in co-cultures of fibroblasts and monocytes with cell-to-cell contact. Treatment of the co-cultures with the p38 MAPK inhibitor SB203580 reduced the expression of both MMP-1 and ICAM-1 by fibroblasts as well as ICAM-1 by monocytes adhered to fibroblasts. These findings suggest that p38 MAPK may be involved in the ICAM-1 stimulated MMP-1 expression in fibroblasts. However, it should be noted that the inhibitory effect of the p38 MAPK inhibitor SB203580 on the expression of MMP-1 may also be due to reduction of other adhesion molecules involved in cell adhesion between fibroblasts and monocytes (Shang et al., 1998).

The involvement of soluble factors in the monocyte-stimulated MMP-1 expression in gingival fibroblasts was investigated by co-culturing fibroblasts and monocytes in transwell co-cultures where the cells were separated by a membrane that only allowed soluble factors to pass. The expression of MMP-1 in the fibroblasts was stimulated in separated co-cultures, indicating that soluble factors, such as cytokines, may stimulate the expression of MMP-1 in fibroblasts. This assumption was also supported by the findings that conditioned medium from monocytes stimulated the expression of MMP-1 in fibroblasts. Notably, MMP-1 expression in monocytes was not stimulated by fibroblasts in separated co-cultures or by treatment with conditioned medium from fibroblasts, indicating that the expression of MMP-1 in the monocytes was not affected by soluble factors from fibroblasts. In fact, with respect to the monocytes, MMP-1 expression was stimulated only in those adhered to fibroblasts: there was a slight increase in the number of such monocytes expressing MMP-1.

THE EFFECT OF CYTOKINES ON MMP-1 AND MMP-3 EXPRESSION

The effect of the cytokines IL-1β, TNFα and EGF on the expression of MMP-1 and MMP-3 in gingival fibroblasts was investigated. The pro-inflammatory cytokines IL-1β and TNFα, produced by monocytes/macrophages, are increased in gingival crevicular fluid and in gingival tissue during periodontal diseases (Rossomando et al., 1990; Page et al., 1997; Roberts et al., 1997; Salvi et al., 1998; Lo et al., 1999; Holmlund et al., 2004).

The findings demonstrated in this study, showing that IL-1β and TNFα coordinately stimulate the expression of MMP-1 and MMP-3 are in line with previous studies in gingival fibroblasts (Wassenaar et al., 1999; Tewari et al., 1994) and implicates that the production of MMP-1 and MMP-3 is stimulated by the cytokines IL-1β and TNFα in gingival fibroblasts at inflammatory sites in the gingival tissue (Birkedal-Hansen, 1993b; Howells, 1995; Giannobile et al., 2003).

Although the role of EGF in the pathogenesis of periodontal disease is not clear, increased levels have been detected in the saliva of patients with aggressive periodontitis (Hormia et al., 1993) and increased expression of EGF receptor has been detected in inflamed gingival tissue (Chang et al., 1996). Thus the present study also investigated the expression of MMP-1 and MMP-3 by gingival fibroblasts challenged with the growth factor EGF. In accordance with IL-1β and TNFα, EGF stimulated the expression of both MMP-1 and MMP-3 in gingival fibroblasts. This is in agreement with previous findings reported in gingival and synovial fibroblasts (Hiraoka et al., 1992; Ravanti et al., 1999). The stimulatory effect of EGF on MMP-1 and MMP-3 expression suggests that EGF may be involved in the degradation of ECM in gingival tissue. This assumption is supported by previous findings demonstrating that EGF synergistically enhances IL-1 stimulated collagen degradation (van der Zee et al., 1996).

Altogether, the results of these experiments showed that expression of MMP-1 and MMP-3 in gingival fibroblasts is stimulated by the cytokines EGF, IL-1β and TNFα, suggesting that these cytokines participate in tissue degradation by inducing MMP-1 and MMP-3 expression in gingival fibroblasts.

SIGNAL PATHWAYS IN THE REGULATION OF MMP-1 AND MMP-3

Cell-adhesion and cytokines induce gene transcription through several signal pathways, including tyrosine kinases, MAPK and PKC. Tyrosine kinases are implicated as signal pathways transducing the response of growth factors and cytokines, including EGF, IL-1β, and TNFα (Vincenti et al., 1996; Yucel-Lindberg et al., 1999a). In the present study the involvement of tyrosine kinase in the cytokine stimulated expression of MMP-1 and MMP-3 was investigated by treatment of the fibroblasts with the src tyrosine kinase inhibitor herbimycin A (Vincenti et al., 1996).

Herbimycin A reduced the stimulatory effect of IL-1β and TNFα on MMP-1 and MMP-3 production, implicating the involvement of tyrosine kinase in the cytokine stimulated MMP-1 and MMP-3 production in gingival fibroblasts. This suggestion is in accordance with previous studies demonstrating that tyrosine kinase is involved in the expression of MMP-1 and MMP-3 in synoviocytes and dermal fibroblasts (Vincenti et al., 1996; Lambert et al., 2001). Moreover, there is also evidence that other kinases such as p38 MAPK and PKC, signal pathways downstream of tyrosine kinase, may also regulate the expression of MMP-1 and MMP-3 in fibroblasts (Westermarck and Kähäri, 1999; Ravanti et al., 1999; Watanabe et al., 2004, Kida et al., 2005).

The p38 MAPK has the ability to transduce signals induced by cytokines, including IL-1β and TNFα, as well as by cell-adhesion molecules, such as ICAM-1 (Matthews et al., 1999; Baud and Karin, 2001; Wang and Doerschuk, 2001; Kida et al., 2005). In the present study the possible role of p38 MAPK in cytokine stimulated expression of MMP-1 and MMP-3 was investigated using the p38 MAPK inhibitor SB203580, which inhibits the p38 MAPK subgroups α and β (Davies et al., 2000; Antonescu et al., 2005). SB203580 reduced IL-1β and TNFα stimulated MMP-1 and MMP-3 production indicating that p38 MAPK is involved in cytokine induced MMP production in gingival fibroblasts, which is in line with previous findings reported in dermal fibroblasts (Ridley et al., 1997).

The involvement of PKC in the production of MMP-1 was studied using the PKC activating phorbol ester PMA. PKC are a family of calcium and phospholipid-dependent enzymes, which exhibit increased activity in inflammatory diseases (Mellor and Parker, 1998; Cataisson et al., 2003). PMA strongly stimulated the expression of MMP-1 in gingival fibroblasts, and this response was inhibited in the presence of the PKC inhibitor BIS. The PMA stimulated MMP-1 production was

synergistically upregulated by IL-1β, which has the capability to activate PKC (Yucel-Lindberg et al., 1999b; Schütze et al., 1994). In addition, the PMA stimulated MMP-1 production was also enhanced in the presence of the calcium-ionophore A23187, suggesting that calcium-dependent PKC isoforms are involved in the stimulation of MMP-1 production (Mellor and Parker, 1998). However, as calcium is reported to stimulate the production of MMP-1 in dermal fibroblasts (Woessner, 1991; Lohi et al., 1994), the stimulatory effect of A23187 on MMP-1 expression in gingival fibroblasts disclosed in the present study may also be attributable to the presence of calcium.

The kinase PKC is reported to be upstream of p38 MAPK and both upstream and downstream, depending on stimuli, of tyrosine kinase (Westermarck and Kähäri 1999; Watanabe et al., 2004). The coeffect of PKC and these kinases in the expression of MMP-1 and MMP-3 was investigated. Interestingly, the tyrosine kinase inhibitor herbimycin A reduced PMA stimulated MMP-1 production, demonstrating that PKC mediated MMP-1 production was partly mediated by tyrosine kinase. However, simultaneous treatment of the cells with PMA and the p38 MAPK inhibitor SB203580 stimulated the production of MMP-1, indicating that the kinase p38 MAPK may inhibit the PMA stimulated MMP-1 production in gingival fibroblasts. This assumption is in line with a previous study showing that activation of p38α MAPK inhibits PMA elicited MMP-1 promotor activation (Westermarck et al., 2001).

Prostaglandin E_2 is reported to exert divergent effects on the expression of MMP-1 and MMP-3. For instance, PGE_2 stimulates the expression of MMP-1 in gingival fibroblasts, but inhibits collagenase production in synoviocytes (Dibattista et al., 1994; Sakaki et al., 2004). Furthermore, PGE_2 reduces MMP-3 expression in fibroblasts from healthy gingiva, but stimulates MMP-3 expression in fibroblasts from gingiva affected by periodontitis (Ruwanpura et al., 2004). One of the key enzymes in prostaglandin synthesis, COX-2, is upregulated in inflamed gingival tissue (Morton and Dongari-Bagtzoglou, 2001, Zhang et al., 2003) and in gingival fibroblasts stimulated with the cytokines IL-1β, TNFα and EGF (Yucel-Lindberg et al., 1999a; Yucel-Lindberg et al., 1999b). In the present study, the COX-2 inhibitor NS-398 reduced IL-1β and TNFα stimulated MMP-1 and MMP-3 production, implicating the enzyme COX-2 in the production of both MMP-1 and MMP-3. These findings also suggest that PGE_2, the result of COX-2 activity, exerts a stimulatory

effect on the expression of MMP-1 and MMP-3 in gingival fibroblasts, a suggestion further supported by recent studies in gingival fibroblasts (Sakaki et al., 2004; Ruwanpura et al., 2004).

In contrast to IL-1β and TNFα, EGF stimulated production of MMP-1 and MMP-3 was not affected by p38 MAPK, tyrosine kinase or COX-2 inhibitors, suggesting that EGF stimulated production of MMP-1 and MMP-3 is independent of these enzymes. These findings are contradictory to the fact that EGF mediates its intracellular signals through its receptor that belongs to the tyrosine kinase family (Venook, 2005) and that EGF activates COX-2 as well as p38 MAPK (Yucel-Lindberg et al., 1999a; Eguchi et al., 2001). However, it should be noted that the signal pathways transducing signals in the cells are numerous and the findings of the present study are based on investigation of only one enzyme in each of the three different signal pathways.

TIMP-1, THE ENDOGENOUS INHIBITOR OF MMP

The activity of MMP-1 and MMP-3 is firmly controlled by endogenous inhibitors known as TIMPs (TIMP-1, -2, -3, -4). TIMP-1 and -2 are found in periodontal lesions (Kubota et al., 1997). TIMP-1 preferably inhibits fibroblasts-derived MMPs including MMP-1 and MMP-3, whereas TIMP-2 preferably inhibits polymorphonuclear granulocyte derived MMPs including MMP-8 (Choi et al., 2004). To balance the activity of MMPs, the same cells that express MMP-1 and MMP-3 usually also express TIMP-1 (Chakraborti et al., 2003).

In the present study, analysis of the cellular expression of MMP-1 and TIMP-1 revealed that the number of fibroblasts expressing MMP-1 greatly exceeded the number of fibroblasts expressing TIMP-1. In healthy conditions, there is a balance between MMP and TIMP levels, to maintain the structure and components of ECM. However, in conditions characterized by tissue destruction, such as periodontal diseases, a shift in balance between MMP and TIMP in favour of MMP has been reported (Reynolds, 1996; Séguier et al., 2001; Soell et al., 2002). Investigation of the expression and regulation of TIMPs, including TIMP-1, is important in furthering our understanding of the regulation of MMPs.

The expression and regulation of TIMP-1 in gingival fibroblasts was investigated in response to cell-interaction with monocytes. In co-cultures with cell-to-cell contact, the expression of TIMP-1 increased in the fibroblasts, but not in the monocytes.

However, of monocytes adhered to fibroblasts, the number of monocytes expressing TIMP-1 increased. The stimulated expression of TIMP-1 in fibroblasts seemed to be dependent on cell-to-cell contact, as co-culturing the cells in separate co-cultures failed to stimulate the expression of TIMP-1 in the fibroblasts. In contrast to MMP-1, treatment of the co-cultures with an anti-ICAM-1 antibody did not reduce TIMP-1 expression in fibroblasts, indicating that the adhesion molecule ICAM-1 is not involved in the stimulated TIMP-1 expression. On the contrary, the number of adhered monocytes expressing TIMP-1 increased in the presence of anti-ICAM-1 antibody, indicating a divergent role of ICAM-1 in the stimulation of MMP-1 and TIMP-1 in interactions between fibroblasts and monocytes.

The effect of the pro-inflammatory cytokine IL-1β was also investigated on the expression of TIMP-1 in gingival fibroblasts. In contrast to the stimulatory effect of IL-1β on MMP-1 and MMP-3 expression, IL-1β did not stimulate the expression of TIMP-1 in gingival fibroblasts. While similar findings have been reported in dermal fibroblasts (Dasu et al., 2003), previous reports in gingival fibroblasts demonstrate a stimulatory effect of IL-1β on TIMP-1 expression (Gogly et al., 1998; Wassenaar et al., 1999). The apparently contradictory results with respect to gingival fibroblasts may be attributable to the duration of exposure of the cells to IL-1β. In this study, demonstrating that IL-1β did not affect TIMP-1 expression, the incubation time was shorter (24 h) than in studies demonstrating that IL-1β stimulate the expression of TIMP-1 (36-72 h) (Gogly et al., 1998; Wassenaar et al., 1999).

The involvement of PKC in the regulation of TIMP-1 was also investigated. The expression of TIMP-1 was increased in the presence of the PKC activator PMA, an effect reduced in the presence of the PKC inhibitor BIS. The involvement of PKC in TIMP-1 expression is in accordance with previous findings reported in synovial fibroblasts and endothelial cells (Hanemaaijer et al., 1993; Cornelius et al., 1995). Furthermore, in accordance with MMP-1, the p38 MAPK inhibitor SB203580 synergistically upregulated the PMA stimulated TIMP-1 expression, indicating that p38 MAPK inhibits both PMA stimulated TIMP-1 and MMP-1 expression in gingival fibroblasts. However, in contrast to MMP-1, the PMA stimulated TIMP-1 production was reduced in the presence of the calcium-ionophore A23187. The inhibitory effect of calcium may be explained by the fact that calcium down-regulates TIMP-1 production (Halverson et al., 1998; Bai et al., 2001). Thus, it seems that calcium and IL-1β, which are increased during inflammation, exert divergent effects on MMP-1

and MMP-3 compared to TIMP-1 production, creating an imbalance of these MMPs and TIMP-1 in favour of MMP-1.

CLINICAL ASPECTS

MMPs including MMP-1 and MMP-3 are key enymes in tissue degenerative diseases including periodontal diseases. Therefore, attempts to control the expression and activity of MMPs have gained much attention in treatment of periodontal diseases. Increased knowledge of the expression and regulation of MMP-1 and MMP-3 may lead to the development of new strategies for MMP inhibition. The *in vitro* model used in this thesis allows investigation of the regulation of MMP-1 and MMP-3 in gingival fibroblasts in inflammatory conditions mimicked by the presence of monocytes and cytokines.

The findings in this thesis, that the anti-inflammatory drugs tetracycline doxycycline and the glucocorticoid dexamethasone reduces the monocyte or cytokine induced expression of MMP-1 or MMP-3, suggests that dexamethasone and doxycycline may encounter beneficial effects in reducing tissue degradation in periodontal diseases. This assumption is in line with the findings that dexamethasone reduces collagen degradation, and that doxycycline, known to inhibit the transcription as well as the activity of MMPs, is suggested to prevent tissue degradation during periodontal diseases (Zhu et al., 2001b; Peterson et al., 2004; Gapski et al., 2004; Lee et al., 2004). In addition, the findings of this thesis also suggest that regulation of MMP-1 and MMP-3 in gingival fibroblasts may be achieved by modulation of extra cellular signals such as interactions with monocytes or cytokines as well as modulation of intracellular signals such as signal transduction pathways. Interestingly, previous reports have shown that IL-1β and TNFα antagonists block the progression of inflammatory cell infiltrate as well as periodontal attachment and bone loss in animal models with periodontal diseases (Salvi and Lang, 2005). Furthermore, the results in this thesis, that the signal pathways PKC, MAPK, tyrosine kinase as well as the enzyme COX-2 regulate MMP expression, highlight these signal pathways as therapeutic targets in treatment of inflammatory conditions, which has also been discussed in previous reports (Chakraborti et al 2003, Westermarck et al., 1999; Saklatvala et al., 2004; Salvi and Lang, 2005).

The results from this thesis may be of importance for future treatment strategies of periodontal disease. However, caution should be exercised in the extrapolation of

the results in this study, since the *in vivo* conditions are more complex than strictly controlled laboratory conditions. Therefore, future studies are needed to investigate the *in vivo* expression of MMP-1, MMP-3 and their inhibitors TIMPs in gingival tissue in individuals with periodontal diseases.

CONCLUSIONS

- The expression of MMP-1 in gingival fibroblasts is stimulated by direct cell-to-cell contact with monocytes as well as by soluble factors derived from monocytes in separated co-cultures. Furthermore, the expression of TIMP-1 increased in co-cultures with cell-to-cell contact, but not in fibroblasts in separated co-cultures.

- Cell-to-cell contact between gingival fibroblasts and monocytes stimulates the expression of MMP-1 and TIMP-1 as well as ICAM-1 in gingival fibroblasts. The stimulated MMP-1 expression is, in contrast to TIMP-1, mediated partly by the adhesion molecule ICAM-1 and the p38 MAPK signal pathway.

- The cytokines IL-1β, TNFα and EGF stimulate the expression of MMP-1 and MMP-3 in gingival fibroblasts. The stimulatory effect of IL-1β and TNFα on MMP-1 and MMP-3 expression is mediated partly by the signal transduction pathways p38 MAPK and tyrosine kinase.

- The PKC activator PMA stimulates both the expression of MMP-1 as well as the expression of TIMP-1. However, PMA stimulated MMP-1 and TIMP-1 expression in gingival fibroblasts is regulated differently by IL-1β and calcium.

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