# CHAPTER 2

# GELATINASE A (MMP-2) ACTIVATION BY SKIN FIBROBLASTS: DEPENDENCE ON MT1-MMP EXPRESSION AND FIBRILLAR COLLAGEN FORM


## Summary

The respective requirements of collagen and MT1-MMP in the activation of MMP-2 by primary fibroblast cultures were explored further. Thouree-dimensional gels enriched in human collagens types I and III or composed of recombinant human type II or III collagen, caused increased MT1-MMP production (mRNA and protein) and induced MMP-2-activation. Only marginal induction was seen with dried monomeric collagen confirming the need for collagen fibrillar organisation for activation. To our surprise, relatively low amounts (as low as 25μg/ml) of acid soluble type I collagen added to fibroblast cultures also induced potent MMP-2-activation. However, the requirement for collagen fibril formation by the added collagen was indicated by the inhibition seen when the collagen was pre-incubated with a fibril-blocking peptide, and the reduced activation seen with alkali-treated collagen preparations known to have impaired fibrilisation. Pre-treatment of the collagen with sodium periodate also abrogated MMP-2-activation induction. Further evidence of the requirement for collagen fibril formation was provided by the lack of activation when type IV collagen, which does not form collagen fibrils, was added in the cultures. Fibroblasts derived from MT1-MMP-deficient mice were unable to activate MMP-2 in response to either 3-dimensional collagen gel or added collagen solutions, compared to their littermate controls. Collectively, these data indicate that the fibrillar

structure of collagen and MT1-MMP are essential for the MMP-2-activational response in fibroblasts.

## Introduction

Tissue remodeling is an important process in both physiological and pathological conditions. Among the potential enzymes involved in matrix degradation required for tissue remodeling are the matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases (Woessner, 1994). MMP-2 (Gelatinase A) is an MMP family member with selective ability to degrade type IV collagen, a major structural component of the basement membrane, and gelatin, representing denatured collagen. It shares this capacity with MMP-9 (Gelatinase B) by virtue of a common gelatin-binding domain. However, whilst MMP-9 is highly regulated at the transcriptional level, MMP-2 is not, and its rather general expression is regulated at the level of zymogen activation and/or inhibition by the family of Tissue Inhibitors of MetalloProteinases (TIMPs). Increased levels of active MMP-2 have been associated with invasion and metastasis in various kinds of tumours (Nomura et al., 1995; Koshiba et al., 1998). Activation of the latent pro-MMP-2 zymogen is overwhelmingly effected by membrane type (MT) -MMPs, a novel subfamily of MMPs (Murphy et al., 1999; Seiki, 1999). MT-MMPs contain a furin-like enzyme cleavage motif, and are largely thought to be constitutively activated during cellular transport thourough the Golgi apparatus (Sato et al., 1999). However, endogenous expression of MT-MMP is insufficient to effect MMP-2 activation, and treatment with additional agents is usually required (Murphy et al., 1999; Seiki, 1999). The plant lectin, Concanavalin A (Con A), and a phorbol ester 12-*O*-tetradecanonylphorbol 13 acetate (TPA), potently stimulate activation in a wide variety of cells and thourombin stimulates activation in endothelial cells (Murphy et al., 1999; Seiki, 1999).

Induction of MMP-2 activation in response to thouree-dimensional collagen substratum has been shown by us and others in fibroblasts (Azzam and Thompson, 1992; Seltzer et al., 1994; Gilles et al., 1997; Lee et al., 1997; Tomasek et al., 1997; Boyd and Balkwill, 1999; Preaux et al., 1999), cancer cell lines (Azzam et al., 1993; Gilles et al., 1997; Ellerbroek et al., 1999; Kurschat et al., 1999; Ellerbroek et al., 2000) and endothelial cells (Haas et al., 1998; Haas et al., 1999). As seen with Con A (Yu et al., 1995), collagen-induced MMP-2 activation involves increased steady-state levels of MT1-MMP mRNA and protein (Gilles et al., 1997; Gilles et al., 1998), as well as a non-transcriptional component which can be seen in MCF-7 cells transfected with MT1-MMP driven by the heterologous CMV promoter (Gilles et al., 1998). Co-expression of collagen $\alpha$1(I) and MT1-MMP mRNA transcripts has been detected in fibroblasts around the breast and pancreatic tumours (Gilles et al., 1997; Ellenrieder et al., 2000), suggesting that collagen-regulation of MT1-MMP may be a physiological counterpart to the artificial induction of MMP-2 activation by Con A. This appears to be an important mechanism for fibrillar matrix remodeling, given the over-abundance of collagen seen in mice lacking MT1-MMP (Holmbeck et al., 1999; Zhou et al., 2000).

A number of studies have indicated a specificity for collagen over other matrix components in this induction, and also a requirement for the thouree dimensional structure of the collagen into fibrillar gels, since thin coatings of various collagen preparations, or denatured type I collagen (gelatin) gels, failed to induce MMP-2 activation (Azzam et al., 1993; Gilles et al., 1997; Maquoi et al., 1998). In the current study, we have extended our analysis to preparations enriched in various types of human collagen, (ii) characterised the activation seen in response to direct addition of soluble collagen to the culture medium, (iii) further evaluated the requirement for

collagen fibril formation for induction of MMP-2 activation, and (iv) confirmed an essential role for MT1-MMP in this process. We employed normal human skin fibroblasts, which have shown a similar MMP-2 activational response profile to breast-derived fibroblasts (Azzam and Thompson, 1992), and also primary mouse fibroblast cultures derived from mice which lack MT1-MMP (Holmbeck et al., 1999).

## Experimental Procedures

### 1. Human Collagen Preparation

Fibril-forming collagens were prepared from fetal human skin, obtained with Royal Children Hospital Institutional Ethics approval, using established procedures (Chan et al., 1990). The sample was extracted sequentially with acetic acid and pepsin, and latter subjected to differential salt precipitation with 0.7 M, 0.7-0.9 M, 0.9-1.2 M and 1.2-2.4 M NaCl to prepare different collagen fractions. The composition and purity of these preparations were analysed by 5% SDS-PAGE and protein staining with Coomassie Brilliant Blue R-250, as previously described (Chan et al., 1990). The acid extract contained only type I collagen ($A_I$) while the pepsin extract (P) contained a mixture of type I collagen (70%), type III collagen (30%) and trace amounts of type V collagen. Subsequent salt fractionation with 0.7 M NaCl resulted in a preparation enriched in type III collagen ($P_{III>I}$) that contained about 80% type III collagen; 0.7-0.9 M NaCl fractionation produced a mixture of type I and type III collagens with a proportion similar to the total extract ($P_{I>III}$) with mixture of type I collagen (70%) and type III collagen (30%). The 0.9-1.2 M NaCl fraction contained predominantly type I collagen ($P_I$). Type I collagen in the acid extract contains intact

N- and C- non-helical telopeptides which facilitates *in vitro* fibrillogenesis (data not shown). The acid-extracted collagen also contains more cross-linked β components ($\beta_{1,1}$ and $\beta_{1,2}$) relative to the α-chains components. Collagen concentrations were measured by hydroxyproline assay (Jamall et al., 1981) and collagen preparations were adjusted to 3mg/ml by precipitation with salt and dissolving in an appropriate volume of 0.5 M acetic acid, followed by dialysis against 0.012 M HCl. The final concentration was reconfirmed by hydroxyproline assay. Sterile conditions were maintained in every step of the procedure. Recombinant type III collagen was prepared in a baculovirus expression system (Lamberg et al., 1996), and recombinant type II collagen was purified from transfected HT-1080 fibrosarcoma cells (Fertala et al., 1994). Vitrogen (Collagen Corp. Palo Alto, USA) was used as a positive control, as previously described (Azzam et al., 1993; Gilles et al., 1997). Type IV collagen extracted from human placenta was purchased from Sigma (St. Louis, MO). The purity of the recombinant human type II $[\alpha1(II)]_3$ and purified human type III $[\alpha1(III)]_3$ was confirmed by SDS-PAGE, where they migrated as single bands of appropriate size and lacked cross-link component (data not shown). Vitrogen was confirmed to contain approximately 5 % type III and 95 % type I collagen as reported by the supplier.

**2. Preparation of Collagen into Various Formats**

Collagen preparations were stored in 0.012 M HCl at 4°C. To prepare a 3-dimensional collagen gel, collagen solutions were neutralized to pH 7.0 with 1 M NaOH at 37°C, and allowed to gel in cell culture vessels by incubating the neutralized collagen solution at 37°C for two hours in a cell incubator. Typically, neutralized

collagen solutions were adjusted to 2 mg/ml and gelled at 0.25 ml/cm$^2$. It is difficult to prepare a 3-dimensional gel of pure type III collagen *in vitro* because type III collagen is normally co-polymerized with type I collagen. To allow the presentation of type III collagen within a 3-dimensional structure, Matrigel, a reconstituted basement membrane extract which can also form a 3-dimensional gel but does not induce MMP-2 activation by breast cancer cells or fibroblasts (Azzam and Thompson, 1992; Seltzer et al., 1994; Haas et al., 1998), was spiked with purified type III collagen. To prepare culture vessels coated with monomeric form of collagen, collagen solution was added directly to the culture dishes and the acid solvent was allowed to evaporate, leaving a thin coating. These collagen coatings were washed several times before the experiment to remove unbound collagen.

**3. Preparation of Modified Collagens**

Alkali-treated collagen preparations, which are compromised for fibril-formation (Hattori et al., 1999; Suzuki et al., 1999), were kindly provided in lyophilised form by Dr. Hattori S. Nippi Research Institute of Biomatrix, Tokyo, Japan and are from the same batch as previous reported (Hattori et al., 1999). These were reconstituted in 0.025 % acetic acid at 3 mg/ml, stored at 4°C, and added directly into the fibroblast cultures at 100 μg/ml. Heat-denatured collagen (gelatin) was prepared from Vitrogen by heating to 65°C for 30 minutes. Periodate treatment of collagen was performed as described by Vogel et al. (Vogel et al., 1997). Briefly, Vitrogen was incubated with 10 mM sodium m-periodate (Sigma, MO) for 20 minutes at room temperature in the dark. The reaction was terminated by adding 20mM

sodium bisulphite. Periodate-treated collagen was dialysed sequentially against 10 mM acetic acid, and 0.012 M HCl.

**4. Inhibition of Fibril Formation using a Synthetic Peptide**

An α2 C-telopeptide sequence which was shown previously to inhibit fibril formation *in vitro* (Prockop and Fertala, 1998) was purchased from Auspep (Melbourne, Australia). The purity was confirmed by HPLC to be >95 %, as was stability in our culture conditions over a 3 day period. The peptide (final concentrations 0, 1.5 and 2.5 mM) was dissolved in SFM and pre-incubated with acid-solubilised collagen (30 μg/ml) for 3 hours at room temperature and another 24 hours at 37°C prior to add to fibroblast culture and further incubation for 72 hours. As a control, some cultures were treated with peptide in conjunction with acid-solubilised collagen (100 μg/ml, 72 hours) or Con A (25 μg/ml, 48 hours) without pre-incubation, to control for direct inhibition or toxicity of the peptide.

**5. Cell Culture Conditions for MMP-2 activational studies**

Normal human skin fibroblasts (passage 11-15; Chan et al., 1990), and MT1+/+, +/- and -/- mouse fibroblasts (passage 3-5; Holmbeck et al., 1999) were derived and cultured as previously reported. 24-well culture plates (Falcon, NJ) were first coated with 500 μl of 2 mg/ml of the different collagen types or forms of collagen as described above. Vitrogen gel was used as a positive control, and an uncoated culture dish as negative control. 50,000 cells were then plated in Dulbecco's Modified Eagle medium (DMEM; Life Technology Grand Island, NY) supplemented with 10 % fetal

bovine serum (FBS; CSL Limited Biosciences Parkville Victoria, Australia; batch no. 53501). After an overnight incubation, cells were washed 3 times with unsupplemented medium and replaced with 300 µl of the serum free medium [SFM: Gilles et al., 1997]. In some experiments, acid-soluble ($A_1$) or the modified collagen was added directly to the culture medium at this stage. After incubation for a further 96 hours, conditioned media were collected and analyzed for MMP-2 activation by zymography. Cell number was determined with the colorimetric assay of cell viability, based on the cleavage of tetrazolium salt WST-1 (Boerinhger Mannheim, Germany) by mitochondrial dehydrogenases in viable cells.

**6. Gelatin Zymography**

Analysis of gelatinolytic activity was performed as previously described (Gilles et al., 1997) on non-reducing 10 % SDS-PAGE gels containing 0.15 % gelatin (w/v). Briefly, serum-free conditioned medium samples were diluted 1:3 with Laemmli sample buffer; 62mM Tris/HCl, pH 6.8, 2 % SDS (w/v), 10 % glycerol (v/v), and 0.05 % bromphenol blue (w/v) and loaded onto the gel (5 µl/lane). After electrophoresis, gels were washed twice for 1 hour at room temperature in 2.5 % Triton X-100 solution (v/v) and incubated overnight at 37°C in a developing buffer containing 10mM $CaCl_2$, 50 mM Tris, 0.15 M NaCl, and 1 % Triton X-100. The gels were stained with 0.1 % (w/v) Coomassie Brilliant Blue R-250 in 12.5 % (v/v) ethanol and 7.5 % (v/v) acetic acid, followed by destaining in 12.5 % ethanol, 7.5 % acetic acid. Clear bands against the blue background represent latent (72 kDa), intermediate (62 kDa) and active (59 kDa) forms of MMP-2, respectively.

**7. Northern-blot Analysis**

For analysis of MT1-MMP mRNA expression, cells were recovered from the collagen gel by digestion with bacterial collagenase type II (Clostridium histolyticum; Worthington Biochem Corp., NJ) for 15 minutes at 37°C, and total RNA was extracted using RNAzol B$^{TM}$ (TEL-TEST, Inc., TX). Twenty micrograms of total RNA was used from each sample for Northern-blot analysis. RNA integrity was confirmed by the presence of undegraded ribosomal RNAs, and was transferred onto a positively charged nylon membrane (GeneScreen Plus, Life Science, MA). Prehybridization was carried out at 42°C for at least 2 hours in Hybrisol$^{R}$ I (Intergen, NY) containing 10 μg/ml fish sperm DNA (Boehouringer Mannheim, GmbH, Germany). MT1-MMP transcripts were detected using a 3.4-kb $^{33}$P-radiolabeled cDNA probe, derived by random primer labeling (Beohouringer Mannheim, GmbH, Germany) of the MT1-MMP cDNA (Sato et al., 1994) subcloned into pBluescript SK+. Hybridization was carried out overnight at 42°C in a hybridization oven (Hybaid, UK) with approximately 1x10$^{6}$ c.p.m. of the column-purified probe (Amersham, NJ). The nylon membrane was washed twice for 15 minutes in 2X SSC [0.3 M NaCl, 0.3 M sodium citrate (pH 7)], 0.1 % (w/v) SDS, then thouree times for 10 minutes in 0.2X SSC, 0.1 % (w/v) SDS at 42°C. Radioactive bands were detected and quantified using a Phosphor-Imager (Molecular Dynamics). The concentration of RNA was normalized to the expression of GAPDH mRNA (Southby et al., 1995).

**8. Western-blot Analysis**

Cells cultured in 6-well plates were incubated with various types of collagen (100 μg/ml), washed with PBS, and lysed directly with RIPA buffer (50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 % NP-40, 0.25 % Na-deoxycholate) containing proteinase inhibitors; 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 1 mM NaF, 1 mM NaP and 1 mM $Na_3VO_4$. Protein loading was normalized with respect to cell number, estimated by cell counting from a duplicate well. Samples were mixed with 4X sample buffer (62.5 mM Tris pH 6.8, 2 % SDS (w/v), 10 % glycerol (v/v), 5 % β-mercaptoethanol (v/v), and 0.2 % Bromphenol Blue (w/v), boiled for 10 minutes, separated on 10 % SDS-PAGE, and then transferred onto the PVDF membrane (Immobilon$^{TM}$-P Millipore Corp., Bedford, MA). Transferring was monitored by reversible staining with Ponceau Red (Sigma, MO). The membrane was blocked in 5 % skim milk, 0.1 % Tween-20 in PBS (pH 7.5) for 2 hours before exposure to the primary antibody (Mab 114-1F2, 1 μg/ml; Oncogene Research Products, MA) overnight at 4°C. The membrane was washed and then incubated with a secondary goat anti-mouse IgG antibody conjugated with horseradish-peroxidase (Pierce, IL) diluted 1:10,000. Signals were developed with an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions (Pierce, IL). The filter was stripped with 2 % SDS, 100mM β-mercaptoethanol in 62.5 mM Tris/HCl, pH 6.8, for 30 minutes at 50°C, and reprobed with 1 μg/ml of antibody against human vinculin (VII-F9B11), a gift from Dr. Victor Koteliansky, as described previously (De Nichilo and Yamada, 1996) to normalize the loading amount.

## Results

### Effect of Different Collagen Preparations on the Activation of MMP-2 and MT1-MMP

When used as a 3-dimensional gel, each of the fibril-forming collagen preparations containing either collagens types I, II, or III alone, or mixtures of types I and III collagens induced a similar degree of activation of the endogenous MMP-2 in human skin fibroblast cultures (Figure 2.1A). Time course for induction of MMP-2 activation by the various collagen preparations also revealed no significant differences between each collagen preparation, with MMP-2 activation becoming evident by 24 hours and increasing progressively thourough the 96-hour time course (data not shown). The collagen-stimulated fibroblast cultures tend to only show the fully mature species (~59 kDa) with little or no evidence of the intermediate form (~62 kDa) which is often seen with breast cancer cell lines and HT1080 cells (Azzam and Thompson, 1992).

Given the previous observations by us and others that a 3-dimensional collagen structure was required for induction of MMP-2-activation in diverse cell types, we were surprised at the recent report of ovarian carcinoma cell lines responding to the addition of low levels (from 4 μg/ml) of acid-solubilised collagen into the conditioned medium (Boyd and Balkwill, 1999). We confirmed that each of the fibril-forming collagen preparations which showed activity as a 3-dimensional gel also showed potent induction of MMP-2-activation when added to a final concentration of 100 μg /ml (Figure 2.1B). Again only the fully mature MMP-2 species was seen after

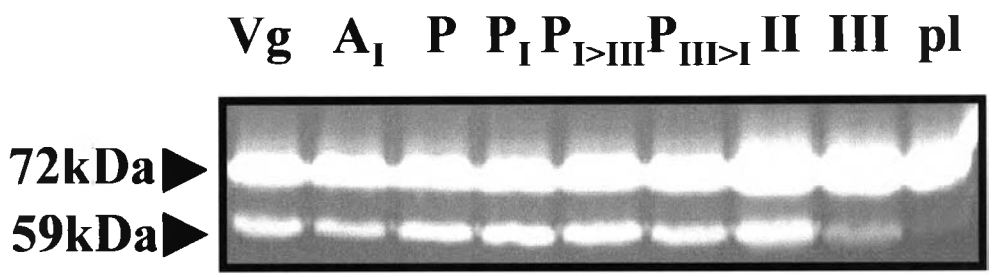
collagen stimulation. No MMP-2 activation was induced by the added type IV collagen.

Western-blot analysis of MT1-MMP levels was performed on the cell lysates from cells treated with each collagen solution (Figure 2.2). As seen in MCF-7-vector control lysates in Figure 2.2A, thouree additional non-specific bands are detected with this antibody using the current protocols, however, the ~60 kDa species was only seen in MT1-MMP-transfected MCF-7 cells, and also specifically depleted from lysates from MT1-MMP -/- mouse fibroblasts (data not shown). This represents the mature form of MT1-MMP, and co-migrates with the form present in unstimulated MDA-MB-231 breast cancer cells (Yu et al., 1995; Pulyaeva et al., 1997). Significant increase in MT1-MMP protein levels was observed in cells treated with each of the different fibril-forming collagen preparations, and were especially increased with the addition of pepsinized preparations enriched in type I (P, $P_I$, $P_{I>III}$) and the recombinant type II collagen.

We further tested the dose-dependence of Vitrogen, acid-solubilised human collagen, or whole pepsin extracted collagen (P) when added in the soluble form (Figure 2.3), reasoning that the telopeptide sequences in the acid-solubilized collagen may facilitate fibril formation at a lower concentration, and thus promote MMP-2-activation earlier. However, a similar dose/ activity profile was seen for each of the purified human collagen preparations, both of which were more active than the Vitrogen. Concentrations of soluble fibril-forming collagen as low as 12.5 μg/ml induced detectable MMP-2 activation and this increased in a dose dependent manner. Higher concentrations (from 25 μg/ml added) resulted in a visible layer of collagen over the cell layer.

**Figure 2.1:** The activation of endogenous MMP-2 by human skin fibroblasts in response to various fibril-forming collagen types in 3-D gel form (*A*) or when added in acid-soluble form directly to the conditioned medium (*B*) was compared by gelatin zymography analysis of 96-hour conditioned media. Cells were plated on plastic (pl) as a control in each case. 72 kDa and 59 kDa indicate latent and fully active forms of MMP-2, respectively. Vitrogen (commercial pepsin extracted bovine collagen preparation enriched in type I collagen) (Vg), an acid-extracted human skin preparation enriched in type I collagen ($A_I$), and pepsin extracted human skin collagen containing a combination of type I+III+V (P), enriched in type I collagen ($P_I$), enriched in type I compared to type III ($P_{I>III}$), or enriched in type III compared to type I ($P_{III>I}$), including the recombinant human types II (II) and III (III) collagen, and commercial purified type IV collagen extracted from human placenta (IV).

**A**


Vg A$_I$ P P$_I$ P$_{I>III}$ P$_{III>I}$ II III pl
72kDa
59kDa

**B**

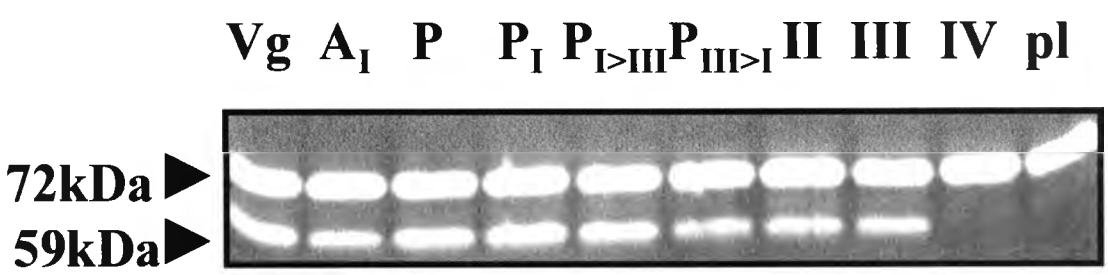

Vg A$_I$ P P$_I$ P$_{I>III}$ P$_{III>I}$ II III IV pl
72kDa
59kDa

**Figure 2.2:** Effect of various collagen preparations on expression of MT1-MMP protein in normal human skin fibroblasts. 100 μg/ml solution form of various types of collagen preparations, including recombinant human type II, purified human type III and human placental extracted type IV, was incubated with culture of human fibroblasts in SFM for 96 hours. Loading of protein lysates were based on an equal number of the cells and also normalized by expression of vinculin. Thirty microlitres of the total cell lysate from either MT1-MMP- or vector control-transfected MCF-7 lysates (*A*) or each of the collagen-treated fibroblasts samples (*B*) was separated using 10 % SDS-PAGE, transferred to the membrane and firstly immunostained with 1 μ g/ml of 114-1F2 MT1-MMP Mab. Three non-specific bands are shown in both MCF-7 lysates as well as the specific band of ~60 kDa active form which is seen only in the MT1-MMP-MCF-7 lysate (A). For the collagen-treated cells (*B*), only the ~60 kDa active MT1-MMP band is shown, as well as the 123kDa band decorated with 1 μg/ml VII-F9B11 vinculin Mab after stripping the membrane for standardization of loading amount. *C.* Densitometric analysis was used to determine the fold-change of MT1-MMP expression in cells treated with various collagens over that of the cells grown on plastic.

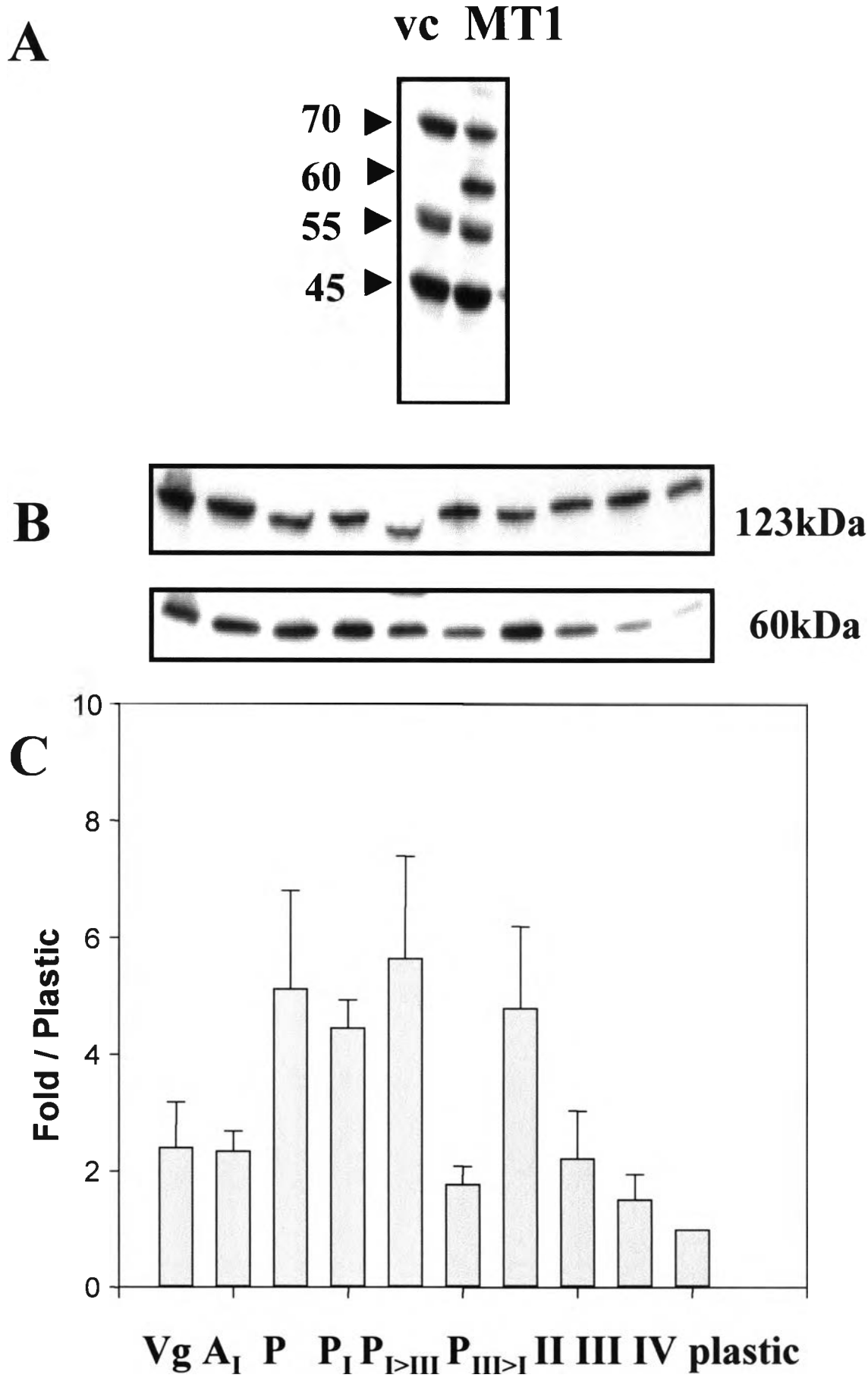
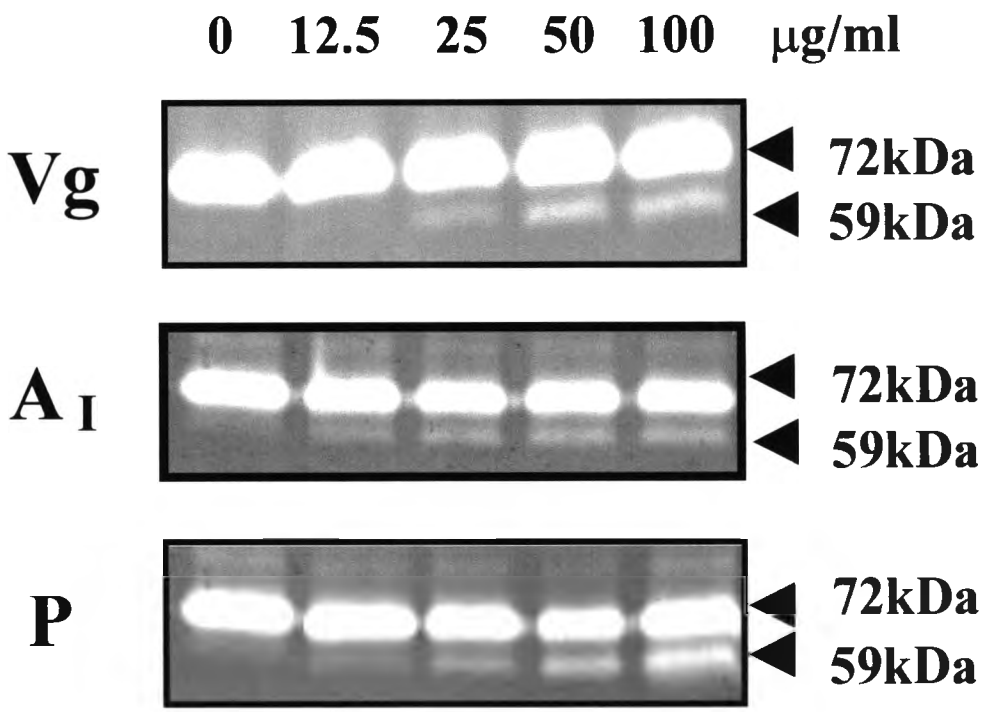
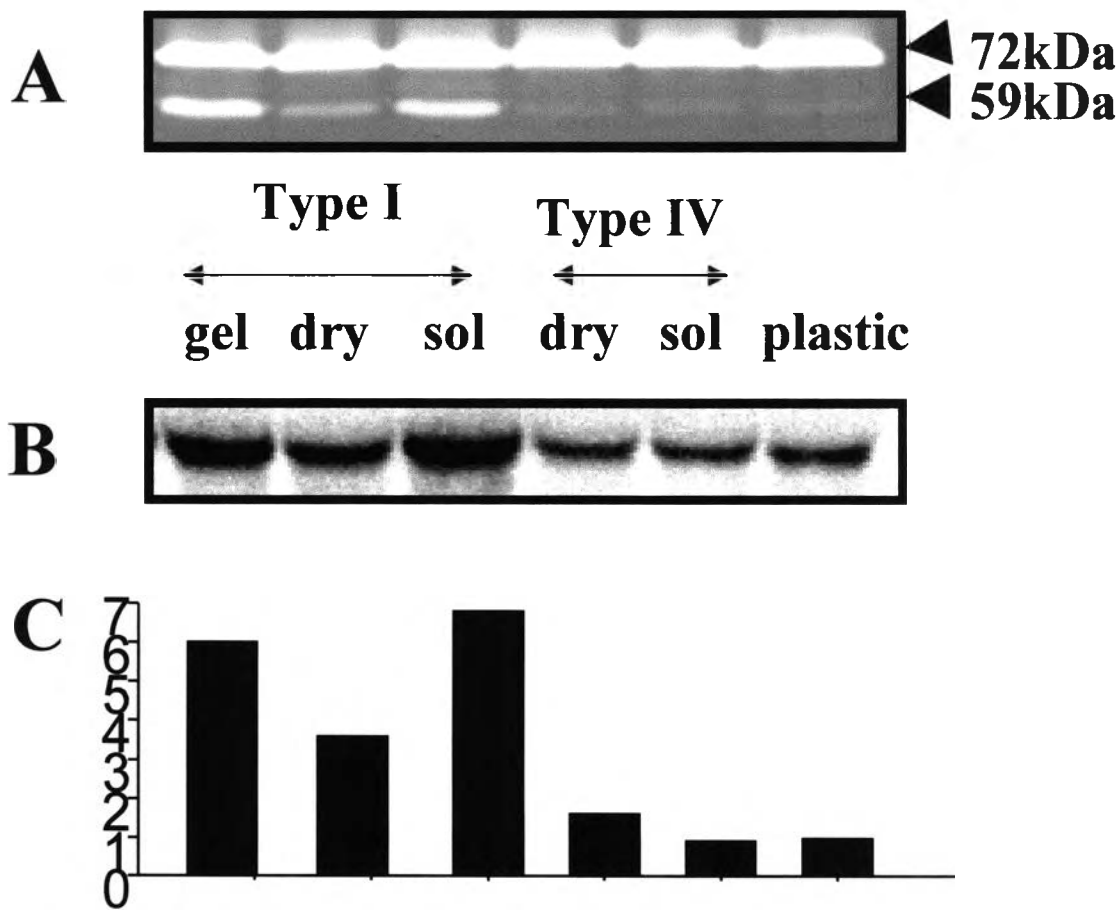

A
vc MT1
70
60
55
45
B
123kDa
60kDa
C
Fold / Plastic
10
8
6
4
2
0
Vg A_I P P_I P_I>III P_III>I II III IV plastic

**Figure 2.3:** Collagen solution induces MMP-2 activation in dose dependent manner. MMP-2 activation analysis by zymography of 96 hour-conditioned media collected from human skin fibroblasts cultured on plastic and stimulated by adding Vitrogen (Vg), acid extracted human collagen ($A_1$), or pepsin extracted human collagen (P) solution at concentration 12.5, 25, 50, and 100 μg/ml. Cells cultured on plastic only used as a control. The Upper and lower bands represent 72 and 59 kDa, respectively.


0 12.5 25 50 100 μg/ml
Vg
72kDa
59kDa
A I
72kDa
59kDa
P
72kDa
59kDa

The activity achieved with addition of collagen solution enabled us to test type IV collagen without the need to form a 3-dimensional gel. As shown in Figure2.4, fibril-forming type I collagen (Vitrogen) in all thouree formats tested (gel, solution, and to a lesser extent, dry) induced the activation of MMP-2 and MT1-MMP mRNA expression. The MMP-2 activation seen with the dried collagen, albeit lower than with the 3D gel, is in contrast to what we and others have previously reported, and this is probably due to our using here an equivalent amount of collagen to that employed in the 3D gel. Our previous work employed significantly less (~10 μg) collagen in the dried monomeric form. The higher levels used here may allow for some of the collagen molecules to release from the plastic surface and fibrilise. In contrast, the network-forming type IV collagen did not induce activation of MMP-2 in any format, even when we increased the total amount of type IV collagen to the level needed for fibril-forming collagens to form a gel. As shown in Figure 2.4, increased MT1-MMP mRNA levels were seen, and these were commensurate with the amount of induced MMP-2-activation. Indeed, all fibril-forming collagen preparations tested stimulated the MT1-MMP mRNA levels to a similar degree (data not shown). Increases of at least 3-fold, and up to nearly 5-fold, were seen after 96 hours of culture, with little difference seen among the different collagen preparations.

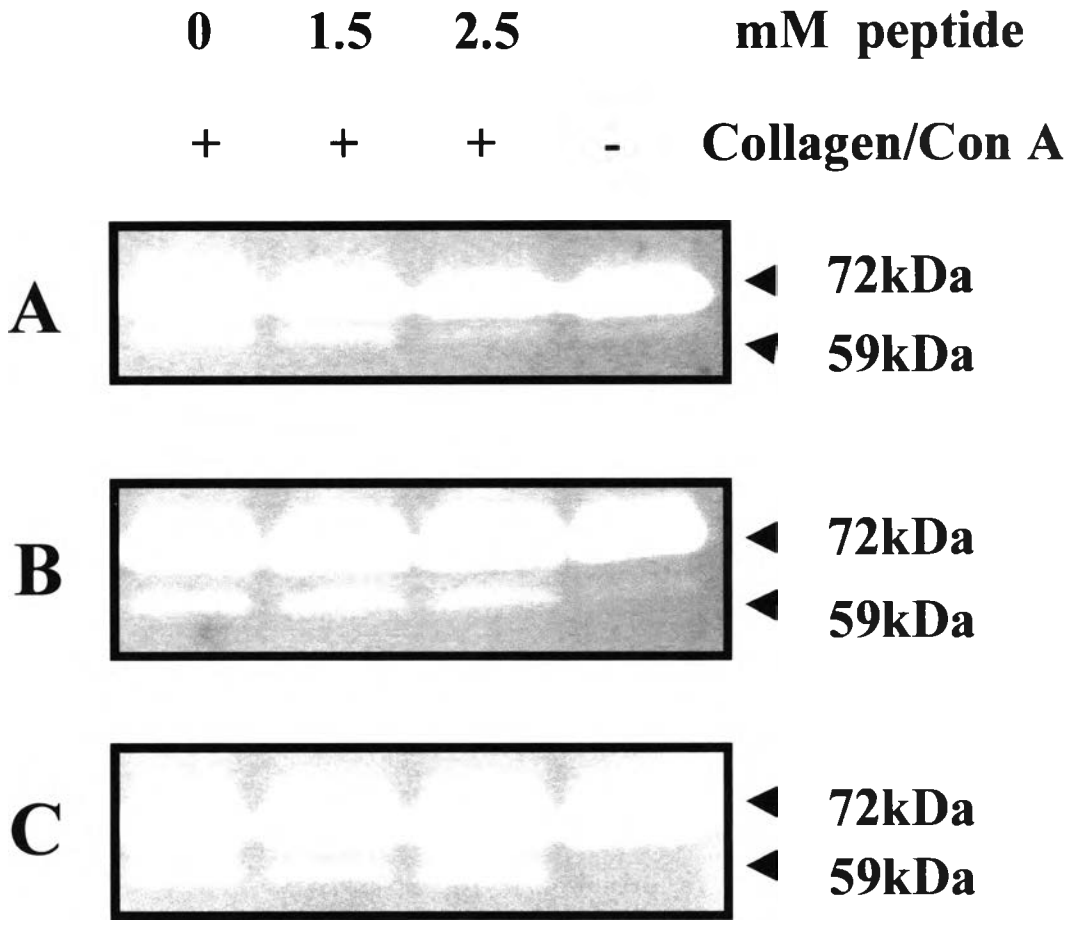
**Figure 2.4:** Collagen type-specificity for MMP-2 activation and role of MT1-MMP. Commercial types I (Vitrogen) and IV (collagen extracted from human placenta) collagen prepared in different formats (fibrillar 3-D gel, dry monomeric coating, and directly added solution) with equal amount of total collagen were introduced to human skin fibroblast culture. *A.* The ability to induce endogenous MMP-2 was analyzed by zymograpghy from 96 hour-collected conditioned media. Exogenous MMP-2 artificially activated with 1 mM aminophenylmercuricacetate (APMA) was used as an MMP-2 standard (not shown). *B.* Autoradiograph of Northern analysis of MT1-MMP. 3.4 kb $^{33}$P-radiolabeled human MT1-MMP cDNA probe was hybridized to the Northern blot containing 20 μg/lane of total RNA extracted from 96 hour-human fibroblast cultured on different formats and types of collagen. Expression level was normalized by expression of 1.3 kb GAPDH (not shown). *C.* Densitometric analysis of *B.* demonstrates as bar graphs representing fold increased above the basal expression level of MT1-MMP mRNA in cells grown on plastic.


A
72kDa
59kDa
Type I
Type IV
gel dry sol dry sol plastic
B
C
7
6
5
4
3
2
1
0

**Specificity for Fibrillar Form of Collagen for Induction of MMP-2 Activation**

Given the differences seen between the levels of MMP-2 activation achieved with collagen presented as a 3-dimensional gel, to that achieved with the dried monomeric, we hypothesized that the fibrillar form is required to induce MMP-2 activation. We used a number of collagen modification strategies to explore this requirement further. The synthetic peptide (GGGYDFGYDGDFYRA) from the C-telopeptide region has been shown to disrupt collagen fibril formation (Prockop and Fertala, 1998). When our acid-extracted collagen ($A_1$) was pretreated under acid conditions with the peptide at 1.5 mM and 2.5 mM before addition to the cell culture, we found a concentration-dependent decrease in the capacity of the collagen to subsequently induce MMP-2-activation (Figure 2.5). Preincubation of the collagen solution was important to allow full equilibration of the monomeric collagen molecules, and as expected, considerably less inhibition was seen when the same amounts of peptide were added to the cultures simultaneously with the collagen solution. This indicates that the peptide was not directly inhibiting MMP-2-activation during the culture period thourough unforeseen toxicity, and this was further supported by the observation that no inhibition was seen with the same amounts of peptide when activation was induced by an unrelated mechanism, (i.e. 30 μg/ml Con A/ 48 hours).

**Figure 2.5:** Inhibition of fibril formation by specific peptide abrogates activation of endogenous MMP-2 induced by acid extracted collagen ($A_I$) solution. *A.* 30 μg/ml of acid extracted collagen ($A_I$) solution was pretreated in SFM with final concentrations of 0, 1.5 and 2.5 mM of peptide inhibitor, respectively, at room temperature for 3 hours followed with 37°C overnight before being transfer to the culture of human skin fibroblasts and culture for 72 hour-period. *B.* Culture of human skin fibroblasts was stimulated with 30 μg/ml of $A_I$ mixed with different concentrations of peptide inhibitor, as described above, without peptide-collagen pre-treatment. *C.* Induction of MMP-2 activation by 30 μg/ml Con A in conjunction with different fibril-forming inhibitory peptide concentrations demonstrates specific inhibitory effect of peptide. 72 hour-conditioned media from *A* and *B*, and 48 hour-conditioned media from *C* were collected and analyzed for MMP-2 activation by zymography.


0
1.5
2.5
mM peptide
+
+
+
-
Collagen/Con A
A
72kDa
59kDa
B
72kDa
59kDa
C
72kDa
59kDa

We also tested collagen treated with alkali for different periods (0, 4 hours, 2 and 15 days), which was previously shown to be compromised for fibril formation (Hattori et al., 1999; Suzuki et al., 1999). SDS-PAGE analysis confirmed that both α1 (I) and α2(I) chains exhibited slower mobility than those of acid- or pepsin-extracted collagen, presumably because of the reduction of the hydrophobicity as the result of deamination of the acid amide group during the alkali treatment (Figure 2.6; Hattori et al., 1999). Alkali pretreatment of the collagen reduced its capacity to induce MMP-2-activation when added directly to the culture medium, with complete abrogation of the effect seen after 2 days alkali treatment.

One specialised receptor pathway known to be periodate-sensitive is the discoidin domain-containing receptor-like tyrosine kinases (DDRs), identified recently as novel signaling receptors for collagens (Vogel, 1999). DDR1 is activated specifically by collagen type I-VI, while DDR2 shows a more limited specificity for collagen types I and III. The native triple helical structure of collagen is also essential for DDR binding and activation (Vogel et al., 1997), however, fibril requirements have not been explored. Periodate treatment of collagen was tested since this abrogates the capacity of collagen to activate the discoidin domain receptors (DDRs). As seen in Figure 2.7, periodate treatment abrogated the MMP-2-inductive capacity of Vitrogen when used either as a 3-D gel, or applied in solution form. To ensure this was not due to oxidative attack by residual periodate, we found (data not shown) that periodate-treated collagen did not inhibit MMP-2-activational responses to either Con A (25 μg/ml, 48 hours) or Vitrogen solution (100 μg/ml, 48 hours). This suggested a potential role of the DDR receptors in this process, and indeed tyrosine kinase activity is required for the stimulation of MT1-MMP expression induced by Con A in MDA-MB-231 breast cancer cells (Yu et al., 1995). However, we were unable to

demonstrate any inhibitory effects of tyrosine phosphorylation inhibitor, Genestein, in either collagen- or Con A-stimulated fibroblast cultures while Con A-treated MDA-MB-231 cells tested in parallel showed the same inhibitory response to Genestein as previously reported (data not shown).

**MT1-MMP Requirement for Collagen-Induced MMP-2 Activation**

Although considerable associations have been reported between (i) the presence of MT1-MMP and collagen-induced MMP-2-activation in breast cancer cell lines, and (ii) the stimulation of MT1-MMP levels by collagen treatment of fibroblasts, endothelial cells and breast cancer cells, a definitive role of MT1-MMP has not been proven. Indeed, collagen-responsive breast cancer cell lines were found to co-express MT3-MMP message, and any of 4 other MT-MMPs could contribute to MMP-2 activational responses to collagen. To explore this further, we examined the MMP-2-activational responses of fibroblasts derived from mice deficient in the MT1-MMP gene (Holmbeck et al., 1999). As shown in Figure 2.8, fibroblasts from littermates with a wild type (+/+) MT1-MMP status cultured on Vitrogen showed similar MMP-2-activational responses to the human fibroblasts cultures, whereas those derived from heterozygous (+/-) littermates showed intermediate levels, and only background levels of activation were seen with the fibroblasts from MT1-MMP-deficient (-/-) mice. Interestingly, the mouse fibroblasts lines all always produced noticeable levels of a gelatinolytic band which migrated slightly slower than human pro-MMP-2.

**Figure 2.6:** Alkali treated collagen loses the ability to induce MMP-2 activation in human skin fibroblasts. Bovine collagen solution treated with alkali [3 % NaOH (w/v), 1.9 % monomethylamine (v/v)] at the different period of time; 0, 4-hour and 2-day and 15-day were used in solution format. *A.* Unique characteristic of alkali-treated collagen was confirmed by 5 % SDS-PAGE with 30 μg protein from each collagen preparation. *B.* Zymography analysis of MMP-2 activation from 96-hour-conditioned media collected from culture of human skin fibroblasts stimulated with 100 μg/ml of different period-alkali treated collagens and their non-alkali-treated collagen control (cont.). Culture of cells with Vitrogen solution 100 μg/ml used as the control of the experiment.

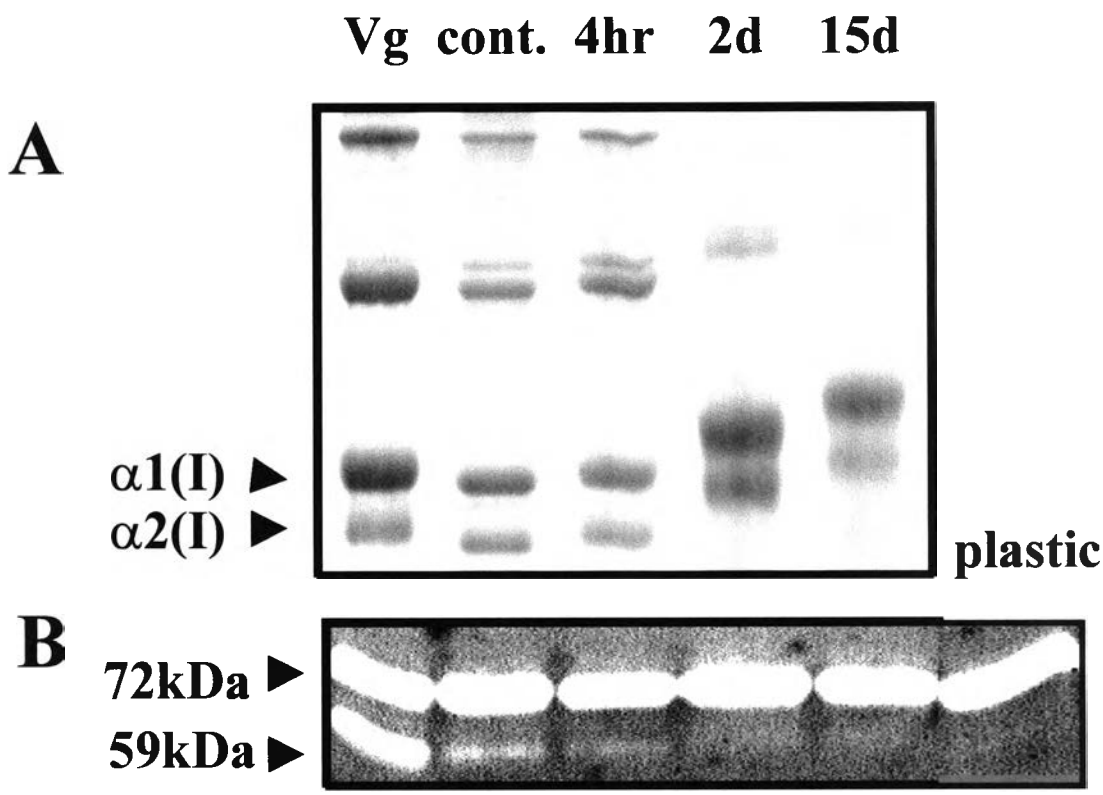

Vg cont. 4hr 2d 15d
A
α1(I) ▶
α2(I) ▶
plastic
B
72kDa ▶
59kDa ▶

**Figure 2.7:** Periodate modified collagen abrogates MMP-2 activation in human skin fibroblasts. Human skin fibroblasts were stimulated with Vitrogen compared to periodate-treated Vitrogen at 0, 12.5, 25, 50, and 100 μg/ml in solution format for 96 hours. Conditioned media were collected and analysed by zymography.

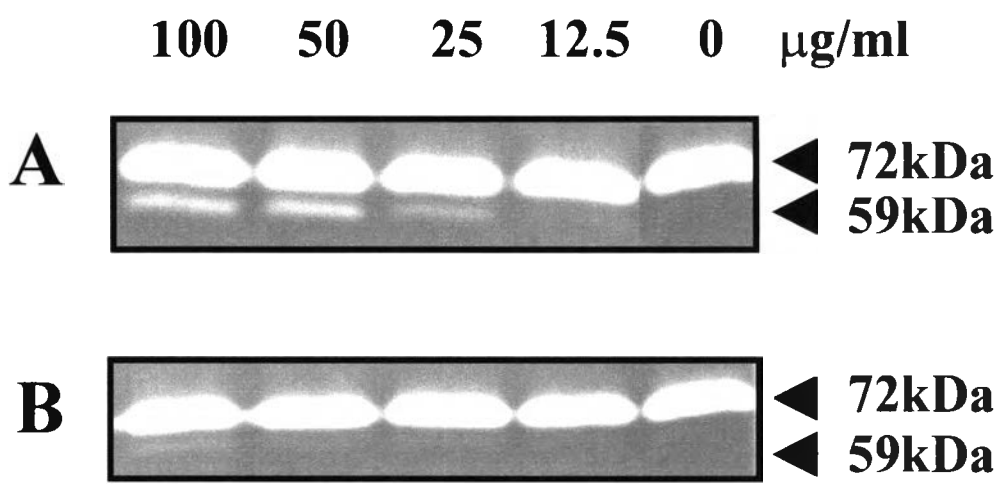
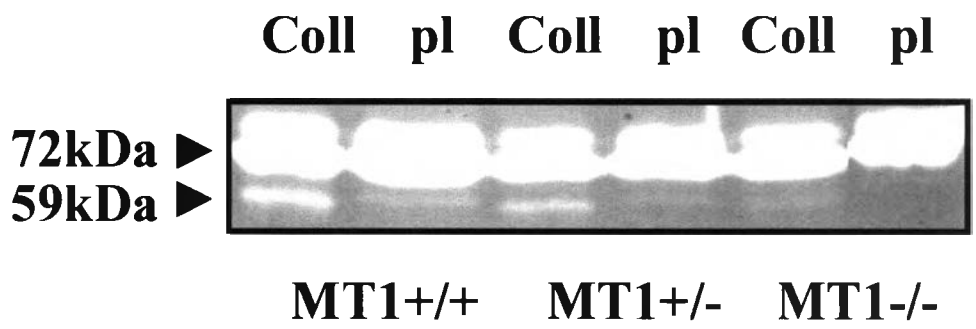

100 50 25 12.5 0 μg/ml
A
72kDa
59kDa
B
72kDa
59kDa

**Figure 2.8:** The important role of MT1-MMP in collagen-stimulated MMP-2 activation demonstrates in MT1-MMP knock out mouse fibroblasts. Conditioned media collected from 96-hour with (Coll) and without (pl) 3-D collagen gel stimulated MT1+/+, MT1+/- and MT1-/- mouse fibroblasts were analysed for MMP-2 activation by zymography.


Coll pl Coll pl Coll pl
72kDa ▶
59kDa ▶
MT1+/+ MT1+/- MT1-/-

## Discussion

The physiological regulation of MT-MMP-mediated MMP-2-activation at the cell surface is not well understood. A growing number of factors, including Con A, phorbol ester and cytochalasin D (Murphy et al., 1999; Seiki, 1999) may mimic certain aspects of the regulation induced by extracellular matrices such as collagen. Collagen-induced activation is well documented in fibroblasts, endothelial cells and invasive cancer cells. Although thin layers of type IV collagen have been shown not to induce activation, this presentation was also seen to be inadequate for fibril-forming collagens. We found here, however, that small amounts of soluble collagen added to the culture medium can induce MT1-MMP expression and MMP-2-activation, presumably by fibrilisation on the cell surface.

### Collagen type specificity

A growing number of studies show the capacity of 3-dimensional gels of type I collagen from various sources for robust induction of both MT1-MMP expression and MMP-2-activation. To systematically examine collagen specificity, we used here differentially precipitated, pepsin-extracted collagen preparations from human skin, collagen prepared from the same material by acid-extraction, thus retaining the telopeptide sequences, and recombinant collagens where available. Each collagen preparation was compared in both the 3-D gel format, and as direct addition to the culture medium. No type-specificity of collagen was seen among fibril-forming collagens with respect to MMP-2 activation. In contrast, type IV collagen, the most abundant non-fibril-forming collagen, was clearly unable to stimulate MMP-2

activation. Thus, induction of the MMP-2 activation process appears to require specific structures rather than general features shared among each type of collagen, such as the triple helical structure or the repeating Gly-X-Y sequence.

**Indications and Implications for Fibril-forming Collagen Requirement**

A number of studies have shown that fibril-forming collagen preparations which can induce MMP-2-activation when presented in the 3-dimensional form are incapable of inducing activation when dried onto the culture plastic in acid solution, thus retaining a monomeric structure (Azzam and Thompson, 1992; Azzam et al., 1993; Maquoi et al., 1998). It came to our attention, however, that we (and others) generally use much lower amounts of monomeric collagen (<10 μg/ml, 10 μg/cm$^2$) compared to that present in the 3-D gel (>1.5 mg/ml, 200 μg/cm$^2$). Nonetheless, we found here that increasing the level of dried collagen to that presented in the 3-D gel was still largely inferior in stimulating MMP-2 activation. Given the apparent need for fibril formation, we were surprised to find that direct addition of collagen solution (acid-solubilized collagen monomer) into the culture medium at low concentrations could induce MMP-2 activation. We explored the hypothesis that the collagen solution formed fibrils in the culture medium which provided the appropriate temperature and pH, perhaps thourough accumulation at the cell surface. Indeed, added collagen concentrations as low as 25 μg/ml resulted in a visible deposition of material around the cell layer, suggesting fibrilisation (data not shown). Furthermore, modifications which retarded fibril formation, like alkali treatment and the inclusion of a specific, fibril-antagonising peptide, decreased MMP-2-activation induction in a dose-

dependent manner. Taken together the results support the requirement of fibril structure in the MMP-2 activation induction.

The requirement for fibrillar collagen form has implications for the recognition and response mechanisms employed by the cell. A number of general characteristics have been shown to be influenced by 3-dimensional collagen gels, including decreased collagen biosynthesis and increased collagen degradation (Lambert et al., 1992), each consistent with the induction of MMP-2-activation we observe. However, in platelets, fibrillar form of the collagen is essential for the release reaction (Santoro et al., 1988). Furthermore, we found that sodium-periodate pretreatment of the collagen, which removes the carbohydrate moieties that would later contribute to covalent cross-linking of the collagen molecules in each fibril, abrogated its ability to induce MMP-2-activation when presented either as a 3-dimensional gel, or in the "solution " form. Although periodate can reduce fibril formation (Brass and Bensusan, 1976), the capacity of periodate-treated collagen to form a 3-dimensional gel here, and in other studies, suggests to us that the major effect lies elsewhere, presumably in the lack of side chain sugars (galactosyl hydroxylysine, and glucosyl-galactosyl-hydroxylysine). Of considerable interest is the observation that the platelet release response is also not seen with periodate-reduced collagen. This may involve the classical integrin $\alpha_2\beta_1$ (GP Ia/IIa) but could also involve the non-classical, divalent cation-independent GPVI and GPIV mechanisms (Nakamura et al., 1998). It is known that adhesion mediated by $\alpha_2\beta_1$ can be seen with both monomeric and fibrillar collagen (Nakamura et al., 1999) so it is unlikely that the specificity for fibrillar collagen form rests with this integrin.

The native triple helical structure of collagen is also essential for DDR binding and activation as has recently been reported (Vogel et al., 1997), however,

fibril requirements have not been explored. Given the similarities in DDR-activational specificities, the MMP-2-activational requirements with respect to collagen type, and our previous observation that the tyrosine phosphorylation inhibitor Genestein could block MMP-2-activational responses of breast cancer cells to Con A, we reasoned that DDRs could be involved in collagen-induced activation. However, repeated efforts failed to show any inhibition of either collagen or Con A-induced activation by Genestein treatment of fibroblast cultures, whereas the expected inhibition was seen in parallel human breast cancer cell cultures. Although this data is not conclusive, it strongly suggests that the DDRs, and tyrosine phosphorylation in general, play at best a minor role in MMP-2-activational response to collagen in these cells.

It is well recognised that MT-MMPs have a role in the activation of MMP-2 (Seiki, 1999). Previous studies in our lab and others have provided substantial correlative evidence for a specific role of MT1-MMP in collagen-induced activation (Gilles et al., 1997; Kurschat et al., 1999), however, in most cases the other MT-MMPs have not been examined. Certainly, in breast cancer cells, MT1-MMP appears a more likely candidate than MT3-MMP, since while the latter is also restricted to invasive, MMP-2-activating cell lines, it is not regulated by collagen (Gilles et al., 1997). MT2-MMP mRNA is not expressed by any of these lines, and MT4-MMP mRNA is expressed by both MMP-2-activating and non-activating cell lines Puente et al., 1996; Gilles et al., 1997). To the best of our knowledge, MT5- and MT6-MMP expression has not been examined in these lines or in fibroblasts. Our observations here again show a compelling regulation of MT1-MMP mRNA and protein levels by collagen, which closely parallel the MMP-2-activational status. Furthermore, the observations with fibroblasts derived from MT1-MMP-deficient mouse skin leave

little doubt about the specific importance of MT1-MMP in this process. Similar indications have already been reported for Con A regulation of MMP-2-activation in the same cell (Holmbeck et al., 1999). This is entirely consistent with the overabundance of collagen in the MT1-MMP-deficient mouse and ensuing skeletal defect (Holmbeck et al., 1999; Zhou et al., 2000). The fact that none of the other MT-MMPs act in a compensatory manner indicated a very high degree of specificity, and supports biochemical observations showing MT1-MMP to be prominent over MT2-MMP in carefully controlled MDCK cells studies (Hotary et al., 2000).

The question arises as to whether fibrillar collagen form is required for both the transcriptional up-regulation of MT1-MMP and the non-transcriptional regulation of MT1-MMP functionality for MMP-2-activation, since these may well be mediated by different receptors or pathways. Clearly the upregulation of MT1-MMP mRNA and protein requires fibrillar form, (Figure 2.4), and we have determined that the non-transcriptional component seen in MT1-MMP-MCF-7 cells is also stimulated to the same extent by Vitrogen added in solution (data not shown). This non-transcriptional response allows for a rapid utilization of pre-existing MT1-MMP stores, while the transcriptional upregulation provides a sustained response (Yu et al., 1997). Collagen stimulation in ovarian cells appears not to cause MT1-MMP upregulation. Instead, activation by collagen of $\beta_1$ integrin leads to a tyrosine kinase mediated protein translation-dependent event (such as *de novo* translation of MT1-MMP) leading to the accumulation of MT1-MMP on the cell surface (Ellerbroek et al., 1999). The Howard laboratory has postulated that the signal induced by collagen interaction may be transduced thourough integrin-mediated focal adhesion complex and actin stress fiber rearrangements, leading to regulation of MT1-MMP and MMP-2 expression, and causing MMP-2 activation (Tomasek et al., 1997). Changes in cell morphology do

effect MMP-2 activation; cell rounding from Con A, SPARC treatment, and fibroblast plated without serum in the medium (personal observation) increased levels of MMP-2 activation. However treatment with nocodazole, which induced microtubule depolymerization and cell shape changes without affecting stress fibers, did not promote MMP-2 activation (Teti et al., 1998), indicating that actin reorganization of the morphology of the cell *per se*, might play an important role in regulation of MT1-MMP for activation of MMP-2 (Tomasek et al., 1997; Teti et al., 1998).

The exact role of the collagen/ MT1-MMP molecular loop is not clear, but refined tailoring of newly deposited collagen is indicated both by observations in the MT1-MMP-deficient mice, as well as the coordinated co-expression of MT1-MMP and $\alpha$1(I) collagen in fibroblasts surrounding human breast (Gilles et al., 1997) and pancreatic (Ellenrieder et al., 2000) tumours. This may well be due to direct action of the MT1-MMP against type I collagen (d'Ortho et al., 1997; Ohuchi et al., 1997), since this capacity is not shared with MT3-MMP (Matsumoto et al., 1997), but it could also involve MMP-13 (Knauper et al., 1996). These mechanisms have relevance to cancer progression from the perspective of desmoplasia, but also the onset of mesenchymal traits which may occur in the cancer cell which undergoes an epithelial-mesenchymal transition. The intense desmoplastic stromal reaction is typified by excessive collagen deposition, and although present in some benign pathological disorders, is most prominent in invasive tumours (Gilles et al., 1997; Ellenrieder et al., 2000). Thus, collagen up-regulation and concurrent effects on MT1-MMP and downstream soluble MMP's highly relevant to the cancer biology, in addition to its broader implications for developmental morphogenesis, tissue fibrosis, wound healing and bone remodeling.