CHAPTER 3

MT1-MMP-DEPENDENT AND INDEPENDENT REGULATION OF GELATINASE A ACTIVATION IN LONG-TERM, ASCORBATE-TREATED FIBROBLAST CULTURES: REGULATION BY FIBRILLAR COLLAGEN

Summary

Addition of collagen to fibroblast cultures stimulates MT1-MMP-mediated activation of pro-MMP-2. Here we analysed the responses of fibroblasts to the collagen deposited in culture after treatment with the essential collagen biosynthesis cofactor, ascorbic acid. Deposition of fibrillar collagen correlated with the appearance of mature MMP-2 species in culture medium. The importance of collagen for this was shown with inhibitors of prolyl 4-hydroxylase, and with fibroblasts derived from the Mov-13 mouse, in which the collagen α1(I) gene is not expressed. However, when Mov-13 fibroblasts were transfected with the wild-type α1(I) gene, type I collagen synthesis was restored with concomitant generation of mature MMP-2. To explore the apparent fibrillar requirement of collagen for MMP-2-activation, Mov-13 cells were transfected with an α1(I) gene mutated at the C-proteinase cleavage site. Although fibrillogenesis of the secreted collagen was inhibited, these cells activated MMP-2. Furthermore, when type I procollagen was purified from these cultures and added exogenously to either Mov13-5 CM or human fibroblast cultures, robust MMP-2-activation occurred. The lathourygen β-aminoproprionitrile blocked the cross-linking of secreted collagen, but did not inhibit generation of mature MMP-2. Taken together these data indicate that fibrillogenesis of collagen *per se* is not required, but some tertiary structure requirements exist. We also tested the role of MT1-MMP in this

MMP-2-activational response using MT1-MMP-deficient fibroblasts. A significant residual activation of MMP-2 was seen in ascorbate-treated MT1-MMP -/- fibroblast cultures, similar to the level remaining after treatment of wild-type cultures with MMP-inhibitors. We were, however, unable to block this MT1-MMP-independent residual activation with a panel of inhibitors specific for serinyl, aspartyl, cysteinyl or metallo-proteinases. These data indicate a novel, MT1-MMP-independent, possibility intracellular pathway for activational processing of pro-MMP-2 in 3-dimensional fibroblast cultures, and confirm an important role for MT1-MMP in mediating fibroblast responses to endogenous collagen.

Introduction

Matrix metalloproteinases (MMPs) are a family of enzymes with substrates ranging from a variety of extracellular matrix components to growth factors, cytokines and other proteinases (Sternlicht and Bergers, 2000). Consequently, this group of enzymes is tightly regulated at the transcription, activation or inhibition level (Birkedal-Hansen, 1995). Gelatinases (MMP-2 and -9) have the specific capacity to digest type IV collagen, a major component of the basement membrane, and also denatured collagens (gelatins). MMP-2 is implicated in various physiologic and pathologic conditions such as organ growth, endometrial cycling, wound healing, bone remodeling, tumour invasion and metastasis, arthouritis, and periodontal disease (Woessner, 1994). The activation of MMP-2 is known to be unique among MMPs, mediated by membrane type MMPs (MT-MMPs) rather than soluble proteinase cascades. So far six MT-MMPs (MT1-6 MMP) have been discovered (Sato et al., 1994; Takino et al., 1995; Will and Hinzmann, 1995; Puente et al., 1996; Pei, 1999; Velasco et al., 2000). MT1-MMP appears a predominant activator of MMP-2 in a variety of studies (Gilles et al., 1996; Ellerbroek et al., 1999; Kurschat et al., 1999; Ellenrieder et al., 2000; Hotary et al., 2000). Pro-MMP-2 is thought to form a trimolecular complex with TIMP-2/MT1-MMP, and its cleavage is initiated by an adjacent MT1-MMP molecule on the cell surface (Strongin et al., 1995; Seiki, 1999). However, the presence of MT1-MMP is not sufficient for MMP-2 activation, which requires further treatment with substances such as the plant lectin, Concanavalin A (Con A), a phorbol ester 12-*O*-tetradecanonylphorbol-13-acetate (TPA; Murphy et al., 1999; Seiki, 1999), or extracellular matrix proteins such as fibrillar collagen (Azzam and Thompson, 1992; Azamm et al., 1993; Gilles et al., 1997), type IV

collagen (Maquoi et al., 2000), SPARC/osteonectin (Gilles et al., 1998), or fibronectin (Stanton et al., 1998).

We are interested in the regulation of MMP-2 activation by collagen, since both are dramatically upregulated around tumours and in various other pathological states. Thouree-dimensional collagen gels have been shown in a number of studies to induce MMP-2 activation in a variety of cells expressing MT1-MMP (Azzam and Thompson, 1992; Azamm et al., 1993; Seltzer et al., 1994; Ellerbroek et al., 1999; Kurschat et al., 1999; Preaux et al., 1999). Collagen has been implicated in both the regulation of the MT1-MMP gene, (Haas et al., 1999) and non-transcriptional regulation of MT1-MMP, as confirmed in MCF-7 cells transfected with MT1-MMP cDNA (Gilles et al., 1998). Our recent *in vitro* study using recombinant collagen showed the requirement for collagen in the fibrillar form for MMP-2 activation, and a strict dependence on MT1-MMP (Ruangpanit et al., 2001). Such a role for fibrillar collagen is consistent with the activation of MMP-2 in strongly desmoplastic tumours.

Here, we have studied the activation of MMP-2 in fibroblasts cultured in 3-dimensional collagen matrices produced by the cells after treatment with ascorbic acid. This model resembles the *in vivo* situation, and was used to test the potential importance of different aspects of collagen structure, and of MT1-MMP, in the induction of MMP-2 activation.

Experimental Procedures

1. Materials

Ascorbic acid, β-aminoproprionitrile, pepsin, cyclohexamide and proteinase inhibitors; aprotinin, pepstatin and cystatin were purchased from Sigma, MO. [^{33}P] dCTP (10 mCi/ml) was purchased from Amersham Australia Pty. Ltd., Sydney, NSW, Australia. MT1-MMP mouse monoclonal antibody clone 114-1F2 was obtained from Oncogene Research Products, MA. Prolyl 4-hydroxylase inhibitors (Compound I and II) were as previously described (Franklin et al., 2001). GM6001, a synthetic inhibitor of matrix metalloproteinases, was purchased from Silenus, Australia. All other chemical were commercially available and of analytical grade.

2. Cells and Culture

Normal human skin fibroblasts (passage 11-15; ref; Chan et al., 1990), were derived and cultured as previously reported (Ruangpanit et al., 2001). The Mov13 cells used here were derived by SV40 transformation of skin fibroblasts from the Mov13 mouse strain in which the collagen α1(I) gene was inactivated by the insertion of Moloney murine leukemia virus into the first intron. These cells, as well as SV40 transformed wide-type fibroblasts, were cultured as previously described (Jaenisch et al., 1983; Schnieke et al., 1983; Lohler et al., 1984). Although transcription of the α2 (I) collagen gene is not affected in the Mov13 cells, in the absence of the α1(I) subunit no stable collagen I is produced. Transfection of wild-type α1(I) collagen cDNA into

Mov13 cells (Mov13-510D5) resulted in the restoration of synthesis and secretion of normal triple helical type I collagen (Schnieke et al., 1987). We also employed Mov13 cells transfected with an α1(I) gene mutated at the C-proteinase cleavage site (Mov13-5 CM). In these cells collagen I synthesis is rescued, but the procollagen is resistant to removal of the C-terminal procollagen propeptide, a process necessary for normal collagen maturation and fibrillogenesis (Fenton and Bateman, unpublished data). Newborn skin fibroblasts from MT1-MMP +/+, +/-, and -/- mice were derived and cultured as previously described (Holmbeck et al., 1999), and used here at passage 4-6.

3. Ascorbate-Treated Fibroblast Cultures

Cells were grown 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) to confluence in 10 cm-diameter culture plates (Falcon) or 6 well Costar plates (designated as day zero). After this, freshly dissolved ascorbic acid (Sigma, MO; 0.25 mM) was added each day until the designated day of harvest. Ninety-six hours before terminating the experiment, the addition of ascorbic acid was stopped and the medium was changed after washing to the serum free medium, [SFM: 1x non-essential amino acid solution, 1x ITS, 0.1 % BSA, 1x vitamin solution, and 1 mM sodium pyruvate (all from Sigma, MO)]. Conditioned media were collected after 96 hours, and analyzed for endogenous MMP-2 activation by zymography as described below. The collagen component was precipitated from conditioned media and/or extracted from the matrix, respectively, as described below. Cell number was estimated with a

colorimetric assay of cell viability, based on the cleavage of the tetrazolium salt WST-1 (Boerinhger Mannheim, Germany), in representative samples.

4. Gelatin Zymography

Zymographic analysis of protease activity was performed as previously described (Ruangpanit et al., 2001).

5. Extraction of Collagen Deposition and Precipitation of Soluble Collagen from the Conditioned Media

The extracellular matrix and cells were scraped from the culture vessels, sonicated, and extracted overnight at 4°C with 5 ml of 50 mM Tris/HCl, 0.15 M NaCl, pH 7.4, containing 5 mM EDTA, 5 mM phenylmethylsulfonylfluoride (PMSF) and 10 mM N-ethylmaleimide (NEM). This removes non-collagenous proteins and newly deposited collagen. The pellet was collected by centrifugation (15,300 g, 20 minutes, 4°C), and in some cases further extracted with 0.5 M acetic acid overnight at 4°C. The extracted soluble material was designated "acid extracted collagen" while the insoluble component was removed by centrifugation (15,300 g, 20 minutes, 4°C). The pellet from this step, or in some cases the material remaining after the Tris/NaCl extraction, was extracted further with 0.1 mg/ml pepsin in 0.5 M acetic acid overnight at 4°C. These acid- or pepsin-soluble collagens were lyophilized and analyzed by 5 % SDS-PAGE as described below.

The conditioned media collected at the various time points from each sample were pooled and precipitated with 25 % saturated $(NH_4)_2SO_4$ at 4°C overnight and

redissolved in 1 ml 50 mM Tris/HCl, 0.15 M NaCl, pH 7.4 containing proteinase inhibitors (5 mM EDTA, 5 mM PMSF and 10 mM NEM). To analyse the collagen component, a 200 µl aliquot was precipitated with 18 % ethanol for 1 hour at 4°C. In some cases, the pellet was further digested with pepsin. Pepsinised or non-pepsinized samples were reconstituted in 1x sample buffer [20 mM Tris/HCl, pH 6.8, 2 % SDS (w/v), 10 % glycerol (v/v), 2 M urea and 0.05 % bromphenol blue (w/v)] for SDS-PAGE analysis.

6. Purification of Secreted Collagen from the Conditioned Medium

Collagen produced by normal human skin fibroblasts, by Mov13-510D5 or by Mov13-5 CM cells in culture was prepared using the same methods developed for the isolation of collagen from conditioned medium as described above, but with sterilie technique. After 18 % ethanol precipitation, the collagen was reconstituted in 1x PBS and protein concentration was measured by using the BCA kit (Pierce, IL). It was immediately used or stored at -20°C.

7. Addition of Partially Purified Endogenous Collagen to the Cell Culture

Human skin fibroblasts or Mov13-5 CM cells were plated (50,000 cells in each well of 48-well plate) overnight in DMEM with 10 % FCS. The medium was changed to SFM containing exogenous MMP-2 (Gilles et al., 1997) and either 100 µ g/ml of collagen or PBS vehicle for 96 hours. Conditioned medium was collected and analysed for the activation of MMP-2.

8. SDS-PAGE Analysis of Collagen

SDS-PAGE analysis of the matrix-extracted collagen samples was performed with thirty microliters from a total of 300 μl. Analysis of collagen in culture medium was performed on an 18 % ethanol precipitation of a 200 μl aliquot from the total of 1 ml. Samples were heated to 95°C for 10 minutes in non-reducing sample buffer, and separated on 5 % SDS-PAGE gel containing 2 M urea using the minigel-apparatus (Bio-Rad, CA). Gels were then stained for 30 min 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 and 7.5 % acetic acid.

9. Northern blot Analysis

For analysis of MT1-MMP mRNA expression, cells were released from matrix by incubation with bacterial *(Clostridium histolyticum)* collagenase type II (Worthington Biochem Corp., NJ) for 30-45 min at 37°C, and total RNA was extracted using RNAzol B™ (TEL-TEST, Inc., TX). RNA concentration was estimated from optical density measurements at 260 and 280 nm, and twenty micrograms of total RNA was analyzed for MT1-MMP mRNA as previously described (Ruangpanit et al., 2001). RNA loading was normalized to the amount of GAPDH mRNA in each sample using densitometric analysis. The 1.3 kb human GAPDH ^{33}P-radioactive probe (Southby et al., 1995) was prepared by the same procedure as the MT1-MMP probe.

10. Western blot Analysis

Cells were 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, and analyzed as previously described (Ruangpanit et al., 2001).

Results

Collagen Deposition, MT1-MMP-Regulation, and MMP-2-Activation in the Ascorbate-treated Fibroblast Cultures

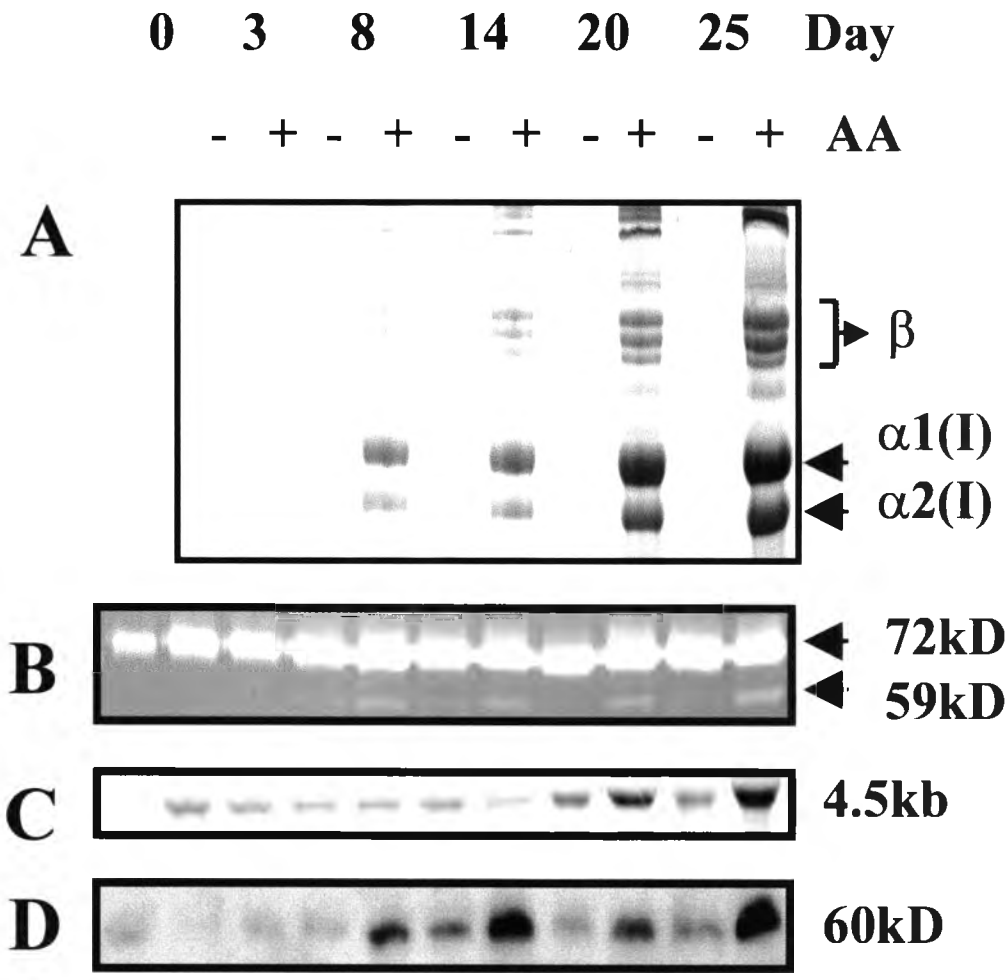
Matrix deposited by the normal human skin fibroblasts grown in the presence and absence of ascorbic acid for different periods was extracted by limited pepsin extraction and separated by SDS-PAGE. As shown in Figure 3.1A, with ascorbic acid supplementation the fibroblast cultures deposited an extensive type I collagen matrix with increasing time of culture, as described in detail previously (Chan et al., 1990). The matrix required pepsin digestion to release the collagen, demonstrating that the *in vitro* fibrillar collagenous matrix was highly crosslinked, as further evidenced by the presence of crosslinked α-chain dimers (β-components). Collagen was detected in the pepsin-extractable matrix of ascorbic acid-treated cultures from day 8, although in certain experiments, evidence of this was sometimes seen as early as day 3. As expected, little or no collagen was seen in any of the cultures without ascorbic acid.

Analysis of the conditioned media showed induction of MMP-2 activation only in the cultures treated with ascorbic acid (Figure 3.1B). This occurred by day 8,

but in certain experiments, we saw the active band by day 3 and in each case it was concomitant with the ability to detect pepsin-extractable collagen. It is interesting to note that the intermediate, 68 kDa form of MMP-2 was barely detected in this cell type when stimulated by collagen. The level of MMP-2 activation increased in long-term culture with ascorbic acid even when standardized for the increased cell number. Thus, activation of MMP-2 by fibroblasts occurred in parallel to collagen produced by the fibroblasts themselves, and the steady increase in the level of MMP-2 activation was correlated with the amount of collagen deposition.

We also examined MT1-MMP mRNA (Figure 3.1C) and protein (Figure 3.1D) expression in this culture model. MT1-MMP mRNA levels were dramatically increased in the ascorbate-treated cultures by day 20 (Figure 3.1C). This correlated with the levels of collagen deposited after 20 days in culture. However, significant collagen deposition and activation of pro-MMP-2 were seen much earlier in ascorbate cultures (Figure 3.1A and 3.1B). Western analysis of MT1-MMP protein from the total cell lysates showed a basal expression level of the protein at confluence. The level increased steadily from day 8 in the presence of ascorbic acid (Figure 3.1D). There was good concordance between increased MT1-MMP protein and observable MMP-2-activation, whereas increased MT1-MMP mRNA levels were not seen until later, allowing us to discriminate between the transcriptional versus non-transcriptional components of collagen regulation of MT1-MMP, as previously described (Gilles et al., 1998; Ruangpanit et al., 2001).

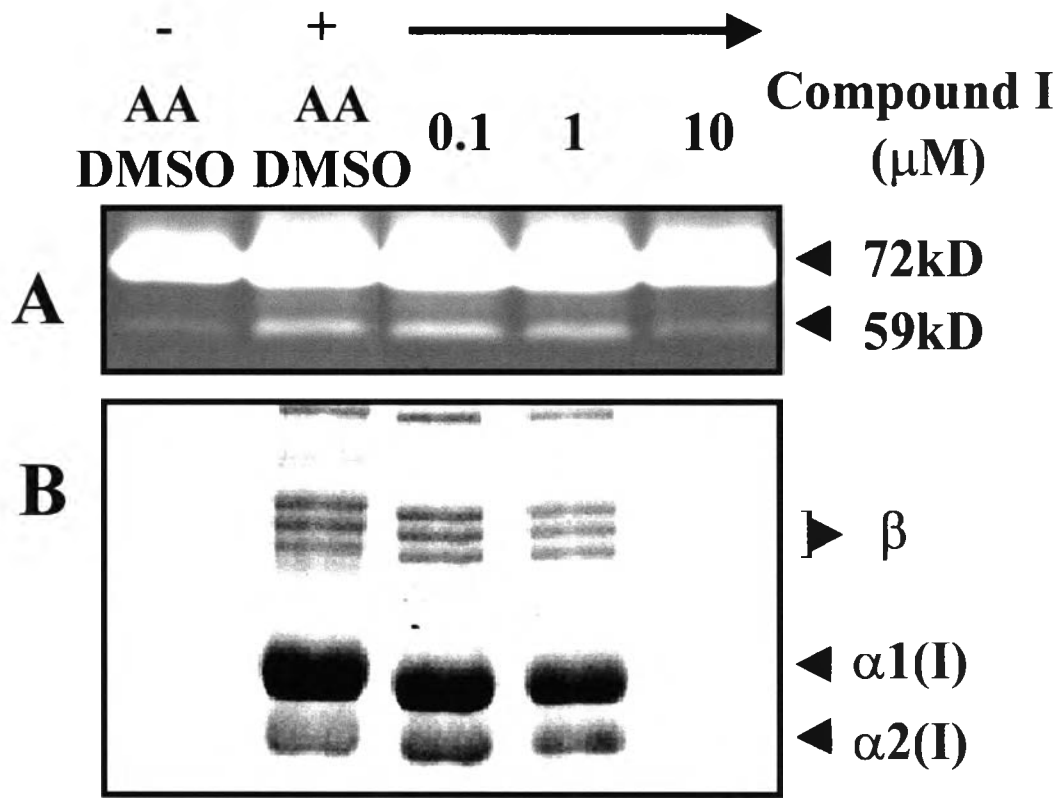
Figure 3.1: Collagen deposition and MMP-2-activation in ascorbate-treated fibroblast cultures. *A.* SDS-PAGE analysis of acid-insoluble (pepsin extracted) matrix deposited by human skin fibroblasts cultured in the presence (+) or absence (-) of 0.25 mM ascorbic acid (AA) for various periods, as shown. Type I collagen α1 chain (α1(I)), α2 chain (α2(I)), and α-chain dimers (β-components) are shown. *B.* Zymographic analysis of endogenous MMP-2 activation in the last 96 hour conditioned medium from the cultures described in *A.* Loading was normalised to cell number. 72 kD and 59 kD represent the latent and fully active forms of MMP-2, respectively. *C.* Northern analysis of 20 μg total RNA extracted from each culture for the expression of MT1-MMP mRNA (4.5 kb) using a ^{33}P-radiolabeled 3.4 kb cDNA probe. *D.* Western analysis of 50 μg total cell lysate from each culture using 1 μg/ml of 114-1F2 mouse Mab. The ~60 kD band shown represents the mature form of MT1-MMP.


0 3 8 14 20 25 Day
- + - + - + - + - + AA
A
β
α1(I)
α2(I)
B
72kD
59kD
C
4.5kb
D
60kD

Prolyl-4-Hydroxylase Inhibitors Abrogate MMP-2 Activation in the Ascorbate-treated Fibroblast Cultures

In order to ensure that MMP-2 activation seen in ascorbic acid treated cultures was due to the collagen production rather than other direct or indirect effects of ascorbic acid, prolyl 4-hydroxylase inhibitors were used to block collagen synthesis. Inhibitors were added every 72 hours, when the culture media was changed. Inhibition of prolyl 4-hydroxylase leads to thermally unstable collagen which is not secreted from the cells. In the untreated culture, sixteen days with ascorbate resulted in a dramatic increase in the extracellular collagen in the pepsin-extractable fraction (Figure 3.2B). As expected, treatment with prolyl 4-hydroxylase inhibitors Compound I (Figure 3.2B) and Compound II (not shown) caused a dose-dependent inhibition of collagen deposition in the pepsin-extractable fraction. The highest dose (10 μM) of Compound I resulted in an almost total lack of collagen in the matrix, and dramatically reduced the levels of collagen secreted into the culture media (not shown). Also, the activation of MMP-2 was dramatically decreased to almost background levels as detected in long-term control cultures (Figure 3.2A). These data confirm an important role of collagen in stimulating MMP-2 activation in the ascorbate-treated fibroblast culture.

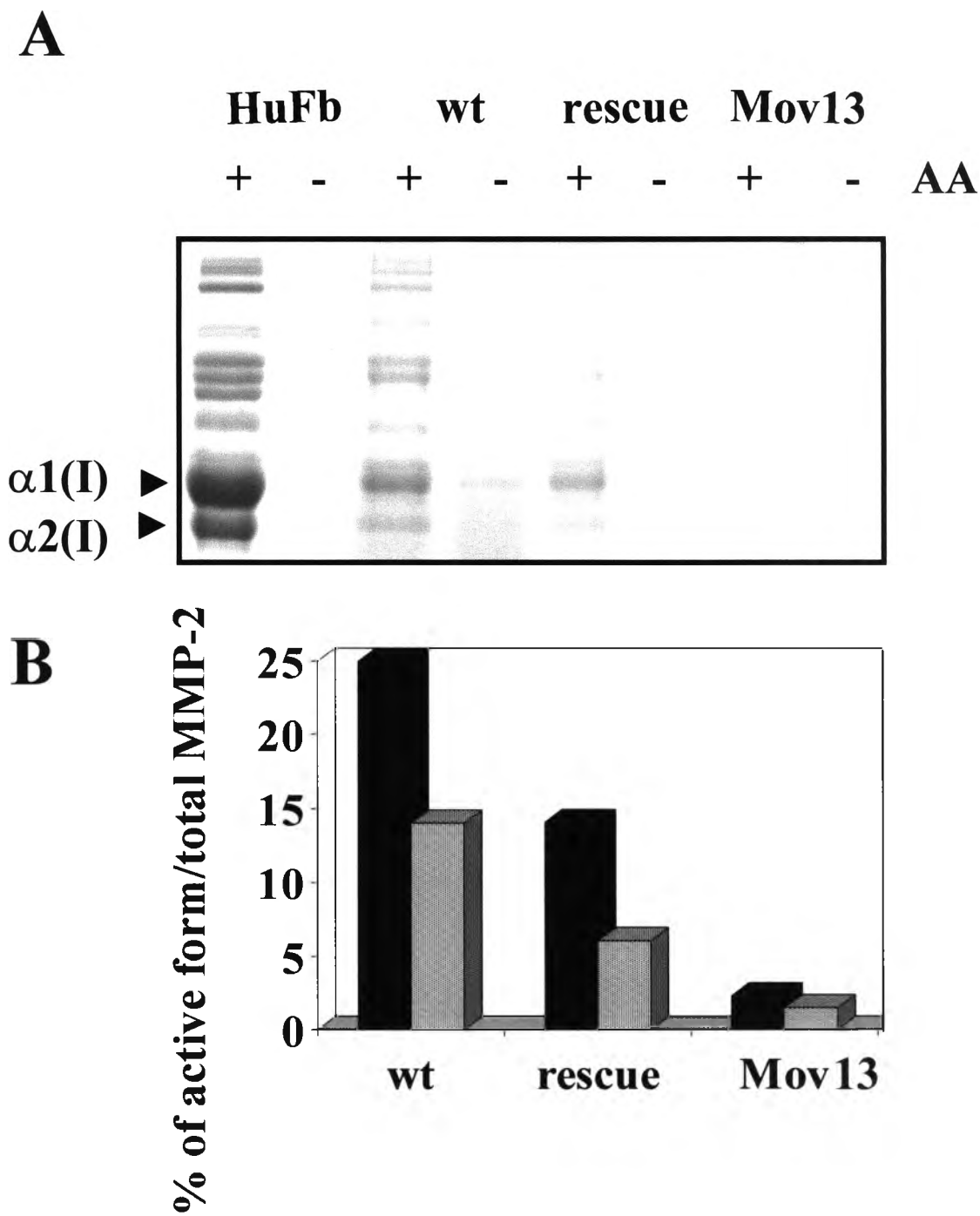
Figure 3.2: Activation of MMP-2 by ascorbate-treated fibroblasts requires collagen. Human skin fibroblasts were cultured either without (-) or with (+) the diary addition of 0.25 mM ascorbic acid (AA) for 15 days, and where indicated, with the prolyl 4-hydroxylase inhibitor (Compound I) at concentrations 0.1, 1, and 10 μM. Fresh Compound I was added with each media change (ea. 3-4 days) *A.* Zymographic analysis of MMP-2 in the last 96-hour conditioned medium. 72 kD and 59 kD represent the latent and fully active forms of MMP-2, respectively. *B.* 5 % SDS-PAGE analysis of pepsin-extracted collagen from each culture. Type I collagen α1(I) and α2(I) chains, and β-components (α-chain dimers; β_{11}, β_{12} and β_{22}) are shown.


-
+
AA
DMSO
AA
DMSO
0.1
1
10
Compound I
(μM)
A
72kD
59kD
B
β
α1(I)
α2(I)

Collagen α1(I) Gene Inactivation Abrogates MMP-2 Activation in the Ascorbate-treated Fibroblast Cultures

The Mov13 system was also used to study the role of type I collagen in the induction of MMP-2 activation. We compared Mov13, Mov13-510D5, and SV40 transformed wide-type normal mouse fibroblasts with primary human fibroblasts (Figure 3.3). No collagen deposition was detected by Mov13 cells in either the presence or absence of ascorbic acid, consistent with their inability to produce the α1 (I) collagen chain. In contrast, SV40-transformed wild-type mouse fibroblasts showed a small amount of collagen deposition in the absence of ascorbic acid, which was dramatically increased after ascorbic acid treatment. M13-510D5 cells transfected with the α1(I) cDNA, produced and deposited collagen in the presence of ascorbic acid (Figure 3.3A). The degree of MMP-2 activation seen by zymography was much weaker in this system than seen in primary human skin fibroblasts (not shown), and densitometric analysis was used to determine the proportion (%) of active MMP-2 to the total endogenous MMP-2 (Figure 3.3B). The level of active MMP-2 found in the SV40-wild-type cells without ascorbic acid treatment was consistent with their ascorbic acid-independent production of collagen, and activation in general paralelled the collagen deposition. These data further support an important role of type I collagen in the activation of MMP-2. Although most of the collagen produced by these cells is type I, the lower level of production of type III collagen expected by the Mov13 cells is apparently not sufficient to induce activation of MMP-2. Addition of larger amounts of purified or recombinant type III collagen do effectively stimulate MMP-2-activation (Ruangpanit et al., 2001).

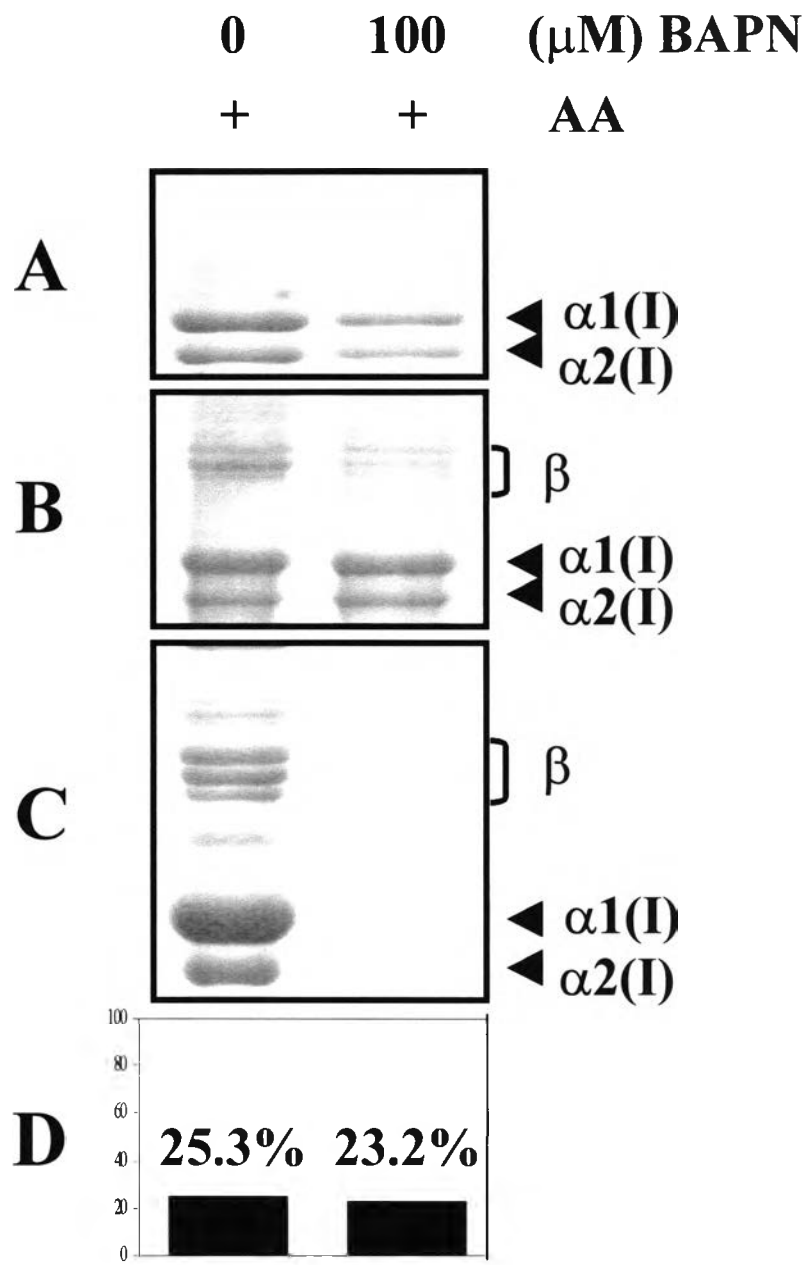
Figure 3.3: Collagen α1(I) gene inactivation abrogates MMP-2 activation by ascorbate-treated fibroblasts. Human skin fibroblasts (HuFb), SV40 transformed wild-type mouse fibroblast (wt), Mov13-510D5 cells (rescue) and Mov13 cells were cultured for 12 days in the presence (+) or absence (-) of 0.25 mM ascorbic acid (AA). *A.* 5% SDS-PAGE analysis of pepsin extracted matrix *B.* Densitometric representation of MMP-2 activation analysed by zymography of the last 96-hour conditioned medium. Each bar shows the proportion (%) of active MMP-2 to the total endogenous MMP-2. Black bars denote the presence, and grey the absence, of ascorbic acid.


A
HuFb
wt
rescue
Mov13
+ - + - + - + - AA
α1(I)
α2(I)
B
% of active form/total MMP-2
25
20
15
10
5
0
wt
rescue
Mov13

Effect of Inhibiting Collagen Cross-linking

We examined other aspects of collagen structure in the induction MMP-2 activation in these cultures. β-aminoproprionitrile (BAPN) irreversibly inactivates lysyl oxidase, an enzyme which otherwise oxidatively deaminates the ε-amino groups of lysyl or hydroxylysyl residues in collagen, resulting in reactive aldehyde residues which lead to inter-molecular cross-linking of the collagen molecules. Pepsin-extracted collagen from conditioned media (Figure 3.4A) and serial extractions of collagen from the matrix with acid (Figure 3.4B) and pepsin (Figure 3.4C), respectively, showed inhibition of the β–components ($\beta_{1,1}$ $\beta_{1,2}$ $\beta_{2,2}$) cross-linked collagen bands, and a consequent reduction in the amount of collagen requiring pepsin treatment for extraction (Figure 3.4C). Very little affect of BAPN was seen on the level of MMP-2 activation in these cultures (Figure 3.4D). Thus, inter-chain cross-linking of collagen fibrils is not required for the induction of MMP-2 activation.

Figure 3.4: Cross-linking of collagen molecules by ascorbate-treated fibroblasts is not required for MMP-2 activation. Human skin fibroblasts were cultured for 15 days with dialy addition of 0.25 mM ascorbic acid (AA), in the absence (0) or presence (100 μM) of BAPN as indicated. *A.* The last 96 hour conditioned medium was analysed 5 % SDS-PAGE for the presence of collagen. *B.* 5 % SDS-PAGE analysis of collagen extracted by 0.5 M acetic acid. β-components represent dimers between α-chains which are the result of cross-linking. *C.* Collagen resisting 0.5 M acetic acid extraction was further digested with 0.1 mg/ml of pepsin and analysed as for B above. *D.* Densitometric representation of MMP-2 activation analysed by zymography of the last 96-hour conditioned medium. Each bar shows the proportion (%) of active MMP-2 to the total endogenous MMP-2.

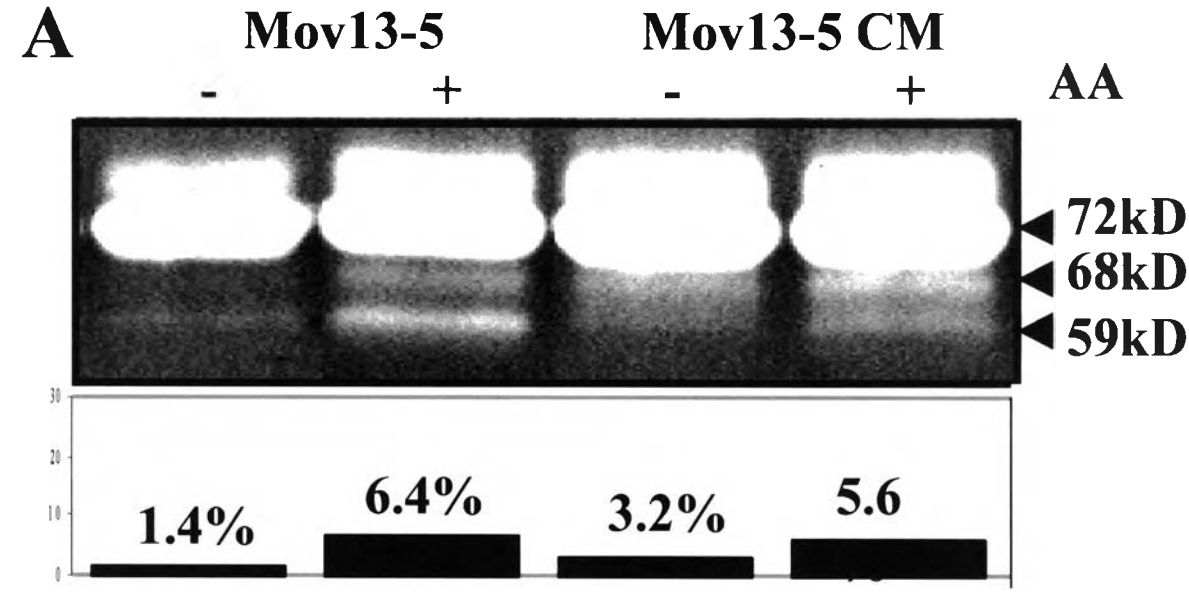

0
100
(μM) BAPN
+
+
AA
A
α1(I)
α2(I)
B
β
α1(I)
α2(I)
C
β
α1(I)
α2(I)
D
100
80
60
40
20
0
25.3%
23.2%

Requirement of Collagen Fibril Structure for the Activation of MMP-2

We recently found that a specific peptide inhibitor of fibril formation could diminish the activation of MMP-2 induced by the addition of exogenous neutralized type I collagen to fibroblast cultures (Ruangpanit et al., 2001). In the present study we explored the fibril requirement further using Mov13 mouse fibroblasts transfected with an α1(I) chain cDNA with a mutation engineered into the procollagen C-proteinase cleavage site (Mov13-5 CM). The bulky, S-S bonded C-propeptide domain of collagen is known to interfere with fibril formation (Miyahara et al., 1982; Kadler et al., 1987). As seen for the SV-40 transformed wild type mouse fibroblasts (Figure 3.3A), the Mov13-5 CM cell line also has some ascorbate-independent collagen secretion, however, as expected, no deposition of the secreted pC-procollagen into either acid- or pepsin- extractable matrix was seen in the short term culture (8 days, data not shown). Coomassie Brilliant Blue staining showed trace amounts of collagen in the matrix of the long-term culture (19 days, data not shown), presumably due to non-specific processing as previously reported (Bateman et al., 1987). To avoid this non-specific processing, we performed certain experiments in short-term (8 days) culture. Unexpectedly, we found a pronounced activation of endogenous MMP-2 in the ascorbate-treated short-term Mov13-5 CM culture lacking matrix deposition (Figure 3.5A), consistent with their production of collagen in the conditioned media. To ensure that this activation was indeed due to the defective collagen produced by these cells, rather than an alternative mechanism which may be clonally activated in these cells, we again used the prolyl 4-hydroxylase inhibitor. Compound I blocked the secretion of collagen by the Mov13-5 CM cells and abrogated the MMP-2 activation (Figure 3.5B). These data suggest either 1) that sufficient collagen is being

alternatively processed to form fibrils, or 2) that the pC-procollagen produced can induce the activation process. Non-reducing SDS-PAGE analysis of collagen partially purified from the conditioned medium confirmed the presence of high molecular weight aggregates (due to the inter-chain disulfide bonds in the C-propeptide domain) which were unable to enter the gel, but did not reveal any processed forms which could potentially form fibrils (data not shown). To study this further, and avoid the possibility that the Mov13-5 CM cells are unique in this response, we purified and concentrated secreted collagens from pooled conditioned medium obtained from normal human skin fibroblasts, Mov13-510D5 cells and Mov13-5 CM cells, and transferred them to either normal human skin fibroblasts or Mov13-5 CM cells in the absence of ascorbic acid. Phase contrast microscopy showed a clear generation of large fibrillar structures when the collagen obtained from either human fibroblasts or Mov13-510D5 cells was added to each culture, but not when the same amount of collagen obtained from the Mov13-5 CM cells was added (not shown). Nonetheless, addition of the Mov13-5 CM collagens induced prominent MMP-2-activation by both recipient human fibroblasts (not shown) and Mov13-5 CM cells (Figure 3.5C). We conclude that while pC-procollagen is unable to form fibrils, it does appear to be able to enhance MMP-2-activation.

Figure 3.5: C-proteinase-resistant collagen induces MMP-2 activation by fibroblasts. *A.* Mov13-5 and Mov13-5 CM cells were cultured for 8 days in the absence (-) or presence (+) of 0.25 mM ascorbic acid (AA). MMP-2 activation of the last 96-hour conditioned medium was assessed by zymography (upper panel), and also expressed as the proportion (%) of active MMP-2 to total MMP-2 after densitometric analysis (lower panel). *B.* Mov13-5 CM cultured ascorbate-free for 15 days and either Compound I (+) or DMSO vehicle (-) was added at each media change. MMP-2 activation of the last 96-hour conditioned media was assessed by zymography (upper panel), and also expressed as the proportion (%) of active MMP-2 to total MMP-2 after densitometric analysis (lower panel). *C.* Mov13-5 CM cells cultured for 96 hours in SFM supplemented with exogenous MMP-2 in the absence (-) or presence (+) of collagen purified from Mov13-5 CM culture medium (100 μg/ml). MMP-2 activation was assessed by zymography (upper panel), and also expressed as the proportion (%) of active MMP-2 to total MMP-2 after densitometric analysis (lower panel).


A
Mov13-5
Mov13-5 CM
-
+
-
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AA
72kD
68kD
59kD
30
20
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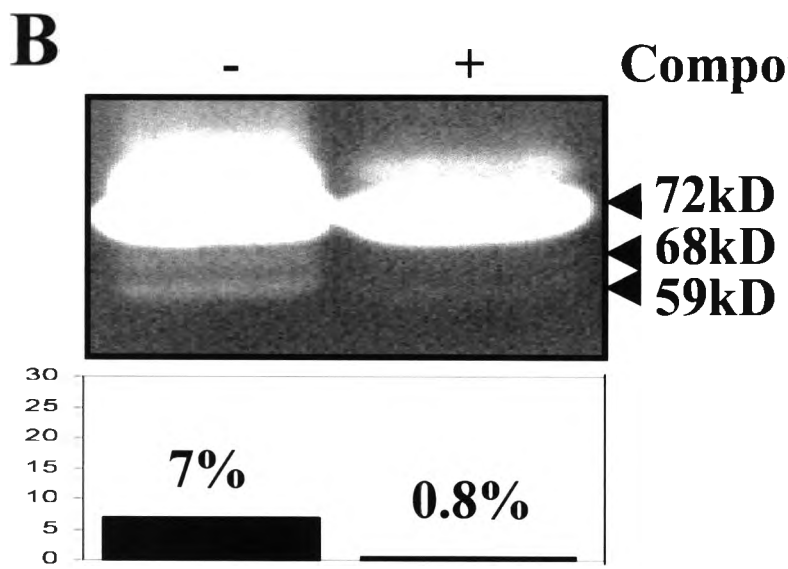
0
1.4%
6.4%
3.2%
5.6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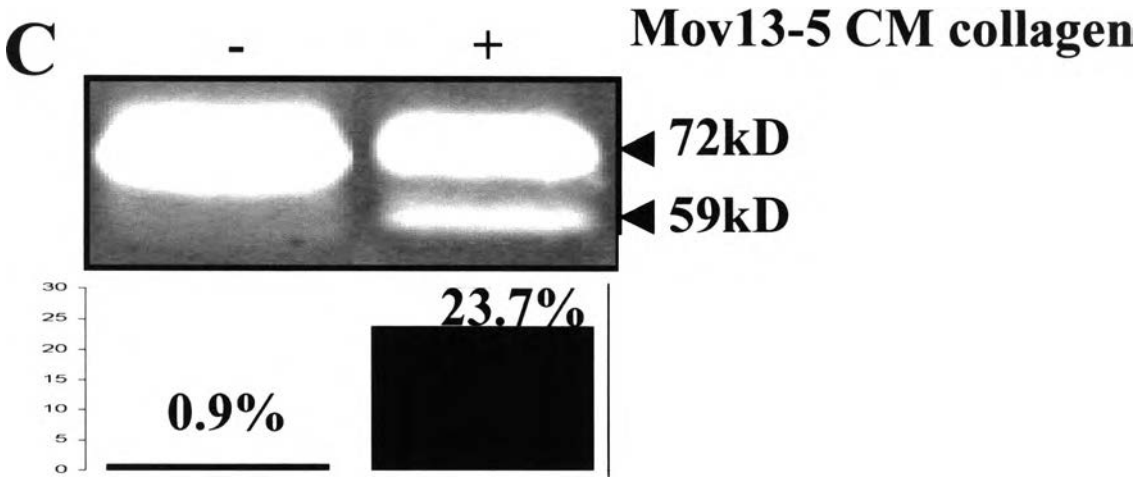
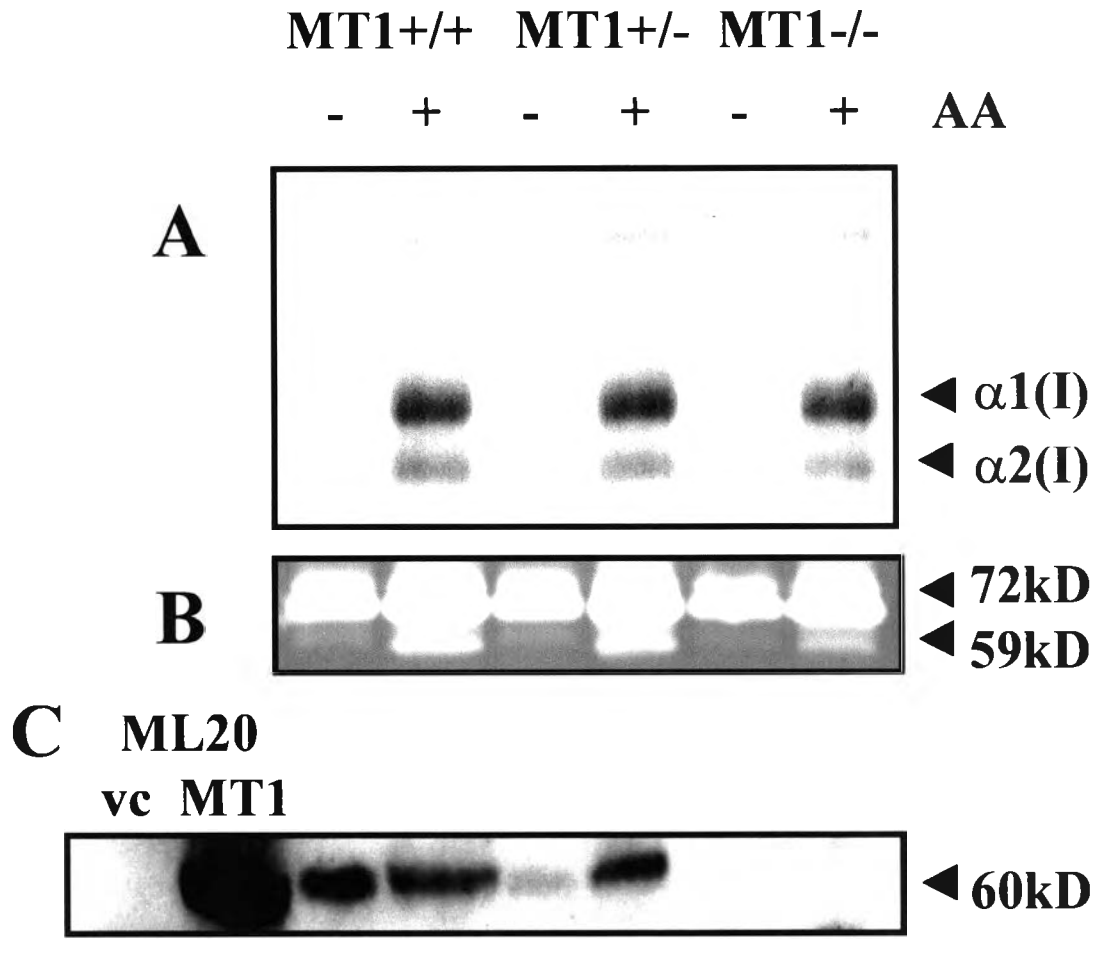

C
-
+
Mov13-5 CM collagen
72kD
59kD
30
25
20
15
10
5
0
23.7%
0.9%

Role of MT1-MMP in MMP-2 Activation

MT1-MMP, working in concert with TIMP-2, has been demonstrated to be the predominant activator of MMP-2. To examine the specific role of MT1-MMP in MMP-2 activation by our endogenous response model, we tested primary cultures (passage 4-6) of fibroblasts derived from the offspring of interbreeding mice which were heterozygous for disrupted expression of the MT1-MMP gene (Holmbeck et al., 1999); MT1+/+, MT1+/- and MT1-/-. After 15 days of culture with ascorbic acid, a 96 hour-conditioned medium was assessed by zymography for MMP-2 activation and by SDS-PAGE analysis for collagen deposition. Preliminary experiments showed that collagen deposition by MT1+/- and MT1-/- fibroblasts was lower than by MT1+/+ fibroblasts (data not shown), so the ascorbate culture period was extended in the MT1+/- (12 days) and MT1-/- (18 days) cultures in order to obtain similar total amounts of collagen deposition to that in the MT1+/+ culture after 9 days (Figure 3.6A). As expected, MMP-2 activation was significantly reduced in cultures of fibroblasts derived from MT1-/- mice, however, to our surprise appreciable activation of MMP-2 persisted (Figure 3.6B). Western analysis for MT1-MMP confirmed increased levels of MT1-MMP in the ascorbate-treated cultures of MT1+/+ and MT1+/- fibroblasts, and its absence in the MT1-/- fibroblast cultures (Figure 3.6C). Although these results confirm an important role for MT1-MMP for activation of MMP-2 in the ascorbate-treated fibroblast culture, they also illustrate a significant residual non-MT1-MMP component.

Figure 3.6: MT1-MMP only partially mediates MMP-2 activation by ascorbate-cultured fibroblasts. MT1-MMP wild-type (MT1+/+), heterozygous (MT1+/-) and knockout (MT1-/-) mouse fibroblasts were cultured in the presence of 0.25 mM ascorbic acid (AA) for 9, 12 and 18 days, respectively, to achieve a similar amount of collagen deposition as shown in *A*. Each culture was extracted with 0.1 mg/ml of pepsin and subjected to 5 % SDS-PAGE. *B*. MMP-2 activation from the last 96-hour conditioned medium was analyzed by zymography. 72 kD and 59 kD represent the latent and fully active forms of MMP-2, respectively. *C*. Western analysis of MT1-MMP in total cell lysates (50 μg/lane) of ML-20 vector control (vc; negative control), MT1-MMP-transfected ML-20 cells (MT1; positive control), and of the fibroblasts cultures described above. The ~60 kDa band shown represents the mature form of MT1-MMP.

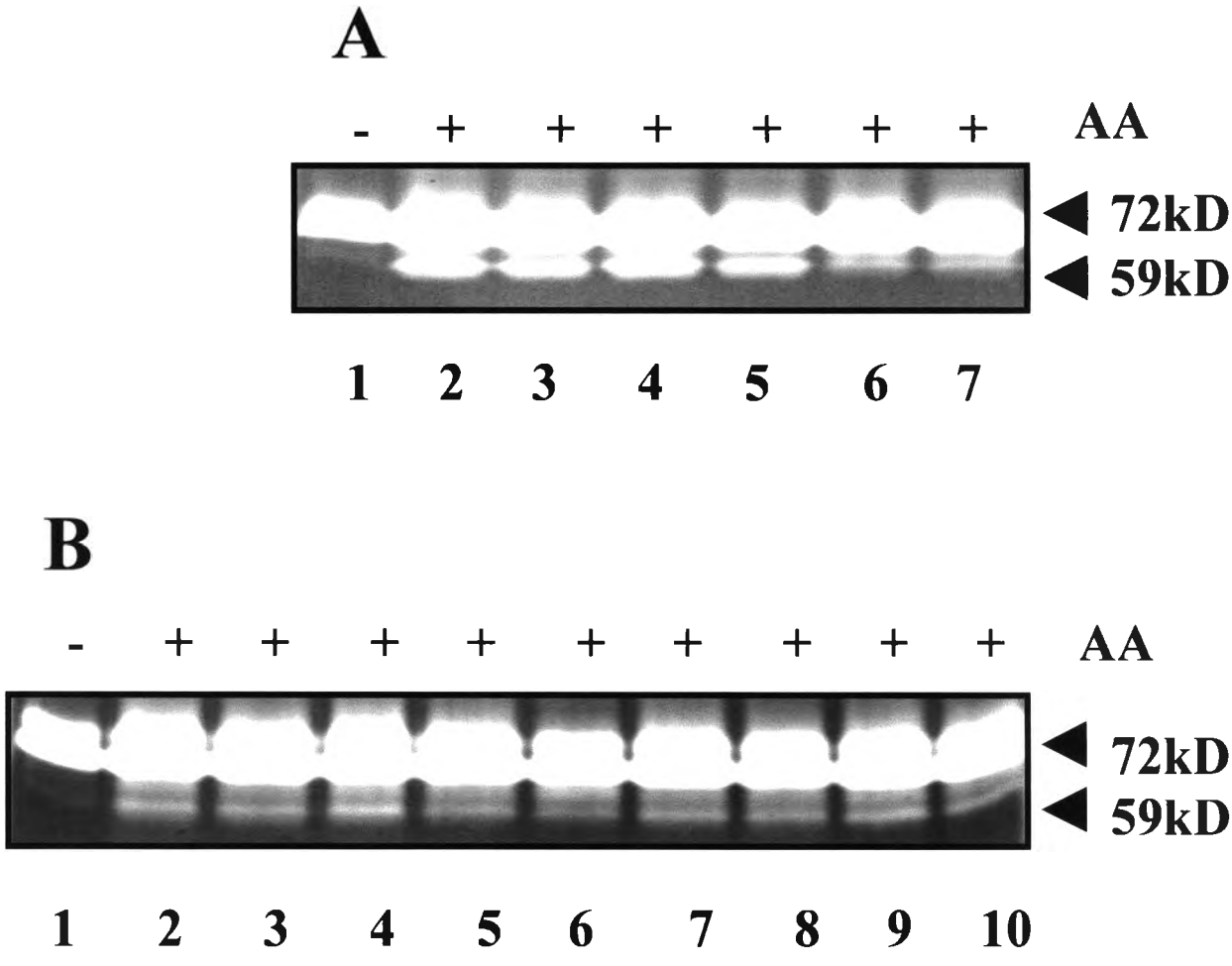

MT1+/+ MT1+/- MT1-/-
- + - + - + AA
A
α1(I)
α2(I)
B
72kD
59kD
C ML20
vc MT1
60kD

We attempted to further define the residual, non-MT1-MMP-mediated MMP-2 activation seen in the MT1-/- fibroblast endogenous response using a panel of inhibitors directed at the major protease families. MT1-/- fibroblasts were treated with ascorbate for 18 days after which the cultures were treated with inhibitors of serine (10 and 20 μg/ml aprotinin), aspartyl (5 μg/ml pepstatin), cysteinyl (10 and 20 μg/ml cystatin), and metallo [GM6001 (10^{-5} and $2x10^{-5}$ M)] proteinases. Again, active MMP-2 persisted in the presence of all of the proteinase inhibitors (Figure 3.7B). This alternative activation also persisted when MT1+/+ fibroblasts were treated with the metalloproteinase inhibitor. (Figure 3.7A).

We also ensured that the partial activation seen in the MT1-/- fibroblast cultures occurred during the final 96-hour period, and was not retained and released from the matrix after prior activation. Cyclohexamide treatment of the cultures for the final 96 hour period dramatically reduced the level of pro-MMP-2 seen after 96 hours, however, no active MMP-2 was seen, confirming that the mature MMP-2 that we see is not simply sequestered MMP-2 which was activated prior to the final 96 hour incubation period (not shown).

The inability to inhibit this residual activation with inhibitors for each of the major classes of proteases led us to question whether it may be occurring intra-cellularly, as suggested previously (Lee et al., 1997). Preliminary observations show a small amount of mature MMP-2 in cytoplasmic extracts from normal human fibroblasts, which is enhanced with ascorbate treatment. Our data nonetheless emphasize an important role for MT1-MMP in the activation of MMP-2 by fibroblasts.

Figure 3.7: Inability to inhibit MT1-MMP-independent activation of MMP-2 with various proteinase inhibitors. (*A.*) MT1-MMP wild-type mouse fibroblasts were cultured for 9 days in the absence (-) or presence (+) of 0.25 mM ascorbic acid (AA) and changed to SFM for 96 hours in the absence (lane 2) or presence of inhibitors for serine (20 μg/ml aprotinin, lane 3), aspartyl (5 μg/ml pepstatin, lane 4), cysteinyl (20 μg/ml cystatin, lane 5), or metallo ($2x10^{-5}$ M GM6001, lane 6; and 10^{-5} M GM6001, lane 7) proteinases. Conditioned media were analysed for the activation of MMP-2 by zymography. 72 kD and 59 kD represent the latent and fully active forms of MMP-2, respectively. (*B.*) MT1-MMP -/- knockout mouse fibroblasts were cultured for 18 days in the absence (-) or presence (+) of 0.25 mM ascorbic acid (AA) and changed to SFM for 96 hours in the absence (lane 2) or presence of inhibitors of serine (10 μg/ml aprotinin, lane 3; 20 μg/ml aprotinin, lane 4), metallo ($2x10^{-5}$ M GM6001, lane 5; 10^{-5} M GM6001, lane 6), aspartic (5 μg/ml pepstatin, lane 7), or cysteine (10 μg/ml cystatin, lane 8; 20 μg/ml cystatin, lane 9) proteinases. Cation chelation (10 nM of EDTA, lane 10), which inhibits metallo-proteinases, was also tested. Conditioned medium were collected and analysed for the activation of MMP-2 by zymography.


A
- + + + + + +
AA
72kD
59kD
1 2 3 4 5 6 7
B
- + + + + + + + + +
AA
72kD
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1 2 3 4 5 6 7 8 9 10

Discussion

The role of collagen in MMP-2 activation is well established, and the importance of this relationship is emphasized by the co-expression of MT1-MMP and α1(I) collagen in stromal cells surrounding breast tumours (Gilles et al., 1997; Ellenrieder et al., 2000) and the association between MT1-MMP expression, MMP-2-activation, and clinical outcome in breast cancer (Ueno et al., 1997). These observations suggest a relationship between fibrillar collagen and MT1-MMP which is important in both normal tissue homeostasis and in the control of neoplasia, consistent with the capacity of both MT1-MMP and MMP-2 to directly degrade type I collagen (Aimes and Quigley, 1995; Ohuchi et al., 1997). Numerous studies, including our own, have documented the MT1-MMP / MMP-2-activation responses of both cancer cells and normal cells (endothelial cells and fibroblasts). Here, we examined a more physiological activation of MMP-2 by fibroblasts producing varying amounts of collagen after diary addition of ascorbic acid (Chan et al., 1990). Fibroblasts grown in monolayer culture frequently show different responses to those grown in a thouree-dimensional collagen gel with the latter resembling the *in vivo* condition (Seltzer et al., 1994). Notably, the ascorbate-treated fibroblasts continued to proliferate thouroughout the 18 day-culture period, and formed a multi-layered, 3-dimensional culture (Chan et al., 1990). In the absence of ascorbic acid the cells were growth inhibited when confluent, and remained in a two-dimensional monolayer.

Increased Collagen Deposition Stimulates MT1-MMP Levels and MMP-2 Activation in Fibroblasts

We found that normal human skin fibroblasts showed activation of endogenous MMP-2 once the deposited collagen had accumulated. We confirmed an essential role for collagen in this response in 2 ways. First, we saw a close relationship between collagen deposition and MMP-2-activation potential using the Mov13 mouse fibroblast system. Mov13 cells, in which the gene encoding the α1(I) collagen chain is disrupted, showed no MMP-2-activation, and re-transfection of wild-type α1(I) collagen chains rescued MMP-2 activation in an ascorbate-dependent manner. Interestingly, the SV40-transformed control fibroblasts in the Mov13 system deposited collagen without ascorbate addition, and showed constitutive activation of MMP-2. Secondly, specific abrogation of collagen secretion by ascorbate-treated human skin fibroblasts with the prolyl 4-hydroxylase inhibitors blocked MMP-2-activation in a parallel way.

The degree of MMP-2 activation was found to relate to the levels of the cell surface MT1-MMP, similar to results obtained in studies of exogenous 3-dimensional collagen (Azzam et al., 1993; Gilles et al., 1997; Ruangpanit et al., 2001). The longer term endogenous response model, however, allowed the temporal resolution of transcriptional and non-transcriptional responses to collagen. Two distinct phases of regulation could be resolved; Firstly, increased MT1-MMP protein levels and MMP-2 activation become evident early in the cultures, from the earliest evidence of collagen deposition, and prior to any increase in MT1-MMP mRNA. Secondly, in the later stage ascorbate-treated cultures (from day 20), MT1-MMP mRNA levels were also increased, with commensurate increases in MT1-MMP protein, and MMP-2-activation. We have documented that both of these responses occur simultaneously when breast cancer cells are cultured on top of a thouree-dimensional gel of pepsin-extracted bovine skin collagen (Gilles et al., 1997), and we suspect the same is true of

fibroblasts cultured in the same way, or stimulated with the addition of collagen in solution (Ruangpanit et al., 2001). The data here suggest that either the maturation of collagen, and/or a higher amount of collagen, is required for the transcriptional response. Along these lines, the addition of BAPN to ascorbate-treated fibroblast cultures appeared to block the up-regulation of MT1-MMP mRNA expression, suggesting that inter- and/or intra-chain cross-links may be essential for the transcriptional response. It should be noted, however, that there was also a reduced amount of total collagen I after BAPN treatment, and this may also have explain the lack of MT1-MMP transcriptional response.

Fibril Requirement for the MMP-2-Activational Responses to Endogenous Collagen

Our recent studies with exogenously added collagen established the importance of the fibrillar form of collagen in inducing MMP-2 activation by fibroblasts (Azzam and Thompson, 1992; Seltzer et al., 1994; Tomasek et al., 1997; Ruangpanit et al., 2001). Further analysis of this requirement could be made in the endogenous response model. Indeed, we were surprised to find that ascorbate-treated Mov13-5 CM cells, which produce defective procollagen unable to form fibrils, showed quite robust MMP-2-activation, which could be blocked by the prolyl 4-hydroxylase inhibitors. Although small amounts of collagen deposition were detected by Coomassie Brilliant Blue in long-term, ascorbate-treated Mov13-5 CM cultures consistent with processing by non-specific cleavage (Bateman et al., 1987), this amount of collagen did not appear sufficient to induce MMP-2 activation. Addition of purified soluble collagen from conditioned medium of Mov13-5 CM to human

fibroblasts or Mov13-5 CM cells confirmed the capability of incompletely processed procollagen (pC-procollagen) to induce activation of MMP-2. Our conclusion is that the pC-procollagen secreted by these cells can adopt a configuration capable of inducing MMP-2 activation. Studies in mice in which the gene encoding BMP-1 (Bone Morphogenetic Protein 1 or C-proteinase) is partially disrupted revealed a 65nm periodicity of collagen fibrils which were thinner and had a barbed wire appearance by TEM (Suzuki et al., 1996). Other evidence has been documented to show collagen with attached C-prodomain being incorporated into fibril, albeit with lower efficiency, although the role of non-specific proteinase cleavage, as seen to a small degree in these cultures, cannot be ruled out (Bateman et al., 1987). It is not possible for us to conclude whether the activation we see is due to the low level of fibril formation in those cultures or due to a non-fibrillar form which can be achieved by this pC-procollagen, but we favour the latter interpretation and hope that it may ultimately shed light onto the molecular mechanisms involved in the collagen induction of MT1-MMP expression and functionality.

Role of MT1-MMP in MMP-2 Activation

Considerable data is mounting to support a specific requirement for MT1-MMP in the cell surface activation of MMP-2, even though many/most of the other MT-MMPs have been shown to have this potential. In support of this, mouse fibroblasts expressing different levels of MT1-MMP (MT1+/+, MT1+/- and MT1-/-) showed a positive correlation between MT1-MMP expression and the level of MMP-2 activation in the endogenous response model. This is consistent with the requirement of MT1-MMP in the MMP-2 activation process seen in the same

fibroblasts treated with exogenous collagen (Ruangpanit et al., 2001). However, significant activation of MMP-2 was detected in MT1-/- mouse fibroblasts embedded in their own collagen. Contributions of various other proteases to MMP-2-activation have been reported, including various soluble MMP's (Takino et al., 1995; Yoshiyama et al., 1998; Pei, 1999), urokinase (Zucker et al., 1995; Baramova et al., 1997; Mazzieri et al., 1997; Nguyen et al., 1999; Nguyen et al., 2000), and maybe other proteases (Belkhiri et al., 1997). To try and determine which class of protease is responsible for the non-MT1-MMP-mediated residual activation, we employed a variety of class specific proteinase inhibitors. However, none of the proteinase inhibitors employed blocked the remnant activation, indicating against an involvement of these enzymes in extracellular activation of the MMP-2. It is noteworthy that treatment of the wild-type ascorbate-treated cultures with the GM6001 synthetic MMP-inhibitor reduced the MMP-2-activation level to an amount similar to that seen in the MT1-MMP -/- cells, confirming that the MMP-independent residual activation was not unique to the MT1-MMP -/- cells. One possibility is that intracellular activation of MMP-2 is occurring, as previously suggested for MT1-MMP (Lee et al., 1997). We did detect activated MMP in cytoplasmic extracts of human fibroblasts, and its increase with ascorbate treatment is consistent with the notion of an intracellular collagen-responsive activation. A much lower degree of residual activation is seen when the MT1-MMP -/- fibroblasts are treated with either Con A (Holmbeck et al., 1999) or exogenous collagen (Ruangpanit et al., 2001), and may be related to that seen in the current study. In comparison however, the residual (non-MT1-MMP) activation seen here is much stronger. Since the long term, matrix-producing cultures better represent the *in vivo* situation, this residual activation may have physiological importance, and warrants further investigation.