MMP-2 release and activation in ovarian carcinoma: the role of fibroblasts

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Summary The matrix metalloproteinase MMP-2 is up-regulated in epithelial cancers and its mRNA localizes to stromal fibroblasts. In this paper we show that co-culture of ovarian carcinoma cells with fibroblasts resulted in an enhanced release of proMMP-2 and TIMP-2 into the culture medium. Cell–cell interaction was a major factor in this response and carcinoma cells stimulated proMMP-2 release from fibroblasts but not vice versa. Collagen I, in a dose-dependent fashion, induced activation of proMMP-2 by tumour-derived, but not normal, fibroblasts. Antibody to β1 integrin also induced proMMP-2 activation by tumour-derived fibroblasts. The activation involved the processing of proMMP-2 by a membrane-bound metalloproteinase. We propose that, in the ovarian tumour microenvironment, interaction between tumour cells and fibroblasts may enhance fibroblast production of the proMMP-2 and TIMP-2. Collagen I, also present in the ovarian tumours, then induces these fibroblasts to activate proMMP-2 even in the presence of TIMP-2. This active MMP-2 can associate with the cell surface of tumour cells and fibroblasts and is used in the processes of tissue remodelling and invasion.

Keywords: ovarian carcinoma; MMP-2; TIMP-2; collagen; fibroblasts

Matrix metalloproteinases (MMPs) are involved in the degradation of the extracellular matrix, a central element of tumour invasion and metastasis (Birkedal-Hansen, 1995). One of these enzymes, MMP-2, is able to degrade type IV collagen, which is a major component of the basement membrane (Liotta et al, 1991). This enzyme can also modulate carcinoma cell adhesion (Ray and Sterlet-Stevenson, 1995).

MMP-2 is released in a pro-form which can be activated by membrane-bound metalloproteinases, MT-MMP-1, -2 and -3 (Sato et al, 1994; Kolenbrock et al, 1997; Shofuda et al, 1997) which themselves require prior activation (Lohi et al, 1996). The active forms of these membrane metalloproteinases cleave the 72 kDa pro-form of MMP-2 at a specific amino acid residue generating an active 64 kDa form (Strongin et al, 1995). The 64 kDa form of MMP-2 can be further processed to a 62 kDa form by autocatalysis (Stetler-Stevenson et al, 1989a; Atkinson et al, 1995) or the plasminogen activator (PA)/plasmin system (Baramova et al, 1997). A number of naturally occurring MMP inhibitors, TIMPs 1–4, are also found in tissues (Stetler-Stevenson et al, 1989b; Greene et al, 1996). The activation of proMMP-2 by active MT-MMPs is blocked by the tissue inhibitor TIMP-2 (Atkinson et al, 1995; Strongin et al, 1995). Paradoxically TIMP-2 is also necessary for the formation of the MT-MMP1 complex which activates MMP-2 (Strongin et al, 1995). The tissue inhibitors TIMPs 1–4 also block the activity of MMP-2 (Stetler-Stevenson et al, 1989b; Greene et al, 1996).

Previous studies have identified the presence of proMMP-2 and its active form in ovarian cancer specimens (Naylor et al, 1994; Garzetti et al, 1995; Tamakoshi et al, 1995). One study has found no association between the level of latent or activated MMP-2 and the grade of ovarian carcinoma, although all of the carcinomas investigated contained latent and active forms of MMP-2 (Naylor et al, 1994).

MMP-2 may be involved in two different aspects of ovarian carcinoma spread. Ovarian tumour cells could use MMP-2 to detach from surface epithelia and migrate into the peritoneal cavity (Niedbala et al, 1987), but they may also use MMP-2 to invade through basement membrane into the ovarian stroma (Campo et al, 1992; Kuwashima et al, 1995).

Fibroblasts at the invasive edge of ovarian tumours show a striking increase in mRNA for MMP-2 and TIMP-2 (Campo et al, 1992; Autio-Harminen et al, 1993; Naylor et al, 1994). While ovarian tumour cells in tumour tissue do not contain MMP-2 or TIMP-2 mRNA, latent and activated MMP-2 protein can be detected at their surface (Afzal et al, 1996). As it is possible that tumour cell lines could utilize fibroblast MMP-2 for invasion (Fishman et al, 1997), interactions between stromal cells and tumour cells may play an important part in ovarian carcinoma pathology in vivo. In this paper we report that fibroblast–carcinoma cell interactions in vitro result in the stimulation of proMMP-2 and TIMP-2 release by fibroblasts. We also describe a mechanism by which collagen I can induce proMMP-2 activation by tumour-derived, but not normal, fibroblasts.

MATERIALS AND METHODS

Materials

ProMMP-2 and antisera against MMP-2 or TIMP-2 were obtained from TCS Biologicals (Buckingham, UK). Anti-EMMPRIN antibody was a kind gift from Dr Bryan P Toole, Tufts University School of Medicine (Boston, MA, USA). The anti-β1 integrin antibody was from Gibco (Paisley, UK); rat tail collagen I from Beckton-Dickinson, Bedford, MA, USA and ECL™ reagent was...
added. Times with medium before fibroblasts or carcinoma cells were 5 min with ice cold 80% methanol. Fixed cells were washed three times with serum-containing medium. In order to detect proMMP-2 processing activity by fibroblast membranes, fibroblasts (2 × 10⁶) were treated with Triton X-114 and 50 µl detergent extract was mixed with 50 µl of TBS, containing 0.02% sodium azide and proMMP-2 (10 ng). ProMMP-2 was also incubated with detergent alone as a control. The reaction mixture was incubated for 48 h at 37°C and the aqueous phase was analysed by gelatin zymography (Lewalle et al, 1995).

Western blotting
Protein in supernatant from the co-culture experiments was precipitated with acetone, run on an SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. The membrane was blocked by 5% milk powder and incubated with specific antibody, revealed with ECL reagents and exposed to X-ray film. Protein of unknown samples was calibrated relative to a fixed amount of standard proMMP-2.

Reverse transcription (RT)-PCR
RNA was extracted from cells using the TRI™-reagent, digested by DNase and converted to cDNA using a Pharmacia ‘Ready To Go Kit’. cDNA was amplified using specific sets of primers and the NIH 1.58 software package was used to quantitate changes in gelatin concentration. The gelatinolytic activity of unknown samples was calibrated relative to a fixed amount of standard proMMP-2.

RESULTS
MMP-2 and TIMP-2 release
Studies of ovarian carcinoma tissue have shown that it consists of nests of tumour cells surrounded by a stroma of fibroblast-like cells (Naylor et al, 1994). Co-culture of dermal fibroblasts and ovarian carcinoma cell lines resulted in similar structures to those seen in vivo with small islands of tumour cells surrounded by a stroma of fibroblast-like cells (Bordier, 1981). Dermal fibroblasts which varied with cell line (Figure 2A). In co-culture experiments with dermal fibroblasts and the ovarian tumour cell lines PEO1, PEO14 or SKOV3, a ratio of 1:5 was chosen because of the leading edge of tumour tissue there is an excess of tumour cells to
fibroblasts (Naylor et al, 1994). A two- to eightfold enhanced release of proMMP-2 was found in such co-cultures (Figure 2A). This enhancement of proMMP-2 release during co-culture was quantified and found to be significant, $P < 0.001$ (Figure 2B).

We then isolated fibroblasts from ovarian tumours and co-cultured them with tumour cells to determine if the tumour fibroblasts behaved in a similar way to normal fibroblasts in co-culture. Co-culture of ovarian carcinoma cell lines with three different batches of fibroblasts derived from ovarian carcinomas resulted in similar structures to those described in Figure 1 and also resulted in a significant stimulation of proMMP-2 release, $P < 0.05$ (Figure 2C). The higher basal release of proMMP-2 from tumour fibroblasts resulted in a lower fold increase of proMMP-2 release compared to that found in dermal fibroblast co-cultures (Figure 2B, C).

Western blotting of protein from cultured supernatants with MMP-2 antisera confirmed the results seen with gelatin zymography (Figure 3); co-culture of dermal fibroblasts and the ovarian carcinoma cell lines enhanced the release of proMMP-2 into the culture media (two- to sixfold increase in three independent experiments). The level of TIMP-2 immunoreactivity in culture medium from co-cultures was also stimulated by a similar degree (Figure 3).

Conditioned medium from the carcinoma cell lines PEO1, PEO14 and SKOV3 did not enhance the release of proMMP-2 from dermal fibroblasts and also had no effect on fibroblast proliferation (data not shown). However, culture of dermal fibroblasts and fixed carcinoma cells resulted in a significant increase in proMMP-2 release from dermal fibroblasts, $P < 0.01$ (Figure 4). Culture of tumour fibroblasts and fixed carcinoma cells also stimulated proMMP-2 release, but fixed fibroblast cell membranes did not enhance proMMP-2 release from the carcinoma cells (data not shown).

**ProMMP-2 activation**

Co-culture of fibroblasts and ovarian carcinoma cell lines did not result in significant activation of proMMP-2 (Figures 2A and 3). A trace amount of the activated 62 kDa form of MMP-2 could be detected in the medium from co-cultures, but it was < 1% of total MMP-2 activity. However, the activated form of MMP-2 is readily detected in ovarian carcinoma tissue (Naylor et al, 1994; Noel et al, 1994; Ito et al, 1995). We therefore investigated the expression of MT-MMP-type enzymes in fibroblast and carcinoma cells, and the ability of cell membranes to activate proMMP-2.
Membrane-bound metalloproteinase activators of proMMP-2

Using RT-PCR we found that fibroblasts (both dermal and tumour), PEO14, SKOV3, but not PEO1, ovarian cells all expressed MT-MMP-1 mRNA. RT-PCR analysis of MT-MMP-1, -2, -3, and -4 revealed no qualitative difference in the expression profile for the dermal and tumour fibroblasts. The fibroblasts, PEO14 and SKOV3 cells also had a low level of MT-MMP-1 protein in their membranes. To elevate the level of membrane MT-MMP-1 (both the 63 and the activated 60 kDa forms) we treated tumour fibroblasts with 50 μg ml⁻¹ concanavalin A (Lohi et al, 1996). However, this did not result in the activation of extracellular proMMP-2 by the tumour fibroblasts (Figure 5A).

Incubation of proMMP-2 with a membrane protein extract of tumour fibroblasts (detergent phase from Triton X-114-treated fibroblast batches 1, 2 and 3) was treated with (+) or without (−) concanavalin A (50 μg ml⁻¹) for 24 h. The fibroblast membranes were extracted with 1.5% Triton X-114, the proteins in the detergent phase run on an SDS-polyacrylamide gel, then blotted. MMP-2 and TIMP-2 were detected by specific antisera. Conditioned medium from dermal fibroblasts (1), PEO1 (2), PEO14 (3), SKOV3 (4), fibroblasts + PEO1 (5), fibroblasts + PEO14 (6) and fibroblasts + SKOV3 (7). The blots are representative of three independent experiments.

Figure 3 Effect of co-culture of the carcinoma cell lines PEO1, PEO14 and SKOV3 with dermal fibroblasts on the release of MMP-2 and TIMP-2 into the culture media. The culture medium was concentrated, run on an 11% SDS-polyacrylamide gel, then blotted. MMP-2 and TIMP-2 were detected by specific antisera. Conditioned medium from dermal fibroblasts (1), PEO1 (2), PEO14 (3), SKOV3 (4), fibroblasts + PEO1 (5), fibroblasts + PEO14 (6) and fibroblasts + SKOV3 (7). The blots are representative of three independent experiments.

Figure 4 Effect of methanol-fixed carcinoma cell lines on the release of proMMP-2 from fibroblasts. PEO1, PEO14 and SKOV3 (12.5 × 10⁴ cells) were incubated overnight in culture medium + serum. The carcinoma cells were fixed by incubation in ice cold methanol for 5 min, dermal fibroblasts (2.5 × 10⁵ cells) were added and cultured overnight in culture medium + serum. After 24 h, the cells were washed and incubated in serum free medium for 48 h. The medium was then analysed by quantitative gelatin zymography. This experiment is representative of three independent experiments done in duplicate. * represents P < 0.01.

Figure 5 (A) Effect of concanavalin A on MT-MMP-1 protein in membranes from ovarian tumour fibroblasts and the activation of proMMP-2. Ovarian fibroblast batches 1, 2 and 3 were treated with (+) or without (−) concanavalin A (50 μg ml⁻¹) for 24 h. The fibroblast membranes were extracted with 1.5% Triton X-114, the proteins in the detergent phase run on an SDS-polyacrylamide gel, blotted and MT-MMP-1 detected using specific antisera. The culture medium from concanavalin A-treated cells was analysed by quantitative gelatin zymography. This experiment is representative of three performed. (B) Activation of proMMP-2 by Triton X-114 membrane protein extract of tumour fibroblasts. All incubations were for 48 h at 37°C. ProMMP-2 with Triton X-114 (1), membrane protein extract with proMMP-2 (2), membrane protein extract with proMMP-2 and BB2116 (30 μM) (3), membrane protein extract plus proMMP-2 complexed with TIMP-2 (4). This experiment is representative of three performed.

64 kDa form of MMP-2. This activation of MMP-2 was blocked by an inhibitor of matrix metalloproteinases, BB2116 (30 μM), suggesting that a MMP-type enzyme was involved in proMMP-2 processing (Figure 5B). As the tumour fibroblasts released TIMP-2 as well as proMMP-2 (data not shown), we incubated MMP-2 complexed with TIMP-2 with the detergent phase from Triton X-114-extracted tumour fibroblasts. No activation of proMMP-2 was seen under these conditions (Figure 5B). The detergent phase from Triton X-114-extracted PEO14 and SKOV3, but not PEO1, cells was also able to activate proMMP-2 and this activation of MMP-2 was blocked by 30 μM BB2116 (data not shown).

Taken together, these results suggest that MT-MMP-type enzymes in the membrane of both tumour cells and fibroblasts can activate proMMP-2, but in co-culture their action is blocked, most likely by TIMP-2. We therefore investigated factors in the extracellular environment of tumours which could stimulate proMMP-2 activation and account for the presence of active MMP-2 in ovarian cancer biopsies. One potential candidate was collagen I, which is a major component of ovarian carcinoma tissue (Zhu et al, 1994). Previous studies showed that culture of normal fibroblasts in collagen gels results in proMMP-2 activation (Azzam and Thompson, 1992; Seltzer et al, 1994; Gilles et al, 1997).

Collagen I treatment of fibroblasts

Treatment of normal fibroblasts with soluble collagen I (100 μg ml⁻¹) did not increase activation of proMMP-2 (Figure 6A). However, incubation of three different batches of
tumour fibroblasts with collagen I (100 mg ml–1) resulted in the appearance of the fully activated 62 kDa form of MMP-2 in the cell culture medium (Figure 6A shows a typical result). A faint gelatinolytic band of 64 kDa could also be detected between the 72 and 62 kDa forms of MMP-2 (Figure 6A). BB-2116 (30 mM) blocked the activation of proMMP-2 by tumour fibroblasts in the presence of collagen I (Figure 6A).

The amount of 62 kDa MMP-2 detected in the culture medium of collagen I-treated cells was increased by 10–2-fold in three experiments. This activated form of MMP-2 could also be detected by Western blotting with specific MMP-2 antiserum (data not shown). The effects of collagen I (4–100 mg ml–1) on proMMP-2 activation were dose-dependent (Figure 6B). The response of tumour fibroblasts to collagen I was only present for the first few passages after isolation (data not shown).

The association of active MMP-2 with cell membranes

We next looked for the presence of active MMP-2 in the membranes of collagen I and concanavalin A-treated tumour fibroblasts. Tumour fibroblasts were incubated with medium alone, collagen I or concanavalin A. Fibroblasts were treated with Triton X-114 and the detergent phase (containing membrane proteins) was analysed for gelatinase activity. The detergent phase from fibroblasts treated with medium alone contained no gelatinase activity. However, the detergent phase from collagen I-treated cells contained a 62 kDa band, whereas the detergent phase from concanavalin A-treated cells contained only a 72 kDa gelatinase band (Figure 7).

Integrin-mediated activation of MMP-2

Collagen I can interact with integrin collagen receptors such as α2β1 and α1β1. Treatment of tumour fibroblasts with specific anti-β1 integrin antibody (10 μg ml–1) also resulted in the activation of proMMP-2 but only the 64 kDa form of MMP-2 could be detected; BB-2116 (30 μM) blocked proMMP-2 activation by both collagen I and anti-β1 integrin antibody (Figure 8). Treatment of normal dermal fibroblasts with the anti-β1 integrin did not result in proMMP-2 activation (Figure 8).
DISCUSSION

In this study, an in vitro co-culture system was used to investigate the mechanisms by which ovarian carcinoma cells stimulate MMP-2 release and activation by fibroblasts. Culture of ovarian carcinoma cells with normal dermal fibroblasts or ovarian tumour fibroblasts resulted in a stimulation of proMMP-2 release into the medium. The stimulation of proMMP-2 release, seen when tumour fibroblasts were cultured with ovarian cancer cells, was lower than that seen with dermal fibroblasts, probably because of higher basal release of proMMP-2 from tumour fibroblasts.

In co-cultures of normal fibroblasts and carcinoma cell lines, TIMP-2 release was also enhanced. The stimulation of proMMP-2 and TIMP-2 release from co-cultures may suggest that the release of these proteins is co-ordinately regulated. This observation is also consistent with the lack of proMMP-2 activation found in co-cultures since TIMP-2 inhibits MT-MMP-1 mediated MMP-2 activation.

Cultured ovarian carcinoma cells or cell lines release both MMP-2 and TIMP-2 (Moser et al., 1994). On the basis of in vitro studies it has been proposed that ovarian carcinoma cells may produce MMP-2 in vivo (Fishman et al., 1997; Westerlund et al., 1997). However, in ovarian cancer biopsies, MMP-2 and TIMP-2 mRNAs localize to the stroma. Epithelial carcinoma cell production of these molecules was not detected (Naylor et al., 1994). This suggests that production of proMMP-2 and TIMP-2 by cultured carcinoma cells may be an adaptation to in vitro culture conditions.

Culture of fibroblasts with fixed carcinoma cells indicates that cell–cell contact plays an important part in the enhancement of proMMP-2 release seen in co-cultures. This is in agreement with the in vivo data where only the fibroblasts close to, or in contact with, carcinoma cells have elevated mRNA for MMP-2 and TIMP-2 (Naylor et al., 1994). The nature of the membrane factor(s) that stimulates proMMP-2 and TIMP-2 release is unknown. EMMPRIN has been proposed as a regulator of MMP-2 release from fibroblasts (Kataoka et al., 1993) and, although this protein could be detected in PEO1 and PEO14 membranes, it could not be detected in SKOV3 cell membranes, yet all three stimulated proMMP-2 release (data not shown).

Both tumour fibroblasts and carcinoma cells express MT-MMP-1 protein (Fishman et al., 1997) and we confirmed this in our study. MT-MMP-1 mRNA is expressed in stromal fibroblast-like cells close to tumour cells in tumour tissue (Okada et al., 1995; Ohtani et al., 1996; Ueno et al., 1997). It has been proposed that MT-MMP-1 is responsible for the activation of proMMP-2 in ovarian carcinoma tissue (Fishman et al., 1997). However, culture of ovarian carcinoma cells and normal or tumour fibroblasts did not result in the activation of MMP-2, probably because extracellular TIMP-2 blocked proMMP-2 activation. Even when the amount of MT-MMP-1 in tumour fibroblasts was increased by concanavalin A treatment, no significant activation of MMP-2 could be seen, in agreement with a recent study on primary human fibroblasts (Lohi et al., 1996). Hence the expression of MT-MMP-1 protein does not always correlate with MMP-2 activation. The ovarian carcinoma microenvironment is rich in TIMPs (Naylor et al., 1994; Tamamkoshi et al., 1995; Kikkawa et al., 1997) and these inhibitors might be predicted to block the activity of MT-MMP-type enzymes. We have found that levels of collagen I as low as 4 μg ml⁻¹ can stimulate the activation of proMMP-2 by tumour fibroblasts, even in the presence of TIMPs. Normal fibroblasts did not display this responsiveness to similar doses of collagen I. Incubation of normal fibroblasts or invasive breast cancer cells in collagen I gels (containing 0.5–1 μg ml⁻¹ of collagen I), results in gross morphological changes and the intracellular activation of proMMP-2 by a metalloproteinase-like enzyme, but no change in TIMP-2 release (Seltzer et al., 1994; Gilles et al., 1997; Lee et al., 1997). Gilles et al. (1997) also reported that three dimensional collagen gel culture up-regulated MT-MMP-1. However, at the lower collagen concentrations used in our study, the tumour-derived fibroblasts seem to exhibit an enhanced responsiveness to collagen.

It is likely that collagen I is acting through the α_1β_1 or α_2β_1 receptors on the tumour-derived fibroblasts to stimulate proMMP-2 activation to the 62 kDa form of MMP-2. Anti-β_1 integrin antibody induced tumour, but not normal, fibroblasts to activate proMMP-2 into the 64 kDa form of MMP-2. This result suggests that the β_1 integrin subunit is involved in the differential response of tumour fibroblasts to collagen I; this could occur by an up-regulation of the β_1 subunit and/or integrins or an enhanced affinity of β_1 integrin for collagen I. It is of interest that treatment of rhabdomyosarcoma cells with anti-α_1 and anti-α_2 integrin antibodies increased their ability to activate MMP-2, and also increased proMMP-2 secretion and invasiveness through matrigel (Kubota et al., 1997).

There are at least three possible mechanisms by which collagen I treatment leads to proMMP-2 activation: collagen I could induce the expression of a metalloproteinase or metalloproteinase activator, it may reduce TIMP-2 levels, or it may relocalize MT-MMP-1 to specific cellular sites. At the site of invasion, β_1 integrin may possibly interact with MT-MMP-1. We also have preliminary data that collagen I does not up-regulate metalloproteinase activity in tumour fibroblasts, again favouring the third hypothesis.

In conclusion, we have found that fibroblast–carcinoma cell interaction in vitro results in the stimulation of proMMP-2 and TIMP-2 release by fibroblasts. This corresponds to our in vivo observations of MMP-2 and TIMP-2 mRNA expression and localization (Naylor et al., 1994). We have also found that ovarian tumour-derived fibroblasts have a novel responsiveness to collagen I with respect to proMMP-2 activation. Collagen I stimulation of proMMP-2 activation by tumour-derived fibroblasts appears to involve the ligation of the β_1 integrin and provides a mechanism by which ovarian tumours could activate MMP-2, despite the presence of TIMPs. The binding of this activated MMP-2 by receptors in the invadopodia of invasive cells, provides a temporal window of opportunity for the utilization of active MMP-2 in cell migration and invasion.

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