Induction of matrix metalloproteinase activation cascades based on membrane-type 1 matrix metalloproteinase: associated activation of gelatinase A, gelatinase B and collagenase 3

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INTRODUCTION

Human procollagenase 3 (MMP-13) cDNA was first isolated from a breast-tumour library and a role was proposed for the protein in tumour progression [1]. Subsequent characterization of recombinant human procollagenase 3 [2] revealed a glycosylated proenzyme of 60 kDa that can be activated by 4-aminophenylmercuric acetate (APMA) or stromelysin 1 (MMP-3) through a 50 kDa intermediate form to a 48 kDa active collagenolytic activity. The subsite specificity of collagenase 3 is similar to that of the gelatinases, and collagenase 3 cleaves collagen as well as type I, II and III collagens and other extracellular-matrix components. A comparison of collagenase sequences from various species showed human collagenase 3 to be more closely related to the interstitial collagenase from rodents than to human procollagenase 1 (fibroblast procollagenase, MMP-1). Like the rodent enzyme, human collagenase 3 cleaves type II collagen more readily than type I and III collagens and its activity is regulated by tissue inhibitors of metalloproteinases (TIMPs)-1, -2 and -3 [2,3].

The human chondrosarcoma cell line SW1353 produces procollagenase 3 mRNA after stimulation with a combination of interleukin 1β (IL-1β) and tumour-necrosis factor-α [4]. Human procollagenase 3 has been demonstrated in osteoarthritic cartilage at both the protein and mRNA levels and is up-regulated upon stimulation with IL-1 [3,5]. The enzyme has also been reported to be produced by synovial cells [6]. The presence of procollagenase 3 in joint cells is significant, since active collagenase 3 is capable of degrading proteoglycan as well as type II collagen [7], and it is possible that some of the cartilage destruction seen in osteo- and rheumatoid arthritis may result from collagenase 3 activity. The activation of procollagenase 3 can be brought about in vitro by stromelysin 1, gelatinase A (MMP-2), MT1-MMP (MMP-14) or plasmin [2,8], but the in vivo activators are not known. The present paper details an investigation of the production and activation of procollagenase 3 by SW1353 cells and describes data on the existence of a potential activation cascade involving collagenase 3, gelatinase A and gelatinase B (MMP-9).

EXPERIMENTAL

Materials

SW1353 cells (human chondrosarcoma cell line) were obtained from the American Type Culture Collection (A.T.C.C. HTB-94). Oncostatin M (Onco M), PMA, transforming growth factor-β1 (TGF-β1), all-trans-retinoic acid and concanavalin A (ConA) were from the Sigma Chemical Co. IL-1β was generously provided by Professor Jeremy Saklatvala, Kennedy Institute of Rheumatology, Hammersmith, London, U.K. Recombinant TIMPs-1, -2 and -3 and recombinant human procollagenase 3

Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; MT1-MMP, membrane-type 1 matrix metalloproteinase; ConA, concanavalin A; Onc M, oncostatin M; DMEM, Dulbecco’s modified Eagle’s medium; IL, interleukin; FBS, fetal bovine serum; APMA, 4-aminophenylmercuric acetate; TGF-β1, transforming growth factor-β1.
were prepared from the culture medium of transfected NS0 myeloma cells essentially as described in [2,9–11].

Cell culture

SW1353 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s F-12 (1:1) plus 10% fetal-bovine serum (FBS) in a humid atmosphere containing 5% CO₂ at 37°C, and passaged with trypsin/EDTA according to standard laboratory procedures. In order to condition the medium, cells were seeded at 1×10⁶ cells/well in a 24-well plate (Linbro™ ICN Biomedicals Ltd., Thame, Oxon., U.K.) in medium containing FBS. After 24 h the cells were washed twice in serum-free medium and cultured for a further 24–72 h in serum-free medium containing 0.2% lactalbumin hydrolysate (300 µl/well).

Characterization of a polyclonal antiserum raised to recombinant human procollagenase 3

A polyclonal antiserum to human procollagenase 3 was raised as described for rabbit collagenase 1 [12] by injecting 100 µg of purified recombinant procollagenase 3 into a sheep, followed by two further injections of 50 µg. Immunoglobulins were prepared from the bleed with the highest titre by (NH₄)₂SO₄ precipitation. Western blotting of the antiserum against purified recombinant procollagenase 3 identified a single band of 60 kDa, and blotting against conditioned media from ConA-stimulated fibroblasts to which recombinant procollagenase 3 had been added identified the 60 kDa band of the proenzyme and the 48 kDa band of the active form [2,8]. Western blotting of conditioned media from SW1353 chondrosarcoma cells stimulated with IL-1β identified the 60 kDa proenzyme (Figure 1, lane 5), and bands at 60 kDa (proenzyme), 48 kDa (active) and two intermediates (56 and 50 kDa) [2] in media from cells stimulated by IL-1 and ConA (Figure 1, lane 6). There was no reaction when the antiserum was used in Western blots of purified recombinant human collagenase 1, neutrophil collagenase (MMP-8), gelatinase A, gelatinase B, TIMP-1, TIMP-2 and TIMP-3. The antiserum detected stromelysin 1 when the enzyme was present at or above 50 ng/lane.

When used for immunolocalization, the anti-(collagenase 3) antibody gave strong staining on NS0 mouse myeloma cells transfected with procollagenase 3, but was negative on NS0 mouse myeloma cells transfected with either human collagenase 1, human gelatinases A and B or human stromelysins 1 and 2.

Western-blot analysis

Conditioned media were analysed for collagenases, stromelysin 1 and TIMP-2 by Western blotting using specific polyclonal antibodies. Polyclonal antibodies raised in sheep against human collagenase 1 [13], human collagenase 3 [8], human stromelysin 1 [14], human membrane-type 1 matrix metalloproteinase (MT1-MMP) [15] and human TIMP-2 [16] were used. Where indicated, media were concentrated using Amicon minicon concentrators (10 kDa cut-off). Samples of media were separated on SDS/10% polyacrylamide gels under reducing conditions and transferred on to a nitrocellulose membrane (Amersham Hyperbond ECL). The membrane was blocked [5%, v/v] dried skimmed-milk powder in 10 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.05% Tween 20; 30 min at room temperature) and incubated with the relevant polyclonal antibody (30–50 µg/ml in blocking buffer, overnight at 4°C). Bound antibody was detected with a donkey anti-sheep horseradish-peroxidase-conjugated antibody (Jackson Immunoresearch Laboratories Inc., West Grove, PA, U.S.A.; 1:10000 in blocking buffer; 1 h at room temperature) and chemiluminescence substrate (Supersignal™ CL-HRP Substrate System; Pierce, Rockford, IL, U.S.A.). Luminescence was recorded on Amersham Hyperfilm ECL.

Collagenolytic activity

Conditioned media were tested for activity against type I collagen by incubation with 14C-labelled type I collagen fibrils [17], without activation by APMA.

Gelatin zymography

Conditioned media were analysed for gelatinases and TIMPs by gelatin zymography [18]. For gelatinases, samples were separated under non-reducing conditions on SDS/12% polyacrylamide gels incorporating 0.5 mg/ml gelatin. For TIMPs, samples were separated on SDS/12% polyacrylamide gels incorporating 0.5 mg/ml gelatin as described in [16].

Preparation of cell membranes

Membranes were prepared by a modification of the method of Ward et al. [19]. Briefly, monolayers of SW1353 cells with or without ConA-treatment were washed twice in serum-free medium then scraped into ice-cold Ham’s F-12. The cells were centrifuged and resuspended in ice-cold Ham’s F-12 twice, then finally centrifuged, resuspended in ice-cold Ham’s F-12 containing protease inhibitors [1 mM PMSF, 1 µg/ml pepstatin and 2 µg/ml of trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64)] and frozen at −70°C overnight. The cell pellet was resuspended in Ham’s F-12 medium with inhibitors and frozen as described above. Homogenization and further centrifugation steps were as described by Ward et al. [19], except that the inhibitors were included throughout.
RESULTS AND DISCUSSION

Induction of procollagenase 3 expression in SW1353 cells

Conditioned media from SW1353 cell monolayers treated with IL-1β or PMA contained significant levels of latent collagenase 3, detected as a single 60 kDa band by Western blotting (Figure 1, lanes 5 and 8), relative to medium from untreated cells. The response to IL-1α was essentially the same as to IL-1β (results not shown). Oncostatin M (Onco M) alone did not induce procollagenase 3 (results not shown), but potentiated the production of procollagenase 3 by IL-1β-stimulated cells (Figure 1, lane 3). This agrees with findings with bovine nasal cartilage, where Onco M increased IL-1-stimulated collagenolytic activity [20]. Onco M was earlier proposed as a chondroprotective agent when it was found to stimulate TIMP-1 expression in human articular chondrocytes [21]. In synovial lining cells, however, it up-regulates TIMP-1 but down-regulates TIMP-3 [22].

Treatment of cells with ConA in combination with IL-1β or IL-1β and Onco M up-regulated collagenase 3 still further (Figure 1, lanes 4 and 6). The band corresponding to active collagenase 3 (48 kDa) was only present after treatment of cells with the tetravalent lectin ConA. Conditioned media from unstimulated cells or from cells treated for 24 h with a combination of IL-1β plus Onco M had negligible activity against 125I-labelled type I collagen. Collagenolytic activity was detected in media from cells treated with IL-1β and ConA (0.06 unit/ml) or with IL-1β, Onco M and ConA (0.16 unit/ml). IL-4 lowered the amount of procollagenase 3 secreted into the medium after IL-1β/Onco M treatment (Figure 2, lane 2). This effect was less apparent when ConA was also present (Figure 2, lane 4). IL-4 has been shown to induce the IL-1 receptor antagonist in mononuclear phagocytes [23] and references cited therein. The induction of IL-1 receptor antagonist in SW1353 cells could have decreased the sensitivity of the cells to IL-1, thereby down-regulating procollagenase 3 synthesis. Conditioned medium from cells treated with ConA alone contained low levels of both latent and active collagenase 3, which could be detected by immunoblotting of concentrated media (Figure 3). Concentrated medium from unstimulated cells did not contain procollagenase 3.

Retinoic acid, which induces matrix loss in human cartilage explants [24], did not induce procollagenase 3 expression in SW1353 cells (results not shown). TGF-β (100 ng/ml) affected neither the induction nor the activation of procollagenase 3 by SW1353 cells (results not shown), although TGF-β1, -β2 and -β3 were all capable of counteracting the retinoic acid-induced degradation of proteoglycan in calf articular cartilage [25]. In human transformed epidermal keratinocytes TGF-β induced the expression of procollagenase 3; this induction was partially prevented by retinoic acid [26]. Thus the regulation of procollagenase 3 clearly differs between the two cell lines.

Secretion and activation of gelatinases A and B

The secretion of gelatinases into the culture medium was determined by zymography, since this method was found to be more sensitive than Western blotting. Zymography enabled the activation status of the gelatinases to be assessed individually without interference by TIMPs. Progelatinase A was produced constitutively by SW1353 cells (Figure 4A, lane 1), and remained latent after treatment of the cells with either PMA (not shown) or with the combination of IL-1β and Onco M (Figure 4A, lane 2). Con A treatment led to the processing of endogenous progelatinase A to a molecular mass corresponding to active gelatinase A (Figure 4A, lanes 3, 4, 7, 8 and 9). Progelatinase B was up-regulated by IL-1β (with or without Onco M) or by PMA (Figure 4A, lanes 2–11), and some conversion into active gelatinase B was visible on zymograms after stimulation of the cells with a combination of IL-1β, Onco M and ConA (Figure 4A, lanes 3 and 7). Active gelatinase B was seen only when active collagenase 3 and active gelatinase A were present (Figure 4A, lanes 3 and 7); however, after treatment of cells with ConA alone, the level of progelatinase B remained low and no active gelatinase B was detected (results not shown). Since both gelatinase A [27] and collagenase 3 [28] can activate progelatinase B in vitro, one or both of these enzymes may be responsible for progelatinase B activation by SW1353 monolayers. In order to identify the role of collagenase 3 in the activation of progelatinase B, cells were stimulated with PMA. The induction of progelatinase B by PMA is comparable with that by IL-1 and Onco M, but induction of procollagenase 3 by PMA is much lower. Although gelatinase A was activated by ConA treatment of
PMA-stimulated cells, the level of active collagenase 3 was low and no active gelatinase B was detected (Figure 4A, lane 8). These results suggest that collagenase 3 is involved in pro-gelatinase B activation by SW1353 cells. In other studies we have shown that the activation of progelatinase B by active collagenase 3 in vitro occurs at a rate dependent on the concentrations of both the latent and the activating enzymes [28].

Figure 4  Associated activation of collagenase 3 and gelatinases A and B

(A) SW1353 cells were cultured for 48 h in serum-free medium with additions of IL-1β (10 ng/ml), Onco M (50 ng/ml), PMA (20 ng/ml), ConA (50 µg/ml), TIMP-1 (80 nM), TIMP-2 (80 nM) and TIMP-3 (80 nM). Conditioned media were tested for gelatinases by gelatin zymography (upper panel) and for MMP-13 by Western blot (lower panel). Lanes: 1, no addition; 2, IL-1 and Onco M; 3, IL-1, Onco M and ConA; 4, IL-1, Onco M, ConA and TIMP-1; 5, IL-1, Onco M, ConA and TIMP-2; 6, IL-1, Onco M, ConA and TIMP-3; 7, IL-1, Onco M and ConA; 8, PMA and ConA; 9, PMA, ConA and TIMP-1; 10, PMA, ConA and TIMP-2; and 11, PMA, ConA and TIMP-3. (B) SW1353 cells were cultured for 48 h in serum-free medium with additions of IL-1β (10 ng/ml), Onco M (50 ng/ml), ConA (50 µg/ml), aprotinin (2 µg/ml) and plasminogen (5 to 20 µg/ml as indicated). Conditioned media were tested for gelatinases by gelatin zymography (upper panel) and for MMP-13 by Western blot (lower panel). Lanes: 1, no addition; 2, IL-1 and Onco M; 3, IL-1, Onco M and ConA; 4, IL-1, Onco M, ConA and aprotinin; 5, IL-1, Onco M and ConA; 6, and 8, IL-1, Onco M and plasminogen; and 7 and 9, IL-1, Onco M, ConA and plasminogen.

Secretion and activation of procollagenase 1 and prostromelysin

Procollagenase 1 production, as determined by Western blot, was upregulated either by PMA (Figure 1, lane 8) or by IL-1β in combination with Onco M or ConA (Figure 1, lanes 3, 4 and 6). Active collagenase 1 was present in medium from unstimulated SW1353 cells, and the level of active enzyme decreased upon Con A treatment, indicating that the activation of procollagenase 1 was independent of the activation of progelatinase A, progelatinase B and procollagenase 3. Stimulation with PMA led to an increase in active collagenase 1. These results demonstrate the existence of independent activation mechanisms for collagenases 1 and 3 in SW1353 cells. Neutrophil collagenase could not be detected in the medium by Western blotting (results not shown). The presence of active collagenase 1, which can activate progelatinase A in vitro [29], did not correlate with the activation of procollagenase 3 and progelatinase A. This rules out any contribution by collagenase 1 to the activation of procollagenase 3 and progelatinases in SW1353 cells.

Treatment of SW1353 cells with PMA or IL-1β (with or without Onco M) up-regulated prostromelysin 1 (Figure 1), but the addition of ConA did not lead to the activation of this enzyme. Although stromelysin 1 activates procollagenase 3 [2] and progelatinase B [30] in vitro, little active stromelysin 1 was present at the time of procollagenase 3 activation by SW1353 cells. It is therefore unlikely that stromelysin 1 has a role in the activation of procollagenase 3 and progelatinase B in this system.

MT1-MMP in SW1353 membranes

The membrane-bound matrix metalloproteinase MT1-MMP has been linked to the activation of progelatinase A and procollagenase 3 in other cell types [8,31–33], and ConA has been shown to both induce gelatinase A activation and to up-regulate MT1-MMP in human fibroblasts [34].

Crude preparations of membranes made from SW1353 cells with and without 24 h ConA treatment were tested by Western blotting for the presence of MT1-MMP protein. A polyclonal antibody that had been affinity-purified against MT1-MMP catalytic domain detected two bands of 59 kDa and 43 kDa in membranes from ConA-treated cells (Figure 5, lane 3). The 43 kDa species is believed to be generated by further degradation...
of mature MT1-MMP [32]. The 59 kDa band was barely visible in the membranes prepared from unstimulated cells and the 43 kDa band was not detected. This result indicates that MT1-MMP synthesis was up-regulated by ConA treatment. Total RNA extracted from ConA-treated cells was tested for the presence of MT1-MMP and MT2-MMP mRNAs by Northern blotting; only MT1-MMP mRNA was detected (results not shown).

The production of the active form of collagenase 3 was concomitant with progelatinase A activation and up-regulation of MT1-MMP protein synthesis by the cells. ConA-treated fibroblast monolayers and fibroblast membranes that contain MT1-MMP have been shown to activate exogenously added recombinant procollagenase 3, with potentiation by progelatinase A [8]. The activation of endogenous procollagenase 3 by SW1353 cells coincided with an up-regulation in MT1-MMP protein in the cell membranes, and this enzyme may have activated procollagenase 3 either directly or indirectly through gelatinase A. Membranes from ConA-treated SW1353 cells showed an increased ability to activate exogenous recombinant progelatinase A and recombinant procollagenase 3 when compared with the membranes from unstimulated cells (results not shown). Addition of exogenous progelatinase B to the membrane preparations elicited little activation in the presence of progelatinase A relative to that in the presence of procollagenase 3 (results not shown).

Attempts were made to determine the exact order of activation of the MMPs, but no inhibitors were found that were specific to individual MMPs; inhibitory antibodies were not available.

Secretion of TIMP-1 and -2, and the effect of exogenously added inhibitors

Since the TIMPs inhibit active MMPs, SW1353 conditioned media were examined by reverse zymography to see whether the activation of procollagenase 3 coincided with a fall in inhibitor concentration. TIMPs -1 and -2 were made constitutively by these cells, although TIMP-2 was barely detectable by reverse zymography (results not shown). PMA and IL-1 increased the secretion of TIMP-1, but did not alter the TIMP-2 level. There was a slight decrease in the level of TIMP-2 in the culture medium after ConA treatment, detected by both reverse zymography and Western blotting (results not shown). Immuno-localization studies on ConA-treated cells showed TIMP-2 bound at the cell surface (results not shown). MT1-MMP protein localized to cell surfaces [31,35,36], and it has been proposed that TIMP-2 binds to MT1-MMP and the resulting complex forms a receptor for the C-terminal domain of gelatinase A [37]. The decrease in TIMP-2 observed in conditioned medium at the time of gelatinase A activation and MT-MMP up-regulation agrees with observations of Ailenberg and Silverman [38], and may be due to the inhibitor binding to active MT1-MMP at the cell surface. This would be consistent with the increased level of MT1-MMP protein in the SW1353 membranes at the time of progelatinase A and procollagenase 3 activation.

Human recombinant TIMPs were added to SW1353 cells with IL-1, Onco M and ConA to assess their effect on the activation of collagenase 3 and the gelatinases. TIMP-2 and TIMP-3 independently prevented the activation of progelatinase B and procollagenase 3 and decreased the activation of progelatinase A (Figure 4A, lanes 5 and 6). TIMP-1 efficiently inhibited activation of progelatinase B by stimulated SW1353 cells, but in the same experiments was a much less efficient inhibitor of gelatinase A and collagenase 3 activation (Figure 4A, lane 4). TIMP-1 is an inefficient inhibitor of MT1-MMP activity [39], but a very efficient inhibitor of both active gelatinase A and collagenase 3. Together with a lack of evidence for gelatinase B cleavage by MT1-MMP, this result implies a role for MMPs other than MT1-MMP in the activation of gelatinase B in this system. Forms of gelatinase A and collagenase 3 corresponding to intermediate products of propeptide processing increased when TIMP-1 was added. TIMP-1 has been reported to block the final autolytic cleavage of procollagenase 3 during APMA activation [2], generating two intermediate enzyme forms of 56 and 50 kDa. Similarly, TIMP-1 inhibits the final intermolecular autolytic cleavage of gelatinase A activation initiated by MT1-MMP, leading to the accumulation of an intermediate form of 62 kDa [34]. The TIMP inhibition of procollagenase 3 activation demonstrated the involvement of matrix metalloproteinases in the activation process.

The possible role of plasmin in MMP activation by SW1353 cells

Since plasmin can activate both procollagenase 3 and progelatinase B in vitro and in cell models [8,40], conditioned media and cell lysates from SW1353 monolayers were tested for plasminogen-activator activity by plasminogen zymography; none was detected (results not shown). Although the ability of these cells to generate plasmin is therefore doubtful, a further experiment was carried out with the assumption that low levels of plasmin might be present. The plasmin inhibitor aprotinin was included in the culture medium in order to block any contribution made by plasmin towards the activation of MMPs by SW1353 cells. Aprotinin failed to prevent the activation of gelatinase A, collagenase 3 and gelatinase B induced by the combination of ConA, IL-1 and Onco M (Figure 4B, lane 4). Supplementing plasminogen levels in the culture medium did not increase the activation of procollagenase 3 or the progelatinases (Figure 4B).

These results demonstrate that the activation of these MMPs by SW1353 cells is independent of the urokinase-type-plasminogen-activator pathway of plasminogen activation. Collagenase 1 and stromelysin 1, also activated by plasmin in cell models, remained latent at the time of collagenase 3 and gelatinase activation.

Conclusions

Previous experiments using recombinant human procollagenase 3 showed that the enzyme could participate in an activation cascade involving MT1-MMP and gelatinase A, and have shown that active collagenase 3 can activate gelatinase B [8,28]. In the present paper we have demonstrated the co-ordinated activation of endogenous procollagenase 3 and gelatinases A and B by SW1353 cells following MT1-MMP up-regulation. There are a number of physiological situations where these enzymes are co-expressed. MT1-MMP [41], procollagenase 3 [3,5,42], progelatinase B [43,44] and progelatinase A [43] are all expressed by human chondrocytes. The cascade of MMP activation induced by ConA treatment of SW1353 cells led to an increase in collagenolytic activity produced by the cells. If the proposed activation cascade operates in articular chondrocytes, agents causing an increase in active MT1-MMP at the cell surface would also bring about activation of collagenase 3 and the gelatinases. This combination of active enzymes could result in rapid degradation of cartilage matrix. Yan et al. [45] demonstrated that exposure of resting chondrocytes to ConA caused the cells to become hypertrophic, to express type X collagen and to lay down a calcified matrix. Hypertrophic chondrocytes and osteoblastic cells co-expressed mRNAs for MT1-MMP, gelatinase A and collagenase 3, implying a role for these enzymes in the turnover of extracellular matrix associated with endochondral ossification [46]. The demonstration that a cell-associated pro-
teinase, MT1-MMP, is critical to complex interrelated activation cascades of MMPs is analogous to that previously described for cell-surface urokinase-type-plasminogen-activator-generated plasmin-activation mechanisms. The restriction of extracellular-matrix proteolysis to the discrete pericellular environment by sequestration and/or activation of degradative proteinases allows tight cellular control of degradative processes.