Tissue Inhibitor of Matrix Metalloproteinase-2 Regulates Matrix Metalloproteinase-2 Activation by Modulation of Membrane-type 1 Matrix Metalloproteinase Activity in High and Low Invasive Melanoma Cell Lines*

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Activation of pro-matrix metalloproteinase (MMP)-2 on the surface of malignant cells by membrane-bound MT1-MMP is believed to play a critical role during tumor progression and metastasis. In this study we present evidence that MT1-MMP plays a key role for the in vitro invasiveness of malignant melanoma. Melanoma cell lines secreted latent MMP-2 when cultured on plastic. However, when cells were grown in floating type I collagen lattices, only high invasive melanoma cells activated proMMP-2. Activation could be inhibited by antibodies against MT1-MMP, by addition of recombinant tissue inhibitor of metalloproteinases (TIMP)-2 and by inhibition of MT1-MMP cleavage. MT1-MMP protein was detected as an inactive protein in all cell lines cultured as monolayers, whereas in collagen gels, active MT1-MMP protein was detected in the membranes of both high and low invasive melanoma cells. Production of TIMP-2 was about 10-fold higher in low invasive cells as compared with high invasive melanoma cells and was further increased in the low invasive cells upon contact to collagen. Thus, in melanoma cells TIMP-2 expression levels might regulate MT1-MMP-mediated activation of proMMP-2. High invasive melanoma cells displayed increased in vitro invasiveness, which was inhibited by TIMP-2. These data indicate the importance of these enzymes for the invasion processes and support a role for MT1-MMP as an activator of proMMP-2 in malignant melanoma.

Local degradation of connective tissue in the vicinity of the cell surface is thought to be essential for tumor cell invasion and metastasis. Neoplastic cells that invade surrounding tissues and metastasize through the bloodstream or lymphatic system must penetrate several barriers, including basement membranes and the interstitial connective tissue (1, 2). Degradation of extracellular matrix components is accomplished through the combined action of different proteases, primarily of the matrix metalloproteinase (MMP)† (3) and serine protease families (4).

Because of the importance of MMP activity for initiating efficient matrix degradation, MMP expression and activity is tightly regulated and is subject to several levels of control, including gene transcription, post-translational extracellular activation, and inhibition by soluble inhibitors (3, 5). Based production of metalloproteinases is typically low or absent and requires an inducing factor, such as a cytokine, phorbol ester, or contact with components of the extracellular matrix to increase their expression (3). Enzyme activation is achieved by removal of the N-terminal propeptide domain through exogenous or autocalyptic cleavage. In vitro, serine proteases, such as plasmin or trypsin, have been shown to activate most MMPs (3). However, MMP-2 is unique among the MMPs in that its expression is constitutive and its activation can be achieved in a membrane-associated manner as has been shown for fibroblasts (6), for endothelial cells (7, 8), and for tumor cells (9–11). A novel membrane-type matrix metalloproteinase (MT1-MMP) was originally cloned from a breast cancer cDNA library and was characterized as an integral membrane MMP that can activate proMMP-2. Thus far, four different membrane-bound MMPs have been described (12–15). Although MT2-MMP and MT3-MMP were also shown to activate proMMP-2 in vitro (16, 17), MT1-MMP is the one with the best documented correlation to the invasive phenotype of different types of cancer, such as breast and lung carcinomas (9, 18).

In vitro, MT1-MMP expression is up-regulated by concanavalin A, by monensin, cytochalasin D, and by fibrillar type I collagen (19, 20); the latter is, to date, the only known physiological inducer (21). Like other members of the metalloproteinase family, MT1-MMP is synthesized as a proform. The mechanism of MT1-MMP activation and whether cleavage of the N-terminal peptide of proMT1-MMP is a prerequisite for its activity are still a matter of discussion, but both the intracellular protease furin (22) and extracellular plasmin (23) have been reported to be capable of cleave the propeptide. Several studies have demonstrated that activation of proMMP-2 by MT1-MMP depends upon the presence of low amounts of TIMP-2, which is believed to be required for the formation of a membrane-bound ternary complex consisting of MT1-MMP, TIMP-2, and latent MMP-2 (24, 25). A second “free” MT1-MMP
located in proximity to the ternary complex may, in a second step, cleave proMMP-2 bound to the MT1-MMP/TIMP-2 receptor. On the other hand, at high concentrations, TIMP-2 inhibits MMP-2 activation, presumably by blocking the activity of MT1-MMP (25–27).

MMP-2 (28) and MMP-9 (29) have been shown to be overexpressed in many different types of tumors. Furthermore, the expression and activation of these enzymes has been correlated to the invasive and metastatic phenotype of tumors like non-small-cell lung carcinoma (30), gastric and breast carcinomas (31, 32), and squamous cell carcinomas (33), where it is thought to have a major impact on tumor progression. Further, MacDougall and co-workers (34) have shown that MMP-2 is constitutively expressed in malignant melanoma cell lines and Vaisanen et al. (35) have demonstrated that expression of MMP-2 increases with enhanced atypia and dedifferentiation in melanocytic lesions. However, little is known about the role of MMP-2 in neoplastic progression of human melanomas and about the mechanisms underlying the activation of proMMP-2 in vivo.

To examine the hypothesis that variable expression and/or activity of MT1-MMP, MMP-2, and TIMP-2 might be different in melanoma cells of high and low metastatic potential, we analyzed the expression and activation pattern of MMP-2 and MT1-MMP in different melanoma cell lines. Here we report that active MT1-MMP protein is detected in the cell membranes of melanoma cells lines of both high and low invasive potential when cultured in collagen lattices. Activation of proMMP-2, however, is only observed in high invasive melanoma cell lines, which produced significantly lower amounts of TIMP-2 when compared with low invasive cells. Activation of proMMP-2 by high invasive melanoma cells was completely inhibited by antibodies against MT1-MMP, by a synthetic furin inhibitor, as well as by the addition of recombinant TIMP-2. These data indicate that MT1-MMP is responsible for MMP-2 activation in highly invasive melanoma cell lines and that this process is highly dependent on the expression level of TIMP-2.

**MATERIALS AND METHODS**

**Melanoma Cell Lines and Cell Culture**—The following melanoma cell lines were used: MV3 and BLM, known to be highly metastatic with early and frequent formation of metastasis after subcutaneous inoculation in nude mice. The nonmetastatic melanoma cell lines IF6 and 530 were used: MV3 and BLM, known to be highly metastatic with early and frequent formation of metastasis after subcutaneous inoculation in nude mice. The nonmetastatic melanoma cell lines were preincubated for 20 min with the synthetic furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK; Bachem Biosciences, Bubendorf, Switzerland). The filters were coated with 25 μl of Matrigel (Becton Dickinson, Heidelberg, Germany) and placed above the lower compartment, which contained both 200 μl of serum-free RPMI 1640 culture medium (random migration) or fibroblast-conditioned medium. Melanoma cell lines were seeded in serum-free RPMI 1640 (2 × 10^5/ml) and seeded into the upper compartment of the chamber (total volume 0.7 ml). The chambers were incubated at 37 °C for 36 h. Cells attached to the upper side of the filter were then mechanically removed. The filters were then stained with eosin and thiazine dyes (Dade Diff-Quick, Dudgeing, Switzerland). Invasion was determined by counting the cells that had migrated to the lower surface of the filter (100-fold magnification). For proMMP-2 activation experiments, 10 μg of crude plasma membranes were incubated with 20 μl of fibroblast-conditioned medium containing proMMP-2 for 24 h at 37 °C. After digestion with MT1-MMP, the collagen gels were disrupted by mechanical shearing through a 10-ml syringe and subsequent incubation with bacterial collagenase D (Roche Molecular Biochemicals, Mannheim, Germany) at a concentration of 1 mg/ml in PBS, pH 7.4, at 37 °C for approximately 10 min until no collagen fibers were visible in the solution. The digestion was stopped by the addition of 0.5 volume of fetal calf serum, and cells were pelleted by centrifugation (2,000 × g, 10 min, 4 °C). All subsequent steps for preparation of crude plasma membranes were performed at 4 °C and with protease inhibitors. The concentration of TIMP-2 was determined and averaged. Each experiment was performed in triplicate. Invasion assays were also performed in the presence of human recombinant TIMP-2 (5.4 nM) or of 100 μl of medium conditioned by the non-invasive melanoma cell line IF6, which contains 80 ng of TIMP-2, corresponding to a final concentration of 5.4 nM.

**Preparation of Plasma Membranes**—For preparation of crude plasma membranes, the collagen gels were disrupted by mechanical shearing through a 10-ml syringe and subsequent incubation with bacterial collagenase D (Roche Molecular Biochemicals, Mannheim, Germany) at a concentration of 1 mg/ml in PBS, pH 7.4, at 37 °C for approximately 10 min until no collagen fibers were visible in the solution. The digestion was stopped by the addition of 0.5 volume of fetal calf serum, and cells were pelleted by centrifugation (2,000 × g, 10 min, 4 °C). All subsequent steps for preparation of crude plasma membranes were performed at 4 °C and with protease inhibitors. The concentration of TIMP-2 was determined and averaged. Each experiment was performed in triplicate. Invasion assays were also performed in the presence of human recombinant TIMP-2 (5.4 nM) or of 100 μl of medium conditioned by the non-invasive melanoma cell line IF6, which contains 80 ng of TIMP-2, corresponding to a final concentration of 5.4 nM.

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MT1-MMP Expression and MMP-2 Activation in Melanoma Cells

RESULTS

Effect of Cell-Matrix Interactions on the Activity of MMP-2 in Melanoma Cell Lines of High and Low Invasive Potential—Conditioned media prepared from the melanoma cell lines MV3, BLM, IP6, and 530, grown as monolayers and within three-dimensional collagen lattices, were analyzed by gelatin zymography with respect to their production/activation of MMP-2 and MMP-9. In monolayer culture, all cell lines expressed variable amounts of latent MMP-2, whereas activated forms of this enzyme and MMP-9 were not detectable. When cultured in three-dimensional native type I collagen lattices, all melanoma cell lines showed an increase of proMMP-9 and proMMP-2 secretion (Fig. 1). In addition, upon contact with collagen, the high metastastic melanoma cells MV3 and BLM gained the ability to activate proMMP-2 into its 62/59-kDa active forms, whereas the cells of low invasive potential, 530 and IP6, continued to show MMP-2 only as latent proenzyme.

Since activation of proMMP-2 has been reported to occur by a plasma membrane dependent mechanism, we prepared membrane fractions from melanoma cells grown under both culture conditions. As shown in Fig. 2, plasma membranes purified from the two high invasive melanoma cell lines grown in contact to collagen showed activation of exogenous proMMP-2 when plasma membranes were incubated with fibroblast-conditioned medium containing latent MMP-2. In contrast, no activation was obtained by membranes isolated either from the low invasive melanoma cells grown in collagen gels or by membranes purified from any of the melanoma cells cultured on plastic dishes (Fig. 2).

Expression of MT-MMP mRNA in Melanoma Cells Grown as Monolayers or in Collagen Gels—MMP-2 is unique among the MMPs in that it is not activated by serine proteases such as plasmin (43). The putative physiological activators of proMMP-2 are the membrane-type matrix metalloproteinases, e.g. MT1-MMP (12, 24). In order to elucidate the role of MT1-MMP for the activation of proMMP-2 in the high invasive melanoma cell lines, we analyzed MT1-MMP transcript levels in the melanoma cell lines under both culture conditions. All melanoma cells constitutively produced MT1-MMP mRNA in different amounts in monolayer cultures. Upon contact with collagen, a slight increase of the specific mRNA levels was observed with the high invasive melanoma cells (Fig. 3).

In addition to MT1-MMP, MT2- and MT3-MMP have also been reported in vitro to activate proMMP-2 (16, 17), whereas the substrate specificity of MT4-MMP is still unclear. We therefore analyzed mRNA levels for the different MT-MMPs in high and low invasive melanoma cells by semiquantitative RT-PCR analysis. All four MT-MMP mRNAs were detectable in monolayer culture, as well as collagen cultures. Semiquantitative analysis revealed a slight up-regulation of the transcripts for MT1-MMP in the high invasive melanoma cells cultured in collagen gels as already shown by Northern blot analysis. However, as compared with MT1-MMP, transcript levels for MT2-, MT3-, and MT4-MMP were low (data not shown).

Role of MT1-MMP and TIMP-2 in Collagen-induced Activation of proMMP-2 in High and Low Invasive Melanoma Cell Lines—Western blot analysis of MT1-MMP in crude membrane preparations displayed a 63-kDa band corresponding to the unprocessed zymogen (44) in all melanoma cell lines grown as monolayers (Fig. 4A). When cells were grown within three-dimensional type I collagen lattices, high as well as low invasive cell lines showed an additional immunoreactive protein band of 60 kDa, corresponding to furin-activated MT1-MMP (44). This indicates that both high and low invasive cells display activated MT1-MMP protein on their cell surface upon growth in contact with a collagen matrix, although the high invasive melanoma cells only showed activation of proMMP-2.

Preincubation of the invasive melanoma cell line MV3 with increasing concentrations of the synthetic furin inhibitor CMK resulted in complete suppression of collagen-induced proMMP-2 activation (Fig. 4B), whereas the serine protease inhibitor aprotinin did not affect proMMP-2 activation. In addition, detection of MT1-MMP protein in membranes isolated from MV3 cells that were treated with 50 μM CMK for 24 h displayed a significant increase of the latent 63-kDa protein, whereas the amounts of 60-kDa protein were reduced. These data suggest that treatment with the furin inhibitor CMK results in an accumulation of the unprocessed membrane-bound MT1-MMP protein form, providing strong evidence that Golgi-associated furin is mainly responsible for proMT1-MMP processing in melanoma cells. Furthermore, preincubation of membranes with a nonspecific MT1-MMP antibody resulted in a strong reduction of proMMP-2 activation (Fig. 5). These
Role of MT1-MMP, MMP-2, and TIMP-2 for the in Vitro Invasiveness of Melanoma Cell Lines of High and Low Invasive Potential—In order to evaluate whether activated MT1-MMP and MMP-2 contribute to the invasive behavior of melanoma cells, we analyzed the ability of the cells to penetrate a barrier composed of Matrigel in Boyden chamber assays. In contrast to the melanoma cells IF6 and 530 with low penetration of the Matrigel-coated filters, the high invasive BLM and MV3 melanoma cells showed a high rate of penetration (Fig. 7A). The invasive capacity of MV3 cells was reduced by about 50% when the cells were preincubated with 50 μM amounts of the synthetic furin inhibitor CMK, which was shown above to inhibit MT1-MMP processing and to abolish proMMP-2 activation (Fig. 7B). These data suggest a direct involvement of active MT1-MMP in the invasion process. Invasion of Matrigel by MV3 cells was reduced by a similar extent when the invasion assays were performed in the presence of medium conditioned by the non-invasive melanoma cell line IF6, indicating inhibition of Matrigel invasion by soluble factors released by the non-invasive melanoma cells, e.g. TIMP-2. The involvement of TIMP-2 was corroborated by the addition of equivalent concentrations of recombinant TIMP-2 (5.4 nM) to the cell suspension into the Boyden chamber, which resulted in a comparable inhibition of the invasion process (Fig. 7B).

DISCUSSION

Several reports have shown that overexpression of MMP-2 by tumor cells and stromal cells can be correlated with the invasive and metastatic behavior of various tumors (9, 29–33). However, the activity of MMP-2 is tightly regulated, and not only is it dependent on de novo synthesis of the latent enzyme, it also appears to be rather determined by the activation of thezymogen and by the presence of the specific inhibitor TIMP-2. The membrane-associated matrix metalloproteinase, MT1-MMP, which was identified as a potential physiological activator of proMMP-2 (12, 24), could be localized in different tumors and is thought to play a key role in tumor invasion (45). Activation of proMMP-2 and concomitant induction of MT1-MMP gene expression was also found in fibroblasts, endothelial cells, and breast carcinoma cell lines treated with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (31, 44) or concanavalin A (46, 47) or cultivated in collagen gels (19, 20).

During invasion of the dermal connective tissue, melanoma cells interact with different components of the extracellular matrix, including type I collagen. As it has been shown by several investigators that cell-matrix interactions modulate de novo synthesis of matrix-degrading metalloproteinases in different cell types (3, 9, 19, 38), we compared synthesis and activation of proMMP-2 in melanoma cells grown on plastic dishes and within three-dimensional gels composed of fibrillar type I collagen.

In the present study, we used melanoma cell lines, which have been previously characterized by their different invasiveness after subcutaneous injection into nude mice (36, 37). When grown on plastic dishes, both high and low invasive melanoma cells secreted MMP-2 in its latent protein form. Upon contact with the collagenous matrix, only the high, but not the low invasive, melanoma cells gained the capability to convert the latent proenzyme into its 62/59-kDa active forms, a process that could be attributed to the cell surface. This process was completely abolished by the addition of a specific MT1-MMP antibody, indicating that other MT-MMPs, although low transcript levels could be detected by RT-PCR analysis, are not involved in the activation process.

Conversion of latent MMP-2 to its 62/59-kDa active forms by plasma membranes isolated from high invasive cells grown in collagen lattices could be completely abolished by preincubation of the cells with the synthetic furin inhibitor CMK (48), which prevents intracellular activation of proMT1-MMP (49). In addition, crude membranes prepared from furin inhibitor-treated melanoma cells contained MT1-MMP mainly as unprocessed 65-kDa form. Interestingly, similar observations were reported by Maquoi et al. (50), showing that the reduced activation of proMMP-2 by HUT1080 fibrosarcoma cells upon treatment with furin inhibitor is paralleled by suppressed levels of processed MT1-MMP protein. However, in contrast to the melanoma cells used in our studies, in HUT1080 cells, proMMP-2 activation was not completely abolished even at higher concentrations (100 μM). Although Cao et al. (51) have shown by transfection studies that MT1-MMP can activate proMMP-2 without cleavage by a furin-like protease, our data strongly suggest that proteolytic cleavage of the N-terminal

*Fig. 3. Expression of MT1-MMP mRNA in melanoma cell lines grown as monolayer (M) or in collagen lattices (G). For analysis of MT1-MMP mRNA levels, total RNA was extracted after 48 h of culture. 10 μg of total RNA was processed for Northern blot analysis. MT1-MMP transcripts were detected using a 32P-labeled 1.8-kilobase pair MT1-MMP cDNA fragment. RNA loading was visually assessed by ethidium bromide staining of the 18 and 28 S ribosomal RNAs.*
domain of proMT1-MMP is a prerequisite for proMMP-2 activation in melanoma cells. Previously published studies have also demonstrated that the serine proteases plasmin (23) and uPA (52) can extracellularly activate MT1-MMP. Indeed, the melanoma cells MV3 and BLM have been shown to produce uPA (53). However, activation of proMMP-2 is unlikely to be caused by uPA cleavage, as aprotinin added to the culture system did not prevent proMMP-2 processing. In conclusion, our data convincingly demonstrate that furin-activated MT1-MMP mediates proMMP-2 activation in high invasive melanoma cells.

As pointed out by Zucker et al. (27) and Butler et al. (25), the activation of proMMP-2 by the membrane activator MT1-MMP strongly depends on the presence of TIMP-2, which is bound via the interaction of the N-terminal domains of MT1-MMP. This MT1-MMP/TIMP-2 “receptor” in turn binds the C-terminal domain of proMMP-2 via the C-terminal portion of TIMP-2. Cleavage of MMP-2 might then occur by the concerted action of a second, not complexed, MT1-MMP molecule close to this complex (24, 25, 27). We observed that collagen-induced acti-
The mean of cells per microscope field migrated in standard conditions was set as 100%. Data represent the mean ± S.D. of triplicate experiments.

The number of cells that had migrated was determined by visually counting the cells on the lower side of the filter. After 36 h of incubation, the melanoma cell lines (5 × 10⁵/ml) were resuspended in 0.7 ml of serum-free RPMI medium and filled in the upper compartment, while fibroblast conditioned medium was used as a chemoattractant in the lower compartment. At 36 h of incubation, the number of cells that had migrated was determined by visually counting the cells on the lower side of the filter.

Invasion assays were performed with MV3 cells in the absence (open bar) or in the presence of 50 μM synthetic furin inhibitor CMK (gray bar), of 5.4 nM recombinant TIMP-2 (hatched bar), and of 100 μM of IF6 conditioned medium containing 5.4 nM TIMP-2 (black bar). The number of MV3 cells that migrated in standard conditions was set as 100%. Data represent the mean of cells per microscope field ± S.D. of triplicate experiments.

In vitro invasion of Matrigel by melanoma cell lines. A, invasion was assayed in a modified Boyden chamber with Matrigel-coated polycarbonate filters. The melanoma cell lines (5 × 10⁵/ml) were resuspended in 0.7 ml of serum-free RPMI medium and filled in the upper compartment, while fibroblast conditioned medium was used as a chemoattractant in the lower compartment. At 36 h of incubation, the number of cells that had migrated was determined by visually counting the cells on the lower side of the filter. B, invasion assays were performed with MV3 cells in the absence (open bar) or in the presence of 50 μM synthetic furin inhibitor CMK (gray bar), of 5.4 nM recombinant TIMP-2 (hatched bar), and of 100 μM of IF6 conditioned medium containing 5.4 nM TIMP-2 (black bar). The number of MV3 cells that migrated in standard conditions was set as 100%. Data represent the mean of cells per microscope field ± S.D. of triplicate experiments.

The invasion of proMMP-2 by the high invasive melanoma was inhibited by the addition of recombinant TIMP-2, suggesting that TIMP-2 is indeed involved in this process.

To investigate the role of TIMP-2 in proMMP-2 activation in high and low invasive melanoma cells, production of TIMP-2 by the melanoma cell lines was quantified. It turned out that TIMP-2 levels were significantly higher in the high invasive as compared with the high invasive cells. Additionally, we found an increase of TIMP-2 protein production by the low invasive cells in collagen gels, whereas the highly invasive cell lines reduced TIMP-2 production upon collagen contact. Since activated MT1-MMP protein was detected in low invasive melanoma cells upon contact with collagen, the increased TIMP-2 level in these cells might be responsible for the inhibition of proMMP-2 activation by MT1-MMP. Therefore, in low invasive melanoma cells, an altered balance of TIMP-2 and MT1-MMP probably contributes to the inhibition of proMMP-2 activation by blockage of the activator MT1-MMP, and finally leading to a lower invasive potential in the low invasive melanoma cells. On the other side, although TIMP-2 secretion is reduced in the high invasive cells upon contact with collagen, these levels seem to be sufficient to mediate the formation of a ternary complex between MT1-MMP, TIMP-2, and proMMP-2, and to allow proMMP-2 activation. These data are in agreement with the TIMP-2 studies performed by others (17, 24, 27) and underline once more the dual effect of TIMP-2 on proMMP-2 activation (24, 51). To further clarify the role of TIMP-2 in the activation of MMP-2, we treated the high invasive melanoma cells with either recombinant TIMP-2 or with media conditioned by IF6 cells shown to contain high amounts of TIMP-2. As expected, the increased levels of TIMP-2 in the culture medium resulted in suppressed proMMP-2 activation by the high invasive cells.

Furthermore, to test whether MT1-MMP-mediated activation of proMMP-2 might contribute to the invasive ability of malignant melanoma, we analyzed the in vivo invasiveness of high and low invasive melanoma cells using Matrigel as barrier. The results showed that the ability of MV3 cells to penetrate this barrier was greatly reduced in the presence of the furin inhibitor. This reduction is probably a consequence of the reduced MT1-MMP cleavage. Addition of TIMP-2 or of IF6 conditioned media also resulted in a partial inhibition of the invasive capacity of MV3 cells. Taken together, these data indicate that proMMP-2 activation by MT1-MMP significantly contributes to the invasiveness of these melanoma cells, a process that strongly depends on the levels of TIMP-2.

Therefore, as shown in our studies, differences of the invasive phenotype might not become obvious unless more complex in vitro culture systems such as three-dimensional matrices are used.

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