

Front-Cell-specific Expression of Membrane-Type 1 Matrix Metalloproteinase and Gelatinase A during Cohort Migration of Colon Carcinoma Cells Induced by Hepatocyte Growth Factor/Scatter Factor¹

Kazuki Nabeshima, Teruhiko Inoue, Yoshiya Shima, Yasunori Okada, Yoshifumi Itoh, Motoharu Seiki, and Masashi Kono²

Department of Pathology, Miyazaki Medical College, Miyazaki 889-1692 [K. N., T. I., Y. S., M. K.]; Department of Pathology, Keio University School of Medicine, Tokyo 160-0016 [Y. O.]; and Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Tokyo 108 [Y. I., M. S.], Japan

Abstract

Migration of tumor cells is usually assessed as single cell locomotion *in vitro* using Boyden chamber type assays. *In vivo*, however, carcinoma cells frequently invade the surrounding tissue as coherent clusters or nests of cells. We have called this type of movement “cohort migration” and developed a two-dimensional *in vitro* cohort migration model, in which human rectal well-differentiated adenocarcinoma cells (L-10) migrate from piled-up cell islands as coherent sheets of cells when stimulated with hepatocyte growth factor/scatter factor. In this study, we examined whether there is a cohort migration-specific way of expression of matrix metalloproteinases (MMP) and whether degradation of extracellular matrix is necessary for this type of migration. Production of membrane-type 1-MMP (MT1-MMP) and gelatinase A (MMP-2) by L-10 cells was demonstrated by gelatin zymography, immunoblotting, and reverse transcription-PCR. When cohort migration was induced with hepatocyte growth factor/scatter factor, MT1-MMP and MMP-2 were immunolocalized predominantly in the leading edges of the front cells of migrating cell sheets, with the following cells being negative. In addition, during the cohort migration on gelatin-coated substratum, the gelatin matrix was degraded by the cells, in a very organized manner, causing radially arrayed lysis of gelatin matrix at the sites of leading edges. BB94, a synthetic inhibitor of MMPs, tissue inhibitor of metalloproteinases-1 and -2, and the COOH-terminal hemopexin-like domain of MMP-2 inhibited the migration on gelatin matrix. Thus, these data demonstrate that gelatin matrix is reorganized to suit cell migration via leading-edge-of-front-cell-specific localization of MT1-MMP and MMP-2 during cohort migration and suggest that the reorganization is essential for this type of migration.

Introduction

What makes tumors malignant is their ability to invade the surrounding normal tissues and metastasize. Invasive tumors show active migration and high levels of MMP³ expression (1). Among the MMPs, gelatinase A (MMP-2) is the most abundant MMP (2) and frequently correlates with malignant progression and invasive behavior of tumor cells (1). To exert its enzymatic activity, the zymogen of MMP-2 (proMMP-2) requires cleavage and activation by membrane-

type MMPs, including MT1-MMP (3). The expression of MT1-MMP and activation of proMMP-2, which occurs on the cell surfaces, are strongly associated with tumor invasion (3, 4). Moreover, the localization of the proteolytic activity of these two MMPs (MT1-MMP/MMP-2) is confined to the cell surface of invadopodia in human RPMI17951 melanoma cells, and the activity initiates a proteolytic cascade required for cell invasion (5).

Active migration (translocation) of tumor cells includes both single cell locomotion and cohort-type migration (6–9). *In vitro* studies of cell migration are predominantly based on the concept of the single cell locomotion using Boyden chamber-type assays and scattering assays. However, in human surgical specimens, carcinoma cells, especially those of well to moderately differentiated types, frequently invade the stroma as coherent cell nests rather than single cells (9, 10). A time-lapse videomicroscopic study *in vitro* also demonstrated the movement of carcinoma cells as coherent cell clusters in collagen gels (11). We have called this type of carcinoma cell movement “cohort migration” and developed an *in vitro* model to examine the mechanisms involved in this type of migration (6–9, 12, 13). Thus far, in our model of cohort migration induced by 12-*O*-tetradecanoylphorbol-13-acetate or HGF/SF, carcinoma cells migrated keeping close cell-cell contact with one another in the upper portion of cells, and intercellular gaps became wider in the lower portion of cells. This gap formation enabled the cells to extend their leading edges forward onto the substrate beneath the preceding cells and move (8, 13). The process was also associated with increased tyrosine phosphorylation of the E-cadherin/catenin complex, including β -catenin, in 12-*O*-tetradecanoylphorbol-13-acetate-induced migration (6), and with decreased amounts of α -catenin complexed with E-cadherin in HGF/SF-induced migration (8). In addition, production of FN, especially EDA+FN, by migrating carcinoma cells themselves was essential for this type of migration (7). Although degradation and remodeling of ECMs are generally thought to be necessary for cell migration, the role of MMPs in this cohort migration model has never been explored.

In this study, we examined the expression patterns of MMPs, especially MT1-MMP and MMP-2, which play a major role in the pericellular degradation of ECMs and their activity during the cohort migration. The data suggest that gelatin matrix is reorganized to suit cell migration via leading-edge-of-front-cell-specific localization of MT1-MMP and MMP-2 during the migration and that the reorganization is essential for this type of migration.

Materials and Methods

Reagents. Recombinant HGF/SF was purchased from Toyobo (Osaka, Japan), and TIMP-1 and -2 were from Fuji Chemical Industries (Takaoka, Japan).

Cell Cultures. A highly metastatic subline to the liver (L-10) of the human colon adenocarcinoma cell line RCM-1 obtained by *in vivo* selection in nude

Received 12/28/99; accepted 5/11/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (10670212) and a Grant-in-Aid for Research Project from Miyazaki Medical College.

² To whom requests for reprints should be addressed, at Department of Pathology, Miyazaki Medical College, Miyazaki 889-1692, Japan.

³ The abbreviations used are: MMP, matrix metalloproteinase; MT1-MMP, membrane-type 1 MMP; MMP-2, *M*_r 72,000 type IV collagenase/gelatinase A; TIMP, tissue inhibitor of metalloproteinase; HGF/SF, hepatocyte growth factor/scatter factor; CM, conditioned medium; GM, growth medium; FN, fibronectin; EDA, extradomain A of FN; EDA+FN, EDA-containing FN; Gela PEX, the hemopexin-like domain of MMP-2; RT-PCR, reverse-transcription-PCR; ECM, extracellular matrix; mAb, monoclonal antibody.

mice was maintained in GM (for L-10), a 1:1 mixture of RPMI 1640 and Ham's F-12 (Nissui Seiyaku, Tokyo, Japan) supplemented with 5% FCS (12). Another human colon adenocarcinoma cell line, SW837, obtained from Dainihon Seiyaku (Osaka, Japan), was maintained in DMEM supplemented with 10% FCS (GM for SW837).

Cell Motility on a Lab-Tek Chamber Slide. Motility assay was run as described (13), with some modifications. Briefly, L-10 or SW837 cells were seeded into compartments of an 8-well Lab-Tek tissue culture chamber slide (Nunc, Naperville, IL; 1.2×10^5 cells in 0.4 ml of GM) and allowed to attach for 40 h (1 day and overnight) in standard culture conditions. The cells formed interlinked, piled-up cell islands on the tissue culture glass substrate of the Lab-Tek chamber slide. The cells were then exposed to test medium (serum-free medium or GM with or without 20 ng/ml HGF/SF) for various periods up to 24 h. Stimulated cells migrated outward from the cell islands into interisland spaces as coherent cell sheets one-cell thick. Migration was quantified by counting the migrated cells in 10 randomly selected high-power fields ($\times 400$) after the cells were fixed with 3.7% formaldehyde in PBS and stained with hematoxylin. All experiments were performed in duplicate and repeated three times. Means and SEs of the mean were calculated, and statistical differences were analyzed using Student's *t* test for unpaired samples.

In experiments on gelatin-coated substratum, the glass substrate of each compartment of 8-well Lab-Tek chamber slides was coated with 200 μ l of 1 μ g/ml gelatin for 48 h at 4°C (7). Diffuse coating was confirmed by protein staining. Using these coated chamber slides, cell migration was determined as described above.

In experiments with BB-94 (British Biotech Pharmaceuticals, Oxford, United Kingdom), 10 or 50 μ M BB-94 was included in the gelatin solution (1 μ g/ml, 200 ml) and used to coat the glass substrate of chamber slides. Coating was done for 48 h at 4°C. Cell migration was induced as described above, except for a 1-h preincubation of cells with 10 or 50 μ M BB-94 prior to HGF/SF addition and presence of BB-94 during the whole assay period.

In experiments with TIMP-1 and -2 and the Gela PEX, cells were pretreated with TIMPs (10 μ g/ml) or PEX-2 (25 or 50 μ g/ml) for 1 h, followed by a 24-h incubation with HGF/SF (20 ng/ml) in the presence of TIMPs or Gela PEX.

Expression and *in Vitro* Folding of the Gela PEX Protein. Expression vector Gela PEX/pET was constructed by cloning a PCR product of Gela PEX into protein expression vector pET-3a (Stratagene, La Jolla, CA). The PCR product corresponded to bases 1001–1939 (14), and start and stop codons were added at its 5' and 3' ends, respectively. The nucleotide sequences of the Gela PEX product were confirmed by the dideoxy chain-termination method before cloning. The vector Gela PEX/pET was transfected into an *Escherichia coli* BL21(DE3)pLyS host (Stratagene), and expression and *in vitro* folding of the Gela PEX protein were done according to Huang *et al.* (15). The obtained Gela PEX protein was concentrated using Diaflo ultrafiltration membranes (YM5; Amicon, Beverly, MA) and Centriprep 10 (Amicon) and was stored at –20°C until use.

Scattering Assays. L-10 and SW837 cells were added to Lab-Tek tissue culture chamber slides at 5.0×10^3 cells/well in the presence of 50 μ g/ml anti-E-cadherin mAb (HECD-1; Takara) and allowed to grow for 7 days, after which loosely aggregated cell islands were obtained. The cells of these loose islands showed scattering in response to treatment with HGF/SF (20 ng/ml) for 24 h.

Immunofluorescent Staining. After cell motility assays were done as described above, immunofluorescent staining of cells was performed as described (7) using mouse mAb against human MT1-MMP (114-6G6; Fuji Chemical Industries), MMP-2 (75-7F7; Fuji Chemical Industries), E-cadherin or vinculin (VG61110; Affinity Research Products, Nottingham, United Kingdom) as primary antibodies. For E-cadherin immunostaining, all of the solutions contained 1 mM CaCl₂.

Biotin Labeling of Coated Gelatin (Double Staining with Phalloidin). Gelatin-coating of Lab-Tek chamber slide substratum was done as described above. After rinsing with cold PBS twice, the gelatin was biotinylated by incubating in PBS containing water-soluble sulfo-*N*-hydroxysuccinimide-biotin (2 mg/ml; Pierce Chemical Co., Rockford, IL) for 2 h at 4°C, followed by washing with PBS three times and then rinsing in PBS overnight. L-10 cells (1.2×10^5 cells/well) were added and allowed to attach for 40 h, and migration assays were run as described above. After the cells were fixed with 2% paraformaldehyde/0.1 M PB for 30 min at 4°C and permeabilized with 0.1% Triton X-100/PBS for another 30 min at 4°C, the biotinylated gelatin was

visualized with FITC-conjugated streptavidin (Sigma Chemical Co.) for 2 h at room temperature. After rinsing with PBS four times, the cells were stained with rhodamine-phalloidin in PBS (Molecular Probes, Eugene, OR) for 20 min at room temperature.

To obtain footprints of migrating cell sheets, after cohort migration was induced on gelatin-coated substratum, cells were treated with 0.5 mM EDTA/PBS, followed by tapping of chamber slides by fingers. About 30% of cell islands with migrating cell sheets were removed by the treatment, and the remaining footprints were demonstrated by visualization of biotinylated gelatin matrix, as described above.

RT-PCR. mRNA expression for MT1-MMP and MMP-2 in L-10 cells was analyzed using RT-PCR, as described (8). Annealing was done at 60°C for MT1-MMP and 58°C for MMP-2. The sequences of the forward and reverse primers for MMP-2 are 5'-ACCTGGATGCCGTCGTGGAC-3' (1800–1819) and 5'-TGTGGCAGCACCAGGGCAGC-3' (2228–2247), respectively, and the PCR product size is 447 bp (14). The sequences of the forward and reverse primers for MT1-MMP are 5'-CCCTATGCCTACATCCGTGA-3' and 5'-TCCATCCATCACTTGTTAT-3', respectively, and are complementary to bases 598–617 and 1129–1148 (3). The PCR product size is 550 bp.

Preparation of CM and Zymography. After L-10 cells (5.0×10^5 cells in 2 ml of GM) were added and cultured in 35-mm tissue culture dishes (Becton Dickinson, Lincoln Park, NJ) for 24 h, the cells were rinsed in serum-free media three times, then incubated in 1.0 ml of serum-free GM with or without HGF/SF (20 ng/ml) for another 24 h. CM harvested from these cultures was centrifuged twice (110 \times g, 5 min; and 4000 \times g, 20 min) to remove cellular debris, concentrated by 8-fold using centricon 10 (Amicon) and subjected to zymography using gelatin as a substrate, as described previously (16).

Immunoblotting. After L-10 cells were treated with HGF/SF (20 ng/ml) for 24 h, they were then lysed by boiling for 5 min in 2% SDS, 0.1 M dithiothreitol/1 M Tris-HCl (pH 6.8), followed by centrifugation at 11,000 \times g for 5 min. These cell extracts and CM obtained as above were subjected to immunoblotting, as described (8), using mouse mAb to human MMP-2 (75-7F7; Fuji Chemical Industries) or MT1-MMP (114-1F2; Fuji Chemical Industries) as primary antibodies.

Results

Expression of MT1-MMP and MMP-2 in L-10 Cells. We first examined whether MT1-MMP and MMP-2 were expressed in L-10 cells and also whether HGF/SF, which can induce cohort migration of L-10 cells, could affect the expressions. Analyses by RT-PCR and immunoblotting demonstrated the expression of MT1-MMP and MMP-2 in L-10 cells at mRNA and protein levels, respectively (Fig. 1A and B). On immunoblots, stimulation of MMP-2 production with HGF/SF treatment (20 ng/ml) was shown, whereas the MT1-MMP expression was not altered. Zymography of L-10 cell CM revealed the presence of both pro- (M_r 68,000) and active (M_r 62,000) forms of MMP-2 (Fig. 1C). The amounts of samples loaded for zymography were the same as those for immunoblotting, but gel lysis in zymography was so sensitive that a less amount of pro- and active MMP-2 in L-10 cell CM without HGF/SF treatment could cause lysis of gelatin substrate like that by CM with HGF/SF treatment.

Immunolocalization of MT1-MMP and MMP-2 during Cohort Migration. By using immunofluorescent staining techniques, we then investigated the MMP expression patterns during cohort migration of L-10 cells. Without HGF/SF treatment, L-10 cells formed piled-up cell islands with few cells migrated, and MT1-MMP immunostaining was detected as summation of that of each cells in the piled-up cell islands, with some accentuation along the cell borders (Fig. 2A, a). When stimulated with HGF/SF (20 ng/ml, 24 h), L-10 cells migrated outward from the cell islands as coherent cell sheets one-cell thick (Fig. 2A, c and e), as described previously (14). In the migrating cell sheets, MT1-MMP was demonstrated only at their fronts, including the frontmost first to second rows of cells, with the following migrating cells being negative (Fig. 2A, d and f). At a higher magnification, expression of MT1-MMP was observed predominantly

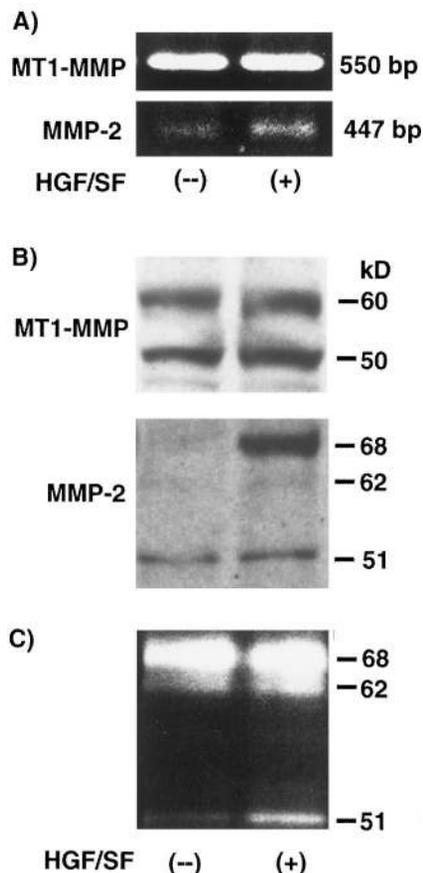


Fig. 1. Expression of MT1-MMP and MMP-2 in L-10 cells. After cells were treated with or without HGF/SF (20 ng/ml), total RNA extracted from the cells was subjected to RT-PCR using primer pairs for MT1-MMP and MMP-2, which form 550-bp and 447-bp products, respectively (A). Total cell extracts were subjected to immunoblotting with anti-MT1-MMP mAb (B; MT1-MMP). The M_r 60,000 band corresponds to the active form of MT1-MMP, and the M_r 50,000 band corresponds to its processed form. Their conditioned media were subjected to immunoblot with anti-MMP-2 mAb (B; MMP-2) and gelatin zymography (C). Bands of M_r 68,000, M_r 62,000, and M_r 51,000 correspond to pro-, active, and further processed forms of MMP-2, respectively.

in the leading edges of the frontmost cells, accompanied by some staining in perinuclear regions (Fig. 2A, b). Negative control with nonimmune mouse IgG did not show any immunofluorescent staining (data not shown). MMP-2 was also demonstrated specifically in the frontmost cells of migrating cell sheets, especially at their leading edges (Fig. 2A, g and h).

To rule out the possibility that mAbs could not reach to the inside cells of the migrating cell sheets, we used other mAbs that should react with both front and inside (following) cells. E-cadherin was shown along the cell borders, even inside the migrating cell sheets (Fig. 2A, i and j). Vinculin also successfully demonstrated focal adhesion plaques in both front and following cells of the migrating cell sheets (data not shown). These results clearly showed that mAbs can reach to not only frontmost cells but also following cells of the migrating cell sheets.

Expression of MT1-MMP and MMP-2 in Scattering L-10 Cells.

As another control, we induced scattering of L-10 cells by stimulating cell migration in the presence of anti-E-cadherin antibody and examined how MT1-MMP and MMP-2 were expressed in the scattering cells. Individual scattering L-10 cells expressed both MT1-MMP and MMP-2 in their leading edges and also within the cytoplasm, especially perinuclear regions (Fig. 2B, a–d). The front cell-specific pattern of expression observed during cohort migration was lost.

Expression of MT1-MMP and MMP-2 in SW837 Cells. To examine whether front cell-specific expression of MT1-MMP and

MMP-2 is unique only to L-10 cells or can be seen in other cell lines, another human colon carcinoma cell line (SW837) was used for an immunolocalization study. This cell line also shows cohort migration in response to HGF/SF treatment (8), and its expression of MT1-MMP and MMP-2 was demonstrated by RT-PCR (data not shown). When cohort migration was induced with HGF/SF treatment, MT1-MMP (Fig. 2C, a and b) and MMP-2 (Fig. 2C, c and d) were demonstrated predominantly in the frontmost cells or sometimes in the first and second rows of cells at the front of the migrating cell sheets. In contrast, when scattering was induced by treatment with HGF/SF in the presence of anti-E-cadherin antibody, all scattering SW837 cells expressed MT1-MMP (Fig. 2C, e and f) and MMP-2 (Fig. 2C, g and h), without preferential localization in the front cells.

Degradation of Gelatin Matrix by Migrating Cells. Using gelatin as a substrate, we studied whether MT1-MMP and MMP-2 expressed at the front of the migrating cell sheets cause ECM degradation. Gelatin labeled with biotin was visualized with avidin-conjugated FITC, and, at the same time, cytoplasmic actin filaments were demonstrated with rhodamine-phalloidin. At the sites of leading edges of the front cells of migrating cell sheets, limited gelatinolysis and consequent partial removal of gelatins occurred, causing radially arrayed lysis of gelatin matrix at the outermost portions (Fig. 3, a–d). Thin, also radially arrayed gelatin matrix was left in between the lysed portions. At the base of the radially arrayed gelatinolysis, an arcuate band-like gelatin matrix was also left beneath the leading edges.

We also tried to demonstrate migrating cell-induced gelatinolysis by removing cells with EDTA treatment and tapping the chamber slides with fingers after cohort migration had been induced, and we called these lysed portions left behind footprints of cells. The footprints also showed a similar organized way of degradation of gelatin matrix at the peripheral portions corresponding to leading edges of the frontmost migrating cells, as radially arrayed lytic marks (Fig. 3e).

An Essential Role of Gelatin Degradation in Cohort Migration.

We finally investigated whether the above organized degradation of gelatin matrix was necessary for cohort migration of carcinoma cells. BB-94, a synthetic inhibitor for MMPs, inhibited HGF/SF-induced cohort migration of L-10 cells in a dose-dependent manner: approximately 34% and 78% inhibition at 10 μ M and 50 μ M, respectively (Fig. 4). Both TIMP-1 and TIMP-2 also inhibited the migration, although TIMP-2 seemed to be more effective than TIMP-1: approximately 72% inhibition by TIMP-1 and 82% inhibition by TIMP-2. Furthermore, Gela PEX effectively inhibited the migration: approximately 60% inhibition at 25 μ g/ml and 74% inhibition at 50 mg/ml. Taken together, these results indicate that MMP-2 activated on the cell surface by MT1-MMP plays a major role in cohort migration on gelatin matrix.

Discussion

The present studies have demonstrated that cohort migration of carcinoma cells has its specific way of expression of MT1-MMP and MMP-2 compared with scattering carcinoma cells, because both MT1-MMP and MMP-2 were demonstrated predominantly at the front cells of the migrating cell sheets, whereas almost all of the scattering single cells showed positive staining for the two enzymes. These MMPs were also demonstrated to be involved in degradation and consequent remodeling of gelatin substrate that were essential for cell migration.

Although the precise mechanisms involved in the front cell-specific localization of MT1-MMP and MMP-2 are currently unknown, there are two possibilities: their localization is regulated (a) at the gene expression level or (b) at the protein level. In the former, only front cells may predominantly express mRNAs for MT1-MMP and

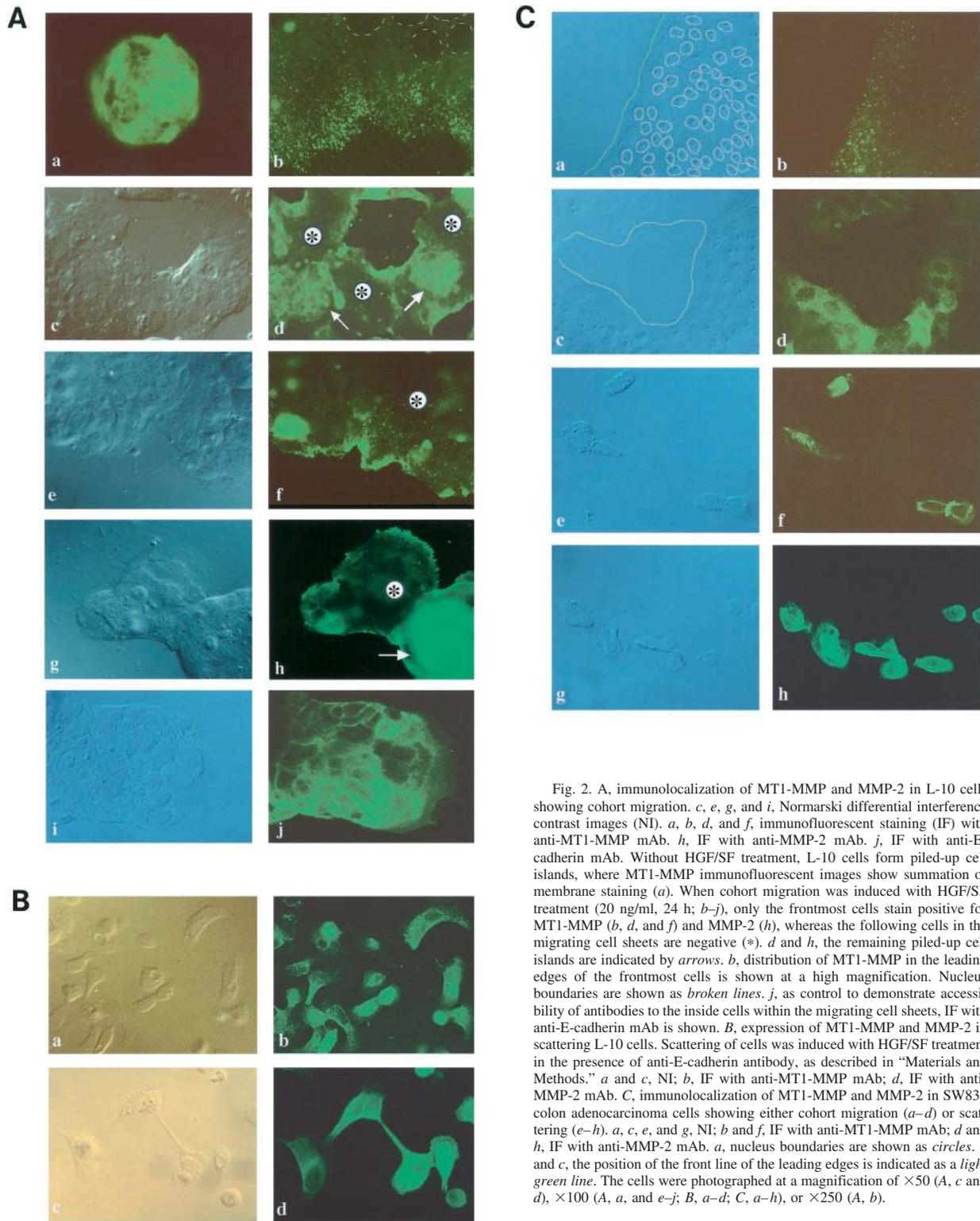


Fig. 2. A, immunolocalization of MT1-MMP and MMP-2 in L-10 cells showing cohort migration. *c, e, g, and i*, Normarski differential interference contrast images (NI). *a, b, d, and f*, immunofluorescent staining (IF) with anti-MT1-MMP mAb. *h*, IF with anti-MMP-2 mAb. *j*, IF with anti-E-cadherin mAb. Without HGF/SF treatment, L-10 cells form piled-up cell islands, where MT1-MMP immunofluorescent images show summation of membrane staining (*a*). When cohort migration was induced with HGF/SF treatment (20 ng/ml, 24 h; *b–j*), only the frontmost cells stain positive for MT1-MMP (*b, d, and f*) and MMP-2 (*h*), whereas the following cells in the migrating cell sheets are negative (*). *d* and *h*, the remaining piled-up cell islands are indicated by *arrows*. *b*, distribution of MT1-MMP in the leading edges of the frontmost cells is shown at a high magnification. Nucleus boundaries are shown as *broken lines*. *j*, as control to demonstrate accessibility of antibodies to the inside cells within the migrating cell sheets, IF with anti-E-cadherin mAb is shown. B, expression of MT1-MMP and MMP-2 in scattering L-10 cells. Scattering of cells was induced with HGF/SF treatment in the presence of anti-E-cadherin antibody, as described in “Materials and Methods.” *a* and *c*, NI; *b* and *f*, IF with anti-MT1-MMP mAb; *d* and *h*, IF with anti-MMP-2 mAb. C, immunolocalization of MT1-MMP and MMP-2 in SW837 colon adenocarcinoma cells showing either cohort migration (*a–d*) or scattering (*e–h*). *a, c, e, and g*, NI; *b* and *f*, IF with anti-MT1-MMP mAb; *d* and *h*, IF with anti-MMP-2 mAb. *a*, nucleus boundaries are shown as *circles*. *a* and *c*, the position of the front line of the leading edges is indicated as a *light green line*. The cells were photographed at a magnification of $\times 50$ (A, *c* and *d*), $\times 100$ (A, *a*, and *e–j*; B, *a–d*; C, *a–h*), or $\times 250$ (A, *b*).

MMP-2. Gene expression of MT1-MMP is reported to decrease in confluent cultures of mouse mammary gland epithelial cells (17), suggesting the regulation of MT1-MMP expression by cell-cell contact in normal epithelial cells. They also suggested the presence of translational control mechanisms for MT1-MMP expression because MT1-MMP immunoreactivity disappeared very early in confluency whereas MT1-MMP mRNA levels started to decline a few days later when cell cultures reached confluency. These possibilities are now

under investigation using *in situ* hybridization. Another possibility, regulation of MT1-MMP localization at the protein level, is suggested in single-cell locomotion: invading human melanoma cells show the accumulation of MT1-MMP at the invadopodia, which are specialized membrane extensions into the FN-coated gelatin matrix (5). However, cytoplasmic docking systems supporting this localization are still unknown. MMP-2 is reported to bind to $\alpha v \beta 3$ integrin (18) or MT1-MMP (2, 19, 20) on cell surfaces, and in breast carcinoma cells

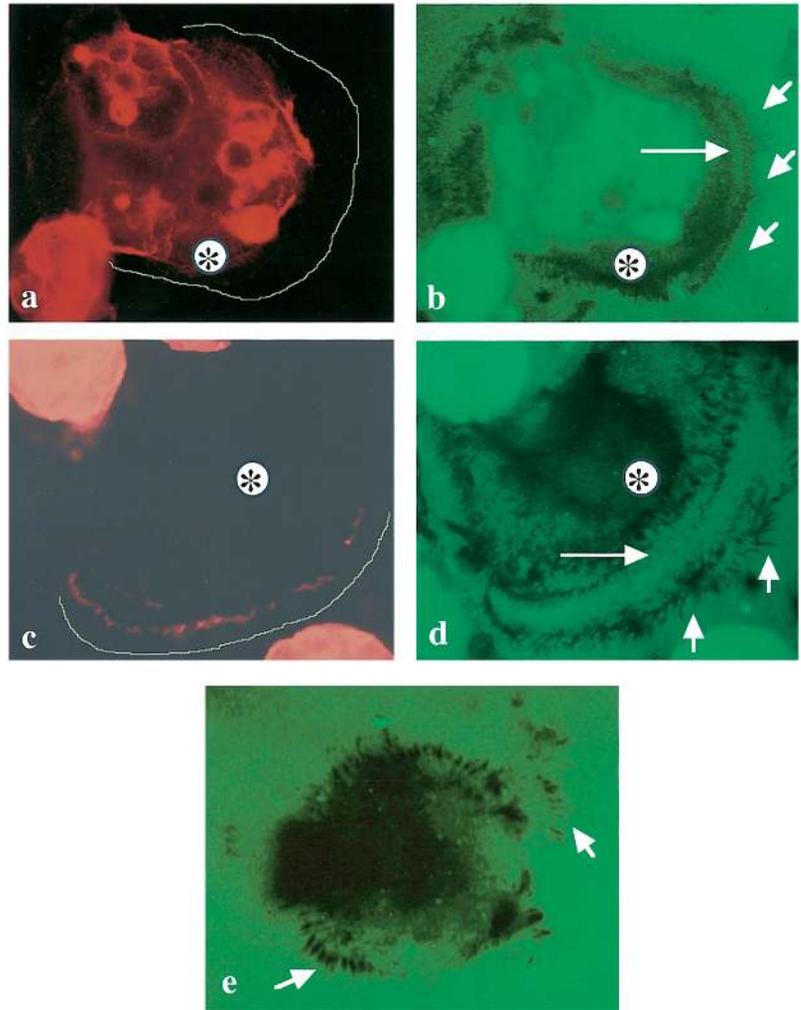


Fig. 3. Organized degradation of coated gelatin substrate by migrating L-10 cells. Cohort migration was induced with HGF/SF treatment (20 ng/ml, 20 h) on FITC-labeled gelatin matrix. *a* and *c*, rhodamine-phalloidin immunofluorescent images. *b*, *d*, and *e*, immunofluorescent images of FITC-labeled gelatin. *b* and *d*, dark areas caused by lysis of FITC-labeled gelatin is seen at the sites corresponding to leading edges (*). The lysis of gelatin is caused in an organized manner: radially arrayed gelatinolysis (*short arrows*) is observed at the frontmost portion of the leading edges, with arcuate band-like gelatin matrix (*long arrows*) being left beneath the main part of the leading edges. Gelatinolysis caused by migrating cells was also demonstrated by removing cells with EDTA treatment and tapping the chamber slides with fingers after cohort migration had been induced, which we called footprints of cells (*e*). Radially arrayed gelatinolysis (*arrows*) is also shown at the frontmost portions. The cells were photographed at a magnification of $\times 100$ (*a-e*).

the cellular binding of MMP-2 reduces in confluent cultures compared with that in sparse cultures (21). Taken together, the presence of abundant cell-cell contact as in confluent cultures seems to suppress MT1-MMP expression and MMP-2 binding, whereas specialized cell-

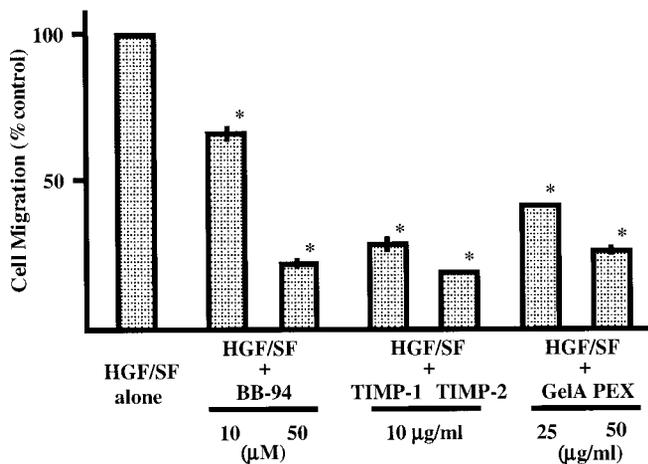


Fig. 4. Effect of BB-94, TIMP-1 and -2, and Gela PEX on HGF/SF-induced cohort migration on gelatin-coated substratum. Cells were treated with BB-94 (10 or 50 μM), TIMP-1 or -2 (10 $\mu\text{g/ml}$), or Gela PEX (25 or 50 $\mu\text{g/ml}$) for 1 h, and then cohort migration was induced with HGF/SF treatment (20 ng/ml, 24 h) on gelatin-coated substratum in the presence of the above inhibitors. The control value was 53.9 ± 1.4 cells/high-power field. The values are means and SEs of the mean ($n = 3$). *, statistically significant compared with control, $P < 0.01$.

ECM contact sites facilitate localization of these enzymes. In our study, it is possible that immunolocalization of MT1-MMP and MMP-2 was detectable positively only when they were aggregated to some extents and the enzymes that were sparsely distributed on the cell surface could not be detected. Thus, the two enzymes may be concentrated in the large leading edges of the front cells of migrating cell sheets but not in short cell protrusions of the following migrating cells that are extended forward beneath the preceding cells (13). This difference may be caused by cell-cell contact all around the following cells and more abundant cell-ECM interactions in the front cells. Because MT1-MMP was distributed diffusely as small dots in the leading edges, its localization was different from that of focal adhesion plaques that were shown as vinculin-positive staining. Radial array of linear gelatinolytic zones beneath the leading edges of front cells was similar to the arrangement of actin in the leading edges that we previously showed (12). Localization of the MT1-MMP/MMP-2 complex might somehow be related to actin distribution.

In general, ECM is thought to be a kind of barrier for tumor cell invasion, and more or less its cleavage and removal are necessary for tumor cells to migrate (1). At the same time, however, ECM components provide cells with good substrate to move on. In our study, removal of gelatin matrix at the leading edges of the front cells of migrating cell sheets was not random or complete. Instead, it was performed in a very coordinate and organized manner, leaving radially arrayed gelatin matrix at the frontmost part. This limited and organized clearing of gelatin matrix was essential for cell migration because MMP

inhibitors efficiently inhibited migration. Thus, an important role of MMP is not just to remove ECM but to rearrange it to suit cell migration. We previously showed that synthesis and deposition of FN, especially EDA+FN, by migrating carcinoma cells were important for cohort-type migration (7), and production of this EDA+FN was stimulated by HGF/SF treatment (22). Because EDA+FN deposition occurs around and beneath the leading edges of front migrating cells (7) and gelatin is removed orderly, rearrangement of ECM by cells for cell migration may include both partial removal of preexisting matrix and the addition of newly synthesized matrix components. MMPs may play a role in the reorganization of the preexisting and newly deposited matrix components to suit cell migration.

The proteinases, which can cleave ECM components on the cell surfaces, may be the most suitable enzymes to rearrange the pericellular matrix. The MT1-MMP/MMP-2 and urokinase-type plasminogen activator/plasmin systems are the most potent examples of those. In our study, the inhibitors specific for MMPs inhibited cohort migration on the gelatin matrix by 70–80%, suggesting the predominant involvement of MMPs in the migration. MT1-MMP not only activates proMMP-2 but also degrades ECM components such as gelatin, FN, and collagen (23, 24), and digestion of collagen matrix by MT1-MMP rather than a MMP-2-mediated effect is reported to play a predominant role in branching tubule formation by Madin-Darby canine kidney epithelial cells in the three-dimensional cultures in collagen matrix (25). Chinese hamster ovary cells transfected with MT1-MMP caused subjacent proteolysis of a gelatin film due to cell surface MT1-MMP rather than via activated MMP-2 (24). In our study, however, Gela PEX effectively inhibited cohort migration on gelatin matrix. Gela PEX, the carboxyl-end domain of MMP-2, is involved in the trimolecular complex formation of MT1-MMP, TIMP-2, and MMP-2 on the cell surfaces (19, 20) and competitively inhibits MMP-2 activation by MT1-MMP (2, 19). Thus, MMP-2 that is activated by MT1-MMP on the cell surfaces seems to play a major role during the cohort migration. Effective inhibition by both TIMP-1 and TIMP-2 also supports this hypothesis because MMP-2 is known to be inhibited by these TIMPs, whereas MT1-MMP is inhibited by TIMP-2 but not by TIMP-1 (24). As shown in our study, in human glioma and fibrosarcoma cells transfected with cDNA encoding MT1-MMP, their ability to contract collagen lattices was shown to be dependent on the MT1-MMP-mediated activation of proMMP-2 and cell surface association of activated MMP-2 (2). Soluble MMP-2 failed to affect gel contraction. Furthermore, in human melanoma cells, the presence of active MMP-2 just on the cell surfaces was not enough, but more specialized localization of MT1-MMP and MMP-2 to the invasion front of cells (invadopodia) was essential for their invasion (5). In cohort migration, the spacial organization of MT1-MMP/MMP-2 into the invasion front of the whole migrating cell sheets occurs. The disturbance of this organization might lead to prevention of efficient tumor invasion.

Acknowledgments

We thank Dr. Y. Nawa (Department of Parasitology, Miyazaki Medical College) for invaluable suggestions and discussion, Y. Shiratani and Y. Nomura for kind help in tissue culture and staining cell culture slides, and T. Miyamoto for help in processing the figures and manuscript.

References

- Stetler-Stevenson, W. G., Aznavoorian, S., and Liotta, L. A. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu. Rev. Cell Biol.*, 9: 541–573, 1993.
- Deryugina, E. I., Bourdon, M. A., Reisfeld, R. A., and Strongin, A. Remodeling of collagen matrix by human tumor cells requires activation and cell surface association of matrix metalloproteinase-2. *Cancer Res.*, 58: 3743–3750, 1998.
- Sato, H., Takino, H., Okada, Y., Cao, J., Yamamoto, E., and Seiki, M. A matrix metalloproteinase expressed on the surface of invasive tumor cells. *Nature (Lond.)*, 370: 61–65, 1994.
- Nakada, M., Nakamura, H., Ikeda, E., Fujimoto, N., Yamashita, J., Sato, H., Seiki, M., and Okada, Y. Expression and tissue localization of membrane-types 1, 2 and 3 matrix metalloproteinases in human astrocytic tumors. *Am. J. Pathol.*, 154: 417–428, 1999.
- Nakahara, H., Howard, L., Thompson, E. W., Sato, H., Seiki, M., Yeh, Y., and Chen, W. T. Transmembrane/cytoplasmic domain-mediated membrane type 1-matrix metalloproteinase docking to invadopodia is required for cell invasion. *Proc. Natl. Acad. Sci. USA*, 94: 7959–7964, 1997.
- Nabeshima, K., Asada, Y., Inoue, T., Kataoka, H., Shimao, Y., Sumiyoshi, A., and Koono, M. Modulation of E-cadherin expression in TPA-induced cell motility: human well differentiated adenocarcinoma cells move as coherent sheets associated with phosphorylation of E-cadherin-catenin complex. *Lab. Invest.*, 76: 139–151, 1997.
- Nabeshima, K., Inoue, T., Shimao, Y., Kataoka, H., and Koono, M. TPA-induced cohort migration of well differentiated human rectal adenocarcinoma cells: cells move in a RGD-dependent manner on fibronectin produced by cells, and phosphorylation of E-cadherin/catenin complex is induced independently of cell-extracellular matrix interactions. *Virchows Arch.*, 433: 243–253, 1998.
- Nabeshima, K., Shimao, Y., Inoue, T., Itoh, H., Kataoka, H., and Koono, M. Hepatocyte growth factor/scatter factor (HGF/SF) induces not only scattering but also cohort migration of human colorectal adenocarcinoma cells. *Int. J. Cancer*, 78: 750–759, 1998.
- Nabeshima, K., Inoue, T., Shimao, Y., Kataoka, H., and Koono, M. Cohort migration of carcinoma cells: differentiated colorectal carcinoma cells move as coherent cell clusters or sheets. *Histol. Histopathol.*, 14: 1183–1197, 1999.
- Sträuli, P., and Weiss, L. Cell locomotion and tumor penetration. Report on a workshop of the EORTC cell surface project group. *Eur. J. Cancer*, 13: 1–12, 1977.
- Friedl, P., Noble, P. B., Walton, P. A., Laird, D. W., Chauvin, P. J., Tabah, R. J., Black, M., and Zanker, K. S. Migration of coordinated cell clusters in mesenchymal and epithelial cancer explants *in vitro*. *Cancer Res.*, 55: 4557–4560, 1995.
- Nabeshima, K., Komada, N., Inoue, T., and Koono, M. A two-dimensional model of cell movement. Well differentiated human rectal adenocarcinoma cells move as coherent sheets upon TPA stimulation. *Pathol. Res. Pract.*, 191: 76–83, 1995.
- Nabeshima, K., Moriyama, T., Asada, Y., Komada, N., Inoue, T., Kataoka, H., Sumiyoshi, A., and Koono, M. Ultrastructural study of TPA-induced cell motility: human well-differentiated rectal adenocarcinoma cells move as coherent sheets via localized modulation of cell-cell adhesion. *Clin. Exp. Metastasis*, 13: 499–508, 1995.
- Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C. S., and Goldberg, G. I. H-ras oncogene-transformed human bronchial epithelial cells (TBE-2) secrete a single metalloproteinase capable of degrading basement membrane collagen. *J. Biol. Chem.*, 263: 6579–6587, 1988.
- Huang, W., Suzuki, K., Nagase, H., Arumugam, S., Van Doren, S. R., and Brew, K. Folding and characterization of the amino-terminal domain of human tissue inhibitor of metalloproteinases-1 (TIMP-1) expressed at high yield in *E. coli*. *FEBS Lett.*, 384: 155–161, 1996.
- Heussen, C., and Dowdle, E. B. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal. Biochem.*, 102: 196–202, 1980.
- Tanaka, S. S., Mariko, Y., Mori, H., Ishijima, J., Tachi, S., Sato, H., Seiki, M., Yamanouchi, K., Tojo, H., and Tachi, C. Cell-cell contact down-regulates expression of membrane type metalloproteinase-1 (MT1-MMP) in a mouse mammary gland epithelial cell line. *Zool. Science (Washington, DC)*, 14: 95–99, 1997.
- Brooks, P. C., Stromblad, S., Sanders, L. C., von Schalscha, T. L., Aimes, R. T., Stetler-Stevenson, W. G., Quigley, J. P., and Cheresch, D. A. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha\beta 3$. *Cell*, 85: 683–693, 1996.
- Strongin, A. Y., Collier, I. E., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. *J. Biol. Chem.*, 270: 5331–5338, 1995.
- Kinoshita, T., Sato, H., Okada, A., Ohuchi, E., Imai, K., Okada, Y., and Seiki, M. TIMP-2 promotes activation of progelatinase A by membrane-type 1 matrix metalloproteinase immobilized on agarose beads. *J. Biol. Chem.*, 273: 16098–16103, 1998.
- Menashi, S., Dehem, M., Souliac, I., Legrand, Y., and Fridman, R. Density-dependent regulation of cell-surface association of matrix metalloproteinase-2 (MMP-2) in breast-carcinoma cells. *Int. J. Cancer*, 75: 259–265, 1998.
- Inoue, T., Nabeshima, K., Shimao, Y., and Koono, M. Hepatocyte growth factor/scatter factor (HGF/SF) is a regulator of fibronectin splicing in MDCK cells: comparison between the effects of HGF/SF and TGF- $\beta 1$ on fibronectin splicing at the EDA region. *Biochem. Biophys. Res. Commun.*, 260: 225–231, 1999.
- Ohuchi, E., Imai, K., Fujii, Y., Sato, H., Seiki, M., and Okada, Y. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *J. Biol. Chem.*, 272: 2446–2451, 1997.
- d'Ortho, M.-P., Stanton, H., Butler, M., Atkinson, S. J., Murphy, G., and Hembry, R. M. MT1-MMP on the cell surface causes focal degradation of gelatin films. *FEBS Lett.*, 421: 159–164, 1998.
- Kadono, Y., Shibahara, K., Namiki, M., Watanabe, Y., Seiki, M., and Sato, H. Membrane type 1-matrix metalloproteinase is involved in the formation of hepatocyte growth factor/scatter factor-induced branching tubules in Madin-Darby canine kidney epithelial cells. *Biochem. Biophys. Res. Commun.*, 251: 681–687, 1998.