Transcellular Invasion of MM1 Rat Ascites Hepatoma Cells Requires Matrix Metalloproteinases Derived from Host Mesothelium

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Summary Degradation of the extracellular matrix by proteases secreted by invading tumor cells is thought to be essential for metastasis. Using an in vitro transcellular migration assay model, we examined the requirement of matrix metalloproteinases (MMP) in the invasion of MM1 rat hepatoma cells through normal mesothelial cell monolayers. Here we show that MM1 cells transmigrate using MMPs not expressed in the tumor cells but secreted by host mesothelial cells. Additionally, the amount of MMP secreted by mesothelial cells was increased by co-culture with hepatoma cells. Our results point to the role of normal host cells in the tumor microenvironment as a source of invasion factors necessary for the metastatic process.

Key words Matrix metalloproteinase, MMP, Tumor cell invasion, Host mesothelium.

Cancer metastasis is a multiple-step process that includes detachment of cancer cells from the primary tumor, invasion into the surrounding tissue, lympho- or hematogenic dissemination, and finally invasion and proliferation in target tissues. During these steps, transcellular migration of tumor cells through layers of host cell is required. We have examined the molecular mechanisms of tumor cell invasion in an experimental carcinomatous peritonitis model using quantitative in vitro migration assay (Akedo et al. 1986). In this assay, cultured rat ascites hepatoma cells were seeded on a rat primary mesothelial cell monolayer, and transmigration activity was evaluated as number of tumor cells that penetrate through the monolayer. The in vitro invasive capacity in this model of several clones obtained from the parental AH130 cells correlates well with their invasiveness in vivo (Mukai et al. 1987).

Cancer cell invasion in 3D matrix environments closely resembles the in vivo situation. However, even such extracellular matrices provide only a passive environment only to be modified by factors produced by the invading cancer cells and ignore the contribution of factors generated by host cells at the site of metastasis.

In addition to cell migration, degradation of the extracellular matrix by matrix metalloproteases (MMPs) is also important in cancer cell invasion. High levels of MMPs have been described in many invasive cancer cell lines (Monsky et al. 1994, Taniguchi et al. 1992, Taniguchi et al. 1994, Bernhard et al. 1990, Bonfil et al. 1989). Single cell motility is classified into 2 types: mesenchymal motility, which is characterized by elongated cell morphology, and amoeboid motility, which is characterized by rounded cell shape with membrane blebs (Sahai 2005). Mesenchymal motility is MMP-dependent, whereas, amoeboid motility is MMP-independent. It has been reported that MM1, a highly invasive clone of AH130 cells, migrate with an elongated shape.

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falling under the mesenchymal type of motility (Mukai et al. 2000).

In the present study, we examined the involvement of MMPs in transcellular migration assay.

Materials and methods

**Materials**

Bovine serum albumin (BSA; fraction V, fatty acid free), 1-oleoyl-sn-lysophosphatidic acid (LPA) and fibronectin from bovine plasma were purchased from Sigma-Aldrich (USA). LPA was dissolved in PBS containing 0.1% (w/v) fatty acid free BSA. GM6001, MMP-2/MMP-9 Inhibitor V, MMP-2 Inhibitor III, and MMP-9 Inhibitor I were purchased from Calbiochem (USA).

**Cells and culture conditions**

Rat mesothelial cells were isolated from mesentery of Donryu rats (Nippon Bio-Supp. Center, Japan) and cultured in Eagle’s MEM containing 2-fold amino acids and vitamins (Nissui, Japan) supplemented with 10% FBS. After the mesothelial cells became confluent, the monolayer was used for the invasion assay. MM1 cells, a highly invasive clone of the AH130 rat ascites hepatoma cell line, were cultured in suspension using modified MEM supplemented with 10% FBS.

**Transcellular migration assay for in vitro invasion**

The assay was performed essentially as previously described (Akedo et al. 1986). Briefly, 1.5×10^5 cells/ml MM1 cells were seeded over a confluent mesothelial cell monolayer and cultured in serum free medium containing 25 μM lysophosphatidic acid (LPA). After 20 h, the supernatant was removed and monolayer was fixed with 10% formalin in PBS. The number of penetrated individual tumor cells and tumor cell colonies was determined using an inverted phase-contrast microscope. The invasive capacity was quantified by the number of invasion foci/cm^2.

**Gelatin zymography**

Confluent mesothelial cell monolayer, MM1 cells, or co-culture of these cells was incubated under FBS-free condition for 20 h. Conditioned media were collected and diluted 1:1 in non-reducing sample buffer and analyzed for proteinase activity by substrate gel zymography. Identical amounts of supernatant were electrophoresed under non-reducing conditions using a 10% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 for 30 min to remove SDS. After incubation at 37°C in developing buffer [1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 10 mM CaCl2, 0.2 M NaCl] for 16 h, the gel was stained with Coomassie Blue. Enzyme activity was visualized by lack of staining indicating gelatin breakdown.

**Reverse transcriptase polymerase chain reaction**

Total RNA was extracted MM1 cells or mesothelial cells using the Isogen isolation reagent (Nippon Gene, Japan) and RNA was reverse-transcribed using the Omniscript RT kit (Qiagen). Primers used for amplification of MMPs were as follows: MMP-2, 5′-AGCTCCCGGAAA-GATTGAT-3′ forward primer, 5′-TCCAGTTAAGGAGCCAGCTCT-3′ reverse primer; MMP-9 5′-CCACCGAGCTATCACCATC-3′ forward primer, 5′-GTCCGGTTTCACGATGTGT-3′ reverse primer; MT1-MMP, 5′-GTACTACCGCTTCAATGAGG-3′ forward primer, 5′-CAGTGCAGTACCAGGAG-3′ reverse primer; TIMP-2, 5′-AGGACCTGACAAGGACATCG-3′ forward primer, and 5′-TGATGCAAGAAAGAACTTGG-3′ reverse primer. Primers used for EMMPRIN were as follows: 5′-GCTGGTTGCTGCTGAGTTTCA-3′ forward primer, 5′-GTACTCTTCCCCAGGCA-AA-3′ reverse primer.
Results and discussion

To study whether MMP activity is required for transcellular migration of hepatoma cells, we used four MMP inhibitors. As shown in Fig. 1a, GM6001, inhibitor of MMP-1, 2, 3, 8, 9, significantly inhibited migration of MM1 cells. As mesothelial cells have basement membrane, we next tested the effect of type IV collagenase inhibitors, MMP-2/MMP-9 Inhibitor V, MMP-2 Inhibitor III, and MMP-9 Inhibitor I. All of these inhibitors markedly inhibited migration of MM1 cells. When MMP-2 inhibited, MM1 cells remained adherent to the mesothelial monolayer and they failed to transmigrate the monolayer (Fig. 2). The same observations were obtained when the cells were treated with other MMP inhibitors (data not shown). Several cell lines which migrate in mesenchymal manner switch to amoeboid migration in presence of MMP inhibitors (Sahai and Marshall 2003, Wolf et al. 2003). However, in the case of MM1 cells, the switch to amoeboid migration was not observed.

We next investigated the expression of MMPs and TIMP-2 in both the mesothelial and MM1 cells. As shown in Fig. 3, in contrast to the expression profile in mesothelial cells, MM1 cells did

![Graphs](image-url)
not express mRNAs for MMP-2 and MMP-9. On the other hand, MT1-MMP and TIMP-2, which are required for activation of MMP-2 (Itoh et al. 2001), were expressed in both mesothelial cells and MM1 cells. These results suggest that MM1 cells transmigrate the mesothelial cell monolayer utilizing MMPs produced by the mesothelial cells.

Gelatinolytic activity of conditioned media from both cell types was analyzed (Fig. 4). In agreement with the results of the RT-PCR experiment, only the mesothelial cells secreted MMP-2, MMP-9. Interestingly, the amount of MMP-9 in medium from co-culture of mesothelial and MM1 cells was much higher than that in medium from the mono-culture of mesothelial cells.

In several cases, interactions between tumor and stromal cells via the matrix metalloproteinase modulator EMMPRIN have been reported (Nabeshima et al. 2006). EMMPRIN plays a critical role in cancer progression inducing several MMPs, including MMP-1, MMP-2, MMP-3, MMP-9, MT1-MMP, and MT2-MMP. RT-PCR revealed that MM1 cells expressed EMMPRIN mRNA (Fig. 4c). EMMPRIN expression by the cancer cells is consistent with the increase of MMP-9 up-regulation observed after co-culture of MM1 and mesothelial cells as shown in Fig. 4a. We were unable to detect active-MMP-2 or active-MMP-9 in conditioned media from these cells. Furthermore, we

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**Fig. 2.** A phase contrast image of transmigrated MM1 cells. Cells were seeded on the mesothelial cell monolayer and cultured for 20 h in the presence of 25 μM LPA. Arrow indicates MM1 cells migrated into the mesothelial monolayer. Scale bar, 50 μm.

**Fig. 3.** Expression of MMP-2, MMP-9, MT1-MMP, TIMP-2 mRNA. cDNAs were amplified 30 cycles by PCR using gene-specific primers. Note the complete lack of MMP-2 and MMP-9 transcripts in MM1 cells.
could not detect the gelatinolytic activity in the cell lysate either (data not shown). The lack of detections could be due to that active-MMPs localize at the leading edge of migrating cell and are diluted in the whole cell lysate beyond the sensitivity of the activity assay. To reconcile the lack of MMP activity with the inhibitory effect of MMP inhibitors shown in Fig. 1, we reason that active-MMPs were below the detection limit of our assay. Nonetheless, it is compelling that MM1 cells invade requiring MMPs secreted by host mesothelial cells even though MM1 is a highly invasive clone.

Fig. 4. Gelatinolytic activity of conditioned medium collected from mesothelial cell monolayer, MM1 cells (1.5×10^5 cells/ml), or co-culture of mesothelial cell monolayer and MM1 cells (1.5×10^5 cells/ml). (a) The cells were cultured in serum/LPA-free medium for 20 h and an equal volume of 5 µl of medium was tested using 0.1% gelatin zymography. Conditioned medium from HT-1080 cells was used to confirm the molecular weight of MMP-2, MMP-9 (left). (b) Quantitative analysis was performed using Image gauge software (Fuji Film, Japan). n=4. (c) Expression of EMMPRIN mRNA. cDNAs were amplified 30 cycles by PCR using gene-specific primers.
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