RESEARCH PAPER

Potentiation of cell invasion and matrix metalloproteinase production by α3β1 integrin-mediated adhesion of gastric carcinoma cells to laminin-5

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Abstract We previously reported that the adhesion of gastric carcinoma cells to the peritoneum mediated by the α3β1 integrin-laminin interaction is a key step in the initial process of peritoneal metastatic dissemination. Carcinoma cells subsequently invade through the intercellular gaps of mesothelial linings. In this study, we examined the role of the interaction of carcinoma cells with laminin-5, which is a major component of submesothelial basement membranes and serves as a high-affinity ligand for α3β1 integrin, in carcinoma cell invasion. Human gastric carcinoma cell lines (MKN1, GT3TKB, and NUGC-4) adhered in an α3β1 integrin-dependent manner to the extracellular matrix deposited by peritoneal mesothelial cells. An in vitro invasion assay using the Boyden chamber system revealed that MKN1 cell migration through the membranes increased when the membranes were coated with matrices produced by mesothelial cells or with laminin-5-containing Matrigel as compared to Matrigel alone. The cell migration promoted by laminin-5-containing Matrigel was inhibited by the presence of anti-α3 integrin antibody. When MKN1 cells were cultured in a laminin-5-coated plate, these cells were promoted to produce matrix metalloproteinase (MMP)-9, as assessed by gelatin zymography, enzyme-linked immunosorbent assay, and reverse transcription-polymerase chain reaction. These results suggest that the production of MMP-9 by MKN1 cells was potentiated by the α3β1 integrin-laminin-5 interaction, which facilitated their invasion via degradation of the matrix.

Abbreviations

BCECF-AM	2′ 7′-Bis(carboxyethyl)carboxyfluorescein tetraacetoxymethyl ester
BSA	Bovine serum albumin
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
MMP	Matrix metalloproteinase
PBS	Phosphate-buffered saline
RT-PCR	Reverse transcription-polymerase chain reaction

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Introduction

Peritoneal dissemination leads to a poor prognosis in patients with gastric cancer. Metastatic dissemination is mediated via exfoliation of carcinoma cells into the abdominal cavity, followed by their adhesion to the mesothelial lining. The adhesion of carcinoma cells to the peritoneum is a key step in the initial process of peritoneal dissemination. Various cell adhesion molecules, including integrins, play crucial roles in adhesion to the peritoneum. The integrin family of cell adhesion molecules serve as adhesion receptors for extracellular matrix (ECM) proteins

and cellular counter-ligands. Integrins are heterodimers of transmembrane glycoproteins (α and β subunits); 24 combinations of α and β subunits have been identified thus far, and individual integrin heterodimers have unique ligand specificities. A number of reports have demonstrated that alterations in integrin expression profiles in cancer cells are frequently associated with malignant phenotypes, including invasive and metastatic potential [1–6]. The immunohistochemical analysis of specimens of gastric cancer resected from more than 100 patients demonstrated that the expression of $\alpha3\beta1$ integrin (CD49c/CD29) was positively correlated with the occurrence of peritoneal and liver metastases, and with increased tumor invasiveness [7], suggesting a causal relationship between the increased expression of this integrin and the malignant phenotypes of cancer cells. Our in vitro study also indicated that $\alpha3\beta1$ integrin expressed on the surface of gastric carcinoma cells mediated their adhesion to the peritoneum excised from mice and to a peritoneal mesothelial cell monolayer culture [8]. It was also suggested that laminin-5 (laminin-332) produced by mesothelial cells served as a major counter-ligand for $\alpha3\beta1$ integrin. In metastatic processes, carcinoma cells initiate invasion through the intercellular gaps of mesothelial linings into the submesothelial tissues following the initial attachment to the peritoneum. Because laminin isoforms, including laminin-5, are localized more densely in the basement membranes underlying mesothelial cells, the $\alpha3\beta1$ integrin-mediated interaction is expected to be involved in the adhesion of carcinoma cells to submesothelial matrices. Therefore, in the present study we focused on the roles played by $\alpha3\beta1$ integrin in the invasion of gastric carcinoma cells into the matrix produced by mesothelial cells. Integrins are also known to contribute to intracellular signaling involved in the control of cell growth and motility, as well as adhesion to the host environment [9, 10]. We also examined the possibility that the $\alpha3\beta1$ integrin-dependent adhesion of cancer cells to laminin-containing matrices induces the production of matrix-degrading enzymes such as matrix metalloproteinases (MMPs), which are considered to play crucial roles in tumor invasion.

Materials and methods

Reagents

Matrigel^{TM} and fibronectin were purchased from BD Biosciences (San Diego, CA) and Chemicon International, Inc. (Temecula, CA), respectively. Laminin-5 was purified from the serum-free conditioned medium of MKN45 human gastric carcinoma cells, as described previously [11]. Bovine serum albumin (BSA), Triton X-100, and

gelatin were products of Sigma (St. Louis, MO). Nonidet P-40 was purchased from Nacalai Tesque (Kyoto, Japan). A fluorescent dye, 2′, 7′-bis(carboxyethyl)carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM), was purchased from Dojindo Laboratories (Kumamoto, Japan). Coomassie brilliant blue R-250 (CBB) was obtained from Merck (Darmstadt, Germany). MMP-2 inhibitor I (oleoyl-N-hydroxylamide, OA-Hy) [12], MMP-9 inhibitor I (a derivative of anthranilic acid) [13], and MMP-2/MMP-9 inhibitor I ((2R)-[(4-biphenylylsulfonyl)amino]-3-phenyl-propionic acid; BiPS) [14] were purchased from Calbiochem (San Diego, CA). The following monoclonal antibodies were used in this study: Anti-$\alpha2$ integrin (Gi9) and anti-$\alpha6$ integrin (GoH3) antibodies were purchased from Beckman Coulter, Inc. (Fullerton, CA). Anti-$\alpha3$ integrin (SM-T1) antibody was prepared in our laboratory [15]. Anti-$\alpha4$ integrin (SG/73), anti-$\alpha5$ integrin (KH/33), and anti-$\beta1$ integrin (SG/19) antibodies were purchased from Seikagaku Corp. (Tokyo, Japan). FITC-labeled anti-mouse IgG and anti-rat IgG antibodies were purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA).

Cell lines

Human gastric carcinoma cell lines MKN1, MKN45, and GT3TKB were supplied by the RIKEN Cell Bank (Tsukuba, Japan), and NUGC-4 was supplied by the Health Science Research Resources Bank (Osaka, Japan). These cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) at 37°C under 5% CO_2.

Flow cytometry

The expression of integrins was measured by a flow cytometer (FACSCalibur, BD Biosciences) as described previously [15], using monoclonal antibodies against human integrin $\alpha2$, $\alpha3$, $\alpha4$, $\alpha5$, $\alpha6$, and $\beta1$ subunits and FITC-labeled secondary antibodies.

Isolation of peritoneal mesothelial cells

The parietal peritoneum and diaphragm were excised from ICR mice under sterile conditions. Mesothelial cells were isolated essentially as described by Nakashio et al. [16]. Briefly, the resected parietal peritoneum and diaphragm were washed with serum-free RPMI 1640 medium and incubated with 0.25% trypsin (GIBCO) in 10 mM phosphate-buffered saline (pH 7.3) (PBS) at 37°C for 20 min. After an equal volume of RPMI 1640 medium/10% FBS was added to the cell suspension, the mixture was passed through nylon mesh and centrifuged at 700 rpm for 5 min. The pelleted cells were suspended in RPMI 1640 medium/

10% FBS (1×10^6 cells/ml), and an aliquot (0.1 ml) was placed in a 96-well culture plate that had been coated with 10% Matrigel solution at 4°C for 16 h. The cells were cultured at 37°C for 2–3 days until growth of a monolayer of polygonal mesothelial cells. This study was conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of Hoshi University.

Preparation of matrix produced by mesothelial cells

Matrices deposited by peritoneal mesothelial cells were prepared according to the method reported by Weizman et al. [17]. Murine peritoneal mesothelial cells were cultured on 10% Matrigel-coated plates for 2–3 days until the cells formed a monolayer, and the monolayer cells were treated with 1% Nonidet P-40 in PBS for 1 min, followed by treatment with 2 M urea/1 M NaCl for 3 min at 4°C. The wells were then washed twice with PBS to remove non-adherent materials.

Cell adhesion assay

Human gastric carcinoma cells were labeled with BCECF-AM (3 μM) at 37°C for 20 min [18], and a suspension of labeled cells (1×10^6 cells/ml in 1% BSA/RPMI 1640, 0.1 ml) was added to the mesothelial cell monolayer or matrix prepared in a 96-well culture plate, as described above. After the plate was incubated at 37°C for 30 min, non-adherent cells were removed by gently washing the plate three times with warm 1% BSA/RPMI 1640. Adherent cells were then lysed with 0.2 ml of 1% Nonidet P-40, and the intensity of fluorescence was measured with a fluorescence spectrophotometer (Ex = 490 nm, Em = 520 nm). For the inhibition experiment, the cells were treated with antibody at 0°C for 30 min before being incubated in a culture plate. The percent adhesion was calculated as follows: (the fluorescence intensity of adherent cells/fluorescence intensity of total cells) × 100.

In vitro cell invasion assay

The in vitro cell invasion assay was performed using the Boyden chamber system, which consists of a 24-well culture plate (Falcon 3504, BD Biosciences) combined with an inner chamber with a polyethylene terephthalate (PET) membrane (pore size: 8 μm; Falcon 3097). The PET membranes were coated with 10% Matrigel containing laminin-5 (5 μg/ml) or 10% Matrigel alone as a control. In some experiments, the membranes were coated with matrices produced by murine peritoneal mesothelial cells, which were prepared by the treatment of a monolayer of mesothelial cells with 1% Nonidet P-40 and 2 M urea/1 M NaCl as described above. A carcinoma cell suspension

(3.5×10^5 cells/ml, 0.2 ml) in serum-free medium (ASF-104, Ajinomoto Co, Inc., Tokyo Japan) was placed in the inner (upper) chamber of the Boyden chamber system. The inner chamber was then combined with an outer (lower) chamber filled with conditioned medium (0.7 ml) of HT-1080 fibrosarcoma cells, and then the chambers were incubated at 37°C for 16 h. The cells migrated into the lower chamber through the membranes were counted under a microscope after being stained with an eosin/thiazine dye-based staining solution (Diff-Quik; International Reagents Corp., Kobe, Japan). For the inhibition experiment, the cells were treated with anti-α3 integrin antibody (SM-T1, 10 μg/ml) at 0°C for 30 min, and then they were placed in the inner chamber.

Gelatin zymography

MKN1 gastric carcinoma cells (2×10^5 cells) were cultured in ASF-104 serum-free medium at 37°C for 24 or 48 h in a 24-well culture plate (Sumitomo, Tokyo, Japan) that had been coated with laminin-5 (5 μg/ml), fibronectin (5 μg/ml), or Matrigel (10). The culture supernatant was concentrated 40-fold with Microcon^{TM} YM-30 (Millipore Corp., Bedford, MA). The gelatinase activity was measured by zymography essentially as described previously [19]. The sample (10 μl) was mixed with an equal volume of SDS-sample buffer (10 mM Tris–HCl, pH 6.8, 1% SDS, 20% glycerol, 0.03% bromophenol blue) and incubated at 37°C for 20 min. Electrophoresis was carried out in 10% acrylamide gel containing 0.15% gelatin and 0.1% SDS at 16°C. The gels were washed twice with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, and 0,02% NaN_3 containing 2.5% Triton X-100 to remove SDS, and then the washed gels were incubated in the same buffer without Triton X-100 at 37°C for 12 h, and stained with 0.3% Coomassie brilliant blue in 30% ethanol/10% acetic acid.

Measurement of MMP-9 by enzyme-linked immunosorbent assay (ELISA)

MMP-9 was measured by ELISA with an MMP-9 Activity Assay Biotrak^{TM} System (GE Healthcare, Piscataway, NJ). MKN1 gastric carcinoma cells (1.7×10^5 cells) were cultured in ASF-104 serum-free medium at 37°C for 48 h in a 6-well culture plate that had been coated with laminin-5 (5 μg/ml), fibronectin (5 μg/ml), or Matrigel (10). The culture supernatant, concentrated 40-fold, was added to an ELISA plate that had been coated with anti-MMP-9 antibody, and the sample was incubated at 4°C for 16 h. After any unbound materials were removed by washing, the proMMP-9 bound to the plate was activated with 1 mM *p*-aminophenylmercuric acetate (APMA) at 37°C for 1.5 h to convert the proform into the active form. The substrate

for MMP-9 and chromogenic reagent were subsequently added, and the mixture was incubated at 37°C for 2 h. The concentration of MMP-9 was estimated by measuring absorbance at 415 nm.

Detection of mRNAs for MMP-9

The mRNA for MMP-9 was detected by a reverse transcription-polymerase chain reaction (RT-PCR) method. The total RNA was isolated from MKN1 cells that had been cultured in an ECM protein-coated plate by the AGPC method [20] using Trizol^{TM} (GIBCO/Invitrogen), and the isolated RNA was reverse-transcribed with SuperScript II reverse transcriptase (GIBCO/Invitrogen) using an oligo-dT primer. PCR was performed with a set of primers: 5′- CCA TTTCGACGATGACGAGTT -3′ (sense primer for human MMP-9 cDNA; 611–631) and 5′- CTTGTCGCTGTCAA AGTTCGA -3′ (antisense primer for human MMP-9 cDNA; 1134–1154, GenBank Accession BC006093). As a control, cDNA for β-actin was also amplified with the following primers: 5′- AAGATGACCCAGATCATGTTTGAG -3′ (sense primer for human β-actin cDNA; 393–416) and 5′- AGG AGGA GCAATGATCTTGATCTT -3′ (antisense primer for human β-actin cDNA; 1017–1040, GenBank Accession X00351). The PCR conditions were as follows: 94°C, 30 s; 60°C, 30 s; 72°C, 30 s; 23 cycles. The products were separated in 2.0% agarose gel in 40 mM Tris–acetate buffer (pH 8.0) containing 1 mM EDTA (1× TAE), followed by ethidium bromide staining.

Results

Adhesion of gastric carcinoma cells to matrix produced by mesothelial cells

We first analyzed the adhesion of gastric carcinoma cells to a monolayer of murine peritoneal mesothelial cells and matrices produced by these cells. All three cell lines tested (GT3TKB, MKN1, and NUGC-4) were found to adhere to both a monolayer of mesothelial cells as well as to the matrices, but adhesion to the matrices was stronger than that to the cell monolayer (Fig. 1). The effects of antibodies against subunits of the β1 integrin subfamily on the adhesion of GT3TKB cells to mesothelial matrix were examined, and it was found that antibodies to the α3 and β1 integrin subunits effectively inhibited adhesion (51 and 74% inhibition by anti-α3 and anti-β1 integrin antibodies, respectively) (Fig. 2). Similar inhibitory effects of these antibodies on the adhesion of the other two cell lines (MKN1 and NUGC-4) were observed. These results strongly suggest that α3β1 integrin plays a central role in the adhesion of gastric carcinoma cells to ECM deposited by mesothelial cells. These results are also in good
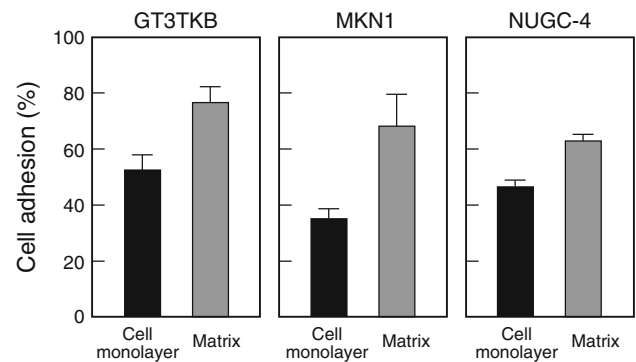

Fig. 1 Adhesion of gastric carcinoma cells to a monolayer of peritoneal mesothelial cells and matrices deposited by these cells. GT3TKB, MKN1, or NUGC-4 cells (1 × 10^6 cells/ml, 0.1 ml) were fluorescently labeled with BCECF-AM and added to a mesothelial cell monolayer (*closed bar*) or matrices (*shaded bar*) prepared in a 96-well culture plate as described in Materials and Methods. After the cells were incubated at 37°C for 30 min, non-adhering cells were removed by gentle washing, and adherent cells were lysed with 0.2 ml of 1% Nonidet P-40. The fluorescence intensity was measured with a fluorescence spectrophotometer (Ex = 490 nm, Em = 520 nm)
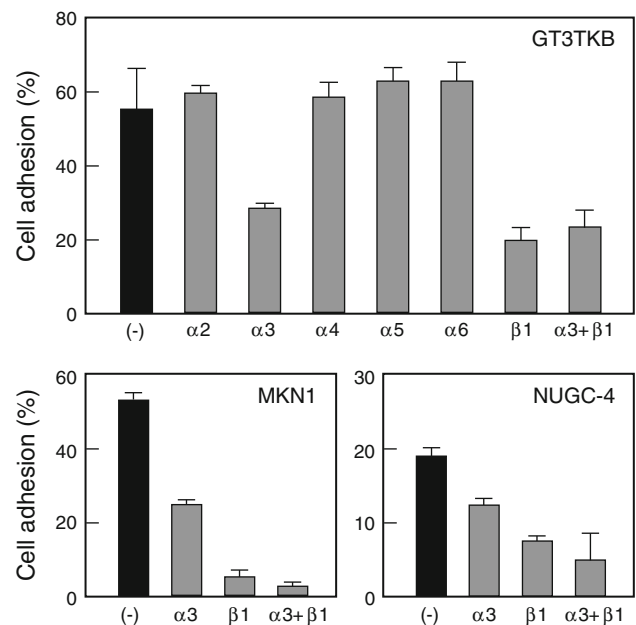

Fig. 2 Effects of antibodies against integrin subunits on the adhesion of gastric carcinoma cells to matrices produced by peritoneal mesothelial cells. Fluorescently labeled GT3TKB, MKN1, or NUGC-4 cells (1 × 10^6 cells/ml, 0.1 ml) were treated with various anti-integrin antibodies (20 μg/ml) at 0°C for 30 min, and the cells were then added to 96-well culture plates on which mesothelial cell-derived matrices were prepared as described in Materials and Methods. After the cells had been incubated at 37°C for 30 min, non-adherent cells were removed by gentle washing, and adherent cells were lysed with 0.2 ml of 1% Nonidet P-40. The fluorescence intensity was measured with a fluorescence spectrophotometer (Ex = 490 nm, Em = 520 nm)

agreement with previous findings that laminin-5 and laminin-10/11, both of which are high-affinity ligands for $\alpha 3\beta 1$ integrin, were produced by mesothelial cells [8]. Flow cytometric analyses confirmed that these three cell lines expressed $\alpha 3\beta 1$ integrin on the cell surface (high levels of expression in the case of GT3TKB and MKN1 cells, and a moderate level of expression in the case of NUGC-4 cells; supplemental data 1).

Invasion of carcinoma cells through reconstituted basement membranes

We next examined the in vitro invasive capacity of MKN1 cells through reconstituted basement membranes using the Boyden chamber system. When the membranes between the lower and the upper chambers were coated with matrices deposited by mesothelial cells, the migration of MKN1 cells into the lower chamber was significantly increased as compared to that across membranes coated with Matrigel used for the mesothelial cell culture as a scaffold (Fig. 3a). The addition of laminin-5 to Matrigel resulted in a similar enhancement of MKN1 cell migration. The increase in cell migration via Matrigel-coated membranes was dependent on the concentration of laminin-5 added to the Matrigel (Fig. 3b). Antibody against the integrin $\alpha 3$ subunit inhibited cell migration through the lammin-5/Matrigel-coated membranes to the control level (Fig. 3c), suggesting that $\alpha 3\beta 1$ integrin plays a crucial role in enhanced cell invasion. It is likely that $\alpha 3\beta 1$ integrin acts as a cellular receptor for laminin-5, and that cells invade into the matrix by using laminin-5 as a scaffold.

Production of MMPs by carcinoma cells

It was already known that various matrix-degrading enzymes, including MMPs produced by tumor cells, play an important role in invasion into the ECM. To assess the contribution of those proteinases, we examined the effects of MMP inhibitors on cell invasion. MMP-2 inhibitor I (oleoyl-N-hydroxylamide, 3.4 μM) [12] and MMP-9 inhibitor I (an anthranilic acid derivative, 1 μM) [13] effectively inhibited the migration of MKN1 cells through the mesothelial matrix-coated or lammin-5/Matrigel-coated membranes (Fig. 4a, b). Cell migration through either membrane was more strongly suppressed by MMP-2/MMP-9 inhibitor I (an N-sulfonylamino acid derivative, 0.31 μM) [14]. These results suggest that both MMP-2 and MMP-9 are involved in MKN1 cell invasion into laminin-5-containing Matrigel. We thus examined the possibility that the interaction of MKN1 cells with laminin-5 or with mesothelial matrix induced the production of MMPs by these cells. The serum-free conditioned medium of
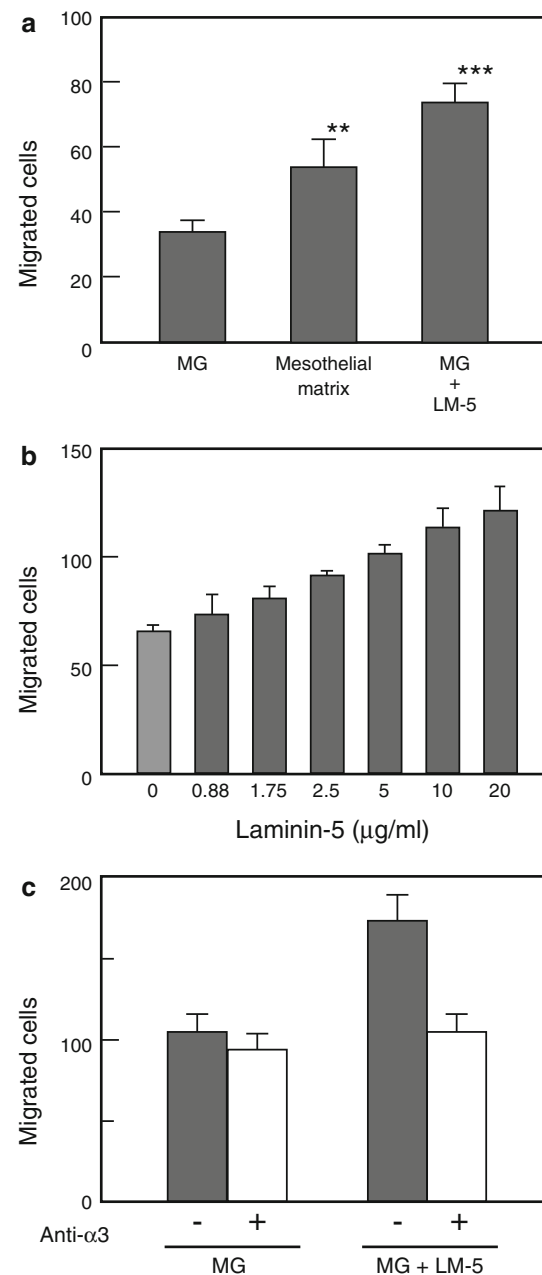
Fig. 3 In vitro cell invasion through reconstituted basement membranes. An MKN1 cell suspension (3.5×10^5 cells/ml, 0.2 ml) in serum-free medium was placed in the upper chamber of a Boyden chamber system and the suspension was incubated at 37°C for 16 h. The number of cells that had migrated into the lower chamber through the membrane was counted under a microscope. **a** The membrane between the upper and lower chambers of the Boyden chamber system were coated with Matrigel alone, Matrigel containing matrices deposited by peritoneal mesothelial cells, or Matrigel containing laminin-5 (5 μg/ml). **b** The membrane was coated with Matrigel containing various concentrations (0–20 μg/ml) of laminin-5. **c** MKN1 cells were pretreated with or without anti-$\alpha 3$ integrin antibody (10 μg/ml) at 0°C for 30 min, and the pretreated cells were then added to the upper chamber containing a membrane coated with Matrigel/laminin-5 (5 μg/ml). *MG*, Matrigel; *LM-5*, laminin-5
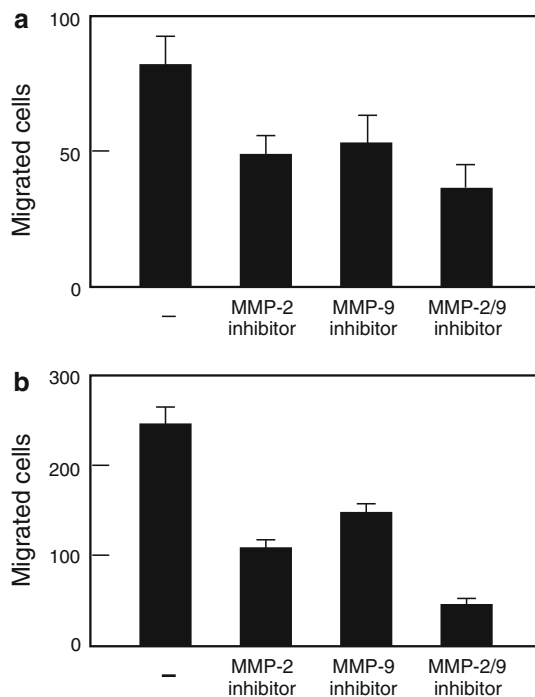
Fig. 4 Effects of inhibitors of MMP-2 and MMP-9 on cell invasion through reconstituted basement membranes. An MKN1 cell suspension (3.5×10^5 cells/ml, 0.2 ml) in serum-free medium was placed in the upper chamber of the Boyden chamber system, and the cell suspension was incubated at 37°C for 16 h in the presence or absence of MMP-2 inhibitor I (3.4 μM) [12], MMP-9 inhibitor I (1 μM) [13], or MMP-2/9 inhibitor I (0.31 μM) [14]. The number of cells that had migrated into the lower chamber through the membranes was counted under a microscope. The details of the experiment are described in Materials and Methods. **a** Membranes coated with matrices deposited by mesothelial cells; **b** Matrigel/laminin-5 membranes

MKN1 cells cultured in a plate that had been coated with the mesothelial matrix was analyzed by gelatin zymography. As shown in Fig. 5a, the supernatant of MKN1 cells cultured in the matrix plate exhibited slightly higher gelatinolytic activity in a region corresponding to the mobility of the proform of MMP-9 (proMMP-9, ~92 kDa) than was observed in the case of control plate; however, almost no enhancement of gelatinolytic activity was observed in the region corresponding to MMP-2 (~72 kDa). MKN1 cells cultured for 24 or 48 h in laminin-5-coated plates also produced higher levels of MMP-9 than those cultured in fibronectin-coated, Matrigel-coated, or control plates (Fig. 5b). The potentiation of proMMP-9 production by laminin-5 was dependent on the concentration of laminin-5 used for immobilization on the plate (Fig. 5c). Measurement by ELISA confirmed the increase in MMP-9 secretion (~4.2-fold) from MKN1 cells cultured in laminin-5-coated plates, as compared to the lower level of MMP-9 secretion observed in MKN1 cells cultured in control plates (Fig. 6a). Moreover, no significant increases in MMP-9 secretion were observed in fibronectin-coated or Matrigel-coated culture
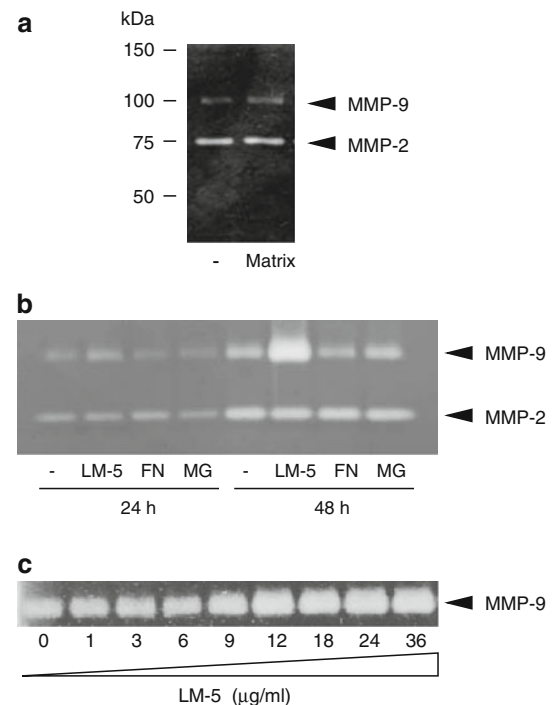

Fig. 5 Detection of MMP activity by gelatin zymography. **a** MKN1 gastric carcinoma cells (2×10^5 cells) were cultured in ASF-104 serum-free medium at 37°C for 48 h in a 24-well culture plate coated with matrices deposited by mesothelial cells. After the culture supernatant was concentrated 20-fold, an aliquot (10 μl) was mixed with an equal volume of sample buffer for SDS–polyacrylamide gel electrophoresis (SDS–PAGE), and the mixture was incubated at 37°C for 20 min, after which it was subjected to gelatin zymography. Electrophoresis was carried out in a 10% acrylamide gel containing 0.15% gelatin and 0.1% SDS at 16°C. **b** MKN1 cells were cultured for 24 or 48 h in culture plates coated with laminin-5 (5 μg/ml) (*LM-5*), fibronectin (5 μg/ml) (*FN*), or Matrigel (10%) (*MG*). The culture supernatants were subjected to gelatin zymography as described above. **c** MKN1 cells were cultured for 48 h in a culture plate that had been coated with various concentrations (0–36 μg/ml) of laminin-5

plates. We then carried out an RT-PCR analysis to investigate changes in mRNA levels in cells cultured with immobilized ECM proteins. When the PCR products were separated by agarose gel electrophoresis, the cells cultured for 24 or 48 h in laminin-5-coated plates yielded more intense bands (544 bp, corresponding to MMP-9 cDNA) than those of cells cultured in fibronectin-coated, Matrigel-coated, or control plates (Fig. 6b). Therefore, these results strongly suggest that the production of MMP-9 is potentiated by laminin-5 at both the protein and the mRNA level.

Discussion

Pathohistological studies of clinical specimens of gastric cancer have suggested a correlation between the enhanced expression of $\alpha 3\beta 1$ integrin on carcinoma cells and their

capacity for peritoneal metastasis. Our previous study indicated that $\alpha3\beta1$ integrin plays a crucial role in the initial adhesion of carcinoma cells to the peritoneum [8]. The mechanisms underlying the progression of peritoneal metastasis of gastric cancer, after an initial attachment to the mesothelial lining, have not been well characterized. In the present study, we further analyzed the interaction between $\alpha3\beta1$ integrin expressed on cancer cells and matrix containing laminin-5, a major ligand for this integrin in the peritoneum. The results indicated that three gastric carcinoma cell lines firmly adhered in an $\alpha3\beta1$ integrin-dependent manner to matrices produced by peritoneal mesothelial cells. These findings are consistent with a previous observation that laminin-5 is a major component of mesothelial basement membranes [8]. The interaction of cancer cells with the mesothelial matrix appears to be of considerable importance in the peritoneal metastasis of gastric cancer, because cancer cells that had exfoliated into the abdominal cavity attached initially to the mesothelial lining, followed by their invasion through the intercellular gaps of mesothelial cells. Mochizuki et al. suggested the importance of the cancer cell-matrix interaction in metastasis to the abdominal wall peritoneum [21]. Micrometastasis selectively forms on the omentum and mesenterium during the early stages of metastasis; subsequently, morphological changes are induced in mesothelial cells, leading to the exposure of the submesothelial ECM at intercellular gaps. It is likely that cancer cells expressing $\alpha3\beta1$ integrin invade into mesothelial tissues by using laminin-5 on exposed mesothelial basement membranes as a scaffold.

It was suggested by a previous study that the $\alpha3\beta1$ integrin-laminin-5 interaction was also involved in hematogenous metastasis. Wang et al. [22] demonstrated the importance of the $\alpha3\beta1$ integrin-mediated arrest of cancer cells by laminin-5 in basement membranes of the vasculature in pulmonary metastasis. On the other hand, Ishii et al. [23] reported an inverse correlation between the level of $\alpha6\beta4$ integrin expression and the frequency of the peritoneal dissemination of gastric cancer. Although $\alpha6\beta4$ and $\alpha3\beta1$ integrins share laminin-5 as a common counterligand, the expression levels of these integrins in gastric cancer apparently were found to have opposite effects. One possible explanation for such opposition would be that these integrins function differently during metastasis; for example, a relatively high level of $\alpha6\beta4$ integrin expression could prevent cancer cells from exfoliation from a primary lesion. Another possibility would be differences in the intracellular signaling events that occur after laminin-5 binds to cells expressing $\alpha6\beta4$ and $\alpha3\beta1$ integrins.

The results of the present study also demonstrated that the invasion of MKN1 gastric carcinoma cells into Matrigel basement membranes was promoted by the addition of
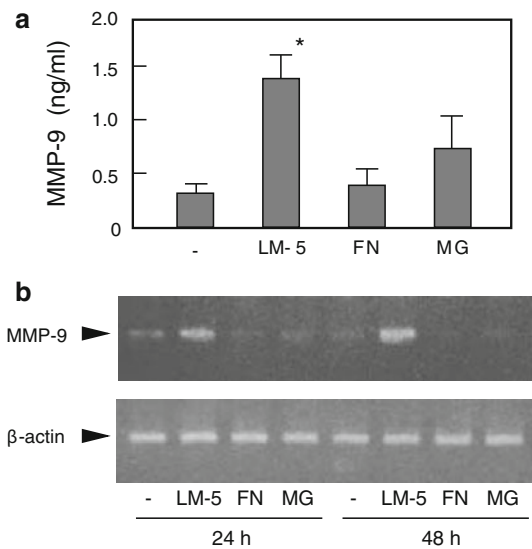

Fig. 6 Increase in MMP-9 production by MKN1 cells upon their adhesion to laminin-5. MKN1 cells (1.7×10^5 cells) were cultured in ASF-104 serum-free medium at 37°C for 48 h in 6-well culture plates coated with laminin-5 (5 µg/ml) (*LM-5*), fibronectin (5 µg/ml) (*FN*), or Matrigel (10) (*MG*). **a** MMP-9 in the culture supernatant was measured by ELISA using an MMP-9 Activity Assay Biotrak™ System (GE Healthcare). **b** The total RNA was isolated from MKN1 cells that had been cultured for 24 or 48 h, and mRNA for MMP-9 was measured by RT-PCR. The products were separated by agarose gel (2.0%) electrophoresis, followed by ethidium bromide staining

laminin-5 to Matrigel, and that the production of MMP-9 by MKN1 cells was potentiated by the interaction of these cells with laminin-5. Because laminin-5 was originally identified as a motility factor-like protein [11], it is reasonable to assume that the cell invasion into Matrigel was promoted by laminin-5. The inhibitory effect of anti-$\alpha3$ integrin antibody on the cell invasion suggests that $\alpha3\beta1$ integrin functions as a cellular receptor. In our recent study, human hepatocellular carcinoma HepG2 cells expressing $\alpha3\beta1$ integrin after cDNA transfection acquired a higher-level migratory phenotype on plates coated with laminin-5, and cells treated in this manner also exhibited more invasive potential with respect to laminin-5-containing matrices [24], suggesting that the $\alpha3\beta1$ integrin-mediated interaction regulates both the migratory and invasive capacity of cancer cells.

Furthermore, the potentiation of MMP-9 production by laminin-5 is also likely to have facilitated cell migration through the Matrigel, because type IV collagen, a major component of Matrigel, is a good substrate for MMP-9. Thus, in cooperation with constitutively produced MMP-2, the MMP-9 secretion promoted by cell adhesion to a laminin-5-containing matrix is thought to cause the degradation of the ECM that leads to enhanced cell invasion. Inhibitors for MMP-2 and/or MMP-9, in fact, effectively suppressed the in vitro cell migration through matrices deposited by mesothelial cells and that through Matrigel

basement membranes (Fig. 4). In this study, MKN1 cells were induced to produce proMMP-9 by their adhesion to laminin-5-containing substratum. Because proMMP-9 is known to be activated by MMP-3 and by serine proteases such as plasmin [25–27], the expression of these processing proteases as well as tissue inhibitors of metalloproteinases (TIMPs) following cell adhesion should also be an important issue in the future studies.

Kubota et al. [28] reported that anti-α3 integrin antibody enhanced proMMP-2 secretion by invasive human rhabdomyosarcoma RD cells, with a concomitant enhancement of cell invasion through Matrigel. These previous results differ somewhat from those of the present study, in which we observed upregulated MMP-9 secretion and unaltered MMP-2 secretion. The discrepancy between results may have been due to differences in the types of cells used, i.e., rhabdomyosarcoma cells were used in the previous study, whereas gastric carcinoma cells were examined in the present study. Moreover, an agonistic antibody was used in their study, whereas an ECM protein ligand (laminin-5) was used in our study, and differences between stimulant types could have led to distinct cellular responses. Their group also reported that the signaling of this cellular activation involved the transient, enhanced interaction of a multifunctional calcium-binding protein, calreticulin, with the cytoplasmic domain of the α3 integrin subunit [29]. The involvement of calreticulin in the signal transduction of laminin-5-mediated enhancement of MMP-9 secretion should be examined in future studies. Analysis of α3 integrin-deficient murine keratinocytes has suggested that MMP-9 secretion is positively regulated by α3β1 integrin expression via the stabilization of MMP-9 mRNA transcripts [30]. Thus, it will also be necessary to examine whether or not the binding of laminin-5 to α3β1 integrin on cancer cells promotes the similar process leading to the potentiation of MMP-9 production.

Recently, Han et al. [31] reported that the expression of MMP-9 in human non-small cell lung carcinoma (NSCLC) cells was stimulated by the α5β1 integrin-mediated adhesion of the cells to fibronectin via extracellular-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3 K) signaling pathways, which could be relevant in the development of malignant phenotypes of NSCLC. The regulation of the production of matrix-degrading enzymes via interactions between carcinoma cells and the ECM will remain an important issue in future studies of the pathobiology of cancer invasion, metastasis and angiogenesis [32, 33]. In conclusion, the present study indicated that α3β1 integrin is intimately involved in the adhesion and invasion of gastric carcinoma cells to the submesothelial ECM, and that the α3β1 integrin-laminin-5 interaction promoted MMP-9 production by these cells, which is most likely to facilitate their invasion and metastasis.

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