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Matrix Metalloproteinase-9 Associated with Heparan Sulphate Chains of GPI-Anchored Cell Surface Proteoglycans Mediates Motility of Murine Colon Adenocarcinoma Cells

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Using murine colon adenocarcinoma-derived clones with different metastatic potentials, the cellular localization of matrix metalloporteinase-9 (MMP-9) and its role in the cell motility were examined. Highly metastatic LuM1 clone aggressively invaded into adjacent tissue *in vivo*, but low metastatic NM11 clone did not. As compared with the NM11 clone, the LuM1 clone expressed and secreted a remarkably large amount of MMP-9, and exhibited higher abilities of cell migration and invasion *in vitro*, which were suppressed by MMP-2/MMP-9 inhibitor IV. MMP-9, exhibiting high affinity to heparin, was demonstrated to be condensed on tips of cellular podia. Treatment of the cells with heparitinase-I or heparin resulted in release of MMP-9 from the cell surface, which caused concomitant suppression of their motility to a similar level to that with the MMP inhibitor. Immunoprecipitation of a LuM1 cell lysate with an anti-MMP-9 antibody resulted in co-precipitation of phosphatidylinositol-specific phospholipase C-susceptible heparan sulphate proteoglycans having 66 and 64 kDa core proteins. Taken together, the present results demonstrate that secreted MMP-9 associates with glypican-like proteoglycans through their heparan sulphate chains, and plays a crucial role in cell motility of LuM1 cells.

Key words: cell motility, cell surface heparan sulphate, glypican, invasion, matrix metalloproteinase-9.

Abbreviations: APMA, *p*-aminophenylmercuric acetate; DAPI, 4,6-diamidino-2-phenylindole; FCS, foetal calf serum; FITC, fluorescein isothiocyanate; GPI, glycosylphosphatidylinositol; MMP, matrix metalloproteinase; MT, membrane-type; PI-PLC, phosphatidylinositol-specific phospholipase C; RT, reverse transcriptase; TBS, Tris-buffered saline; TIMP, tissue inhibitor of metalloproteinases.

Matrix metalloproteinases (MMPs), which have the ability to degrade a variety of matrix proteins, have been reported to be involved in many physiological and pathophysiological processes including embryonic development, tissue remodelling, wound healing, angiogenesis and tumour cell invasion and metastasis (*1*). MMPs are classified into two groups: secreted-type MMPs and membrane-type (MT) ones. Secreted-type MMPs are released into the extracellular space as latent forms, and probably play important roles in various physiological phenomena through complex regulation of their expression (*2*), activation (*3*) and inhibition (*4*).

For invasion and metastasis, it is essential for tumour cells to leave the primary tumour mass and to move into a new environment. The extracellular matrix surrounding tumour cells could act as a barrier against such cell movement. As MMPs in combination can degrade all types of extracellular matrix proteins, they might be enzymes that are involved in removal of this barrier and that help tumour cells to pass through the barrier. In the cases of intravasation at a primary site and extravasation at a secondary site, tumour cells have to degrade the basement membrane. From this viewpoint, Liotta *et al*. (*5*) proposed type IV collagenase activity as an important factor for tumour cells to acquire metastatic potential. Indeed, many tumour cells have been reported to express MMP-2 and/or MMP-9 that have type IV collagenase activity, and their expression is implicated in the malignant phenotypes of tumour cells such as growth, invasiveness, and metastatic potential (*6*, *7*).

Secreted latent proMMP needs to be timely activated at an appropriate site for the enzyme to function. Many proteolytic enzymes can activate MMPs *in vitro*. However, the *in vivo* mechanism for activation of MMPs is not yet fully understood. In the case of MMP-2, a mechanism for activation by MT1-MMP has been proposed in which the MT1-MMP expressed on the cell surface forms a complex with a tissue inhibitor of

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metalloproteinases-2 (TIMP-2) and proMMP-2, and TIMP-2-free MT1-MMP activates proMMP-2 anchored in the MT1-MMP–TIMP-2–proMMP-2 complex. In this mechanism, MMP-2 is expected to be concentrated and activated where it is needed, for example, at the moving front of invasive tumour cells (*8*, *9*).

Although MMP-9 has been reported to be expressed by various tumour cells, and is supposed to be related to the invasive or metastatic capacity of tumour cells (*10*, *11*), it is not yet clear where and by what enzyme secreted proMMP-9 is activated *in vivo*, and how these mechanisms are correlated with the malignant behaviour of tumour cells. Secreted MMP-1, MMP-2, MMP-7, MMP-9 and MMP-13 have been reported to associate with the cell surface. By being anchored on the cell surface, the concentration and regulation of activation of these MMPs are possible at special sites on plasma membranes. As to MMP-9, its association with the cell surface and roles in invasion, metastasis and angiogenesis have been reported (*12*). However, the mode of anchoring of MMP-9 on the cell surface is only poorly understood.

In this study, we intended to clarify the mechanism underlying the function of MMP-9 in the invasion and metastasis of tumour cells, using highly metastatic LuM1 and low metastatic NM11 clones, which were established from murine colon adenocarcinoma, colon 26, cells on the basis of their spontaneous metastatic potentials (*13*, *14*). For this purpose, the cell surface association and localization of MMP-9, and its roles in cell migration and invasion were examined. The results obtained indicate that the heparan sulphate chains of glypican-like proteoglycans interact with MMP-9 on the cell surface, and that this cell surface association of MMP-9 is crucial for the migration and invasion of the cells *in vitro*.

MATERIALS AND METHODS

Enzymes and Antibodies—Heparitinase-I (EC 4.2.2.8) and chondroitinase ABC (EC 4.2.2.4) were purchased from Seikagaku Corp. (Tokyo). Phosphatidylinositol-specific phospholipase C (PI-PLC, EC 3.1.4.10) was a product of Funakoshi Corp. (Tokyo). Goat anti-mouse MMP-9 antibodies were purchased from R&D Systems Inc. (Minneapolis) and other anti-mouse MMP-9 antibodies (C-20) were from Santa Cruz Biotechnology (Santa Cruz). Mouse monoclonal antibodies, F58-10E4 specific to heparan sulphates and F69-3G10 specific to the unsaturated sugar moieties generated on heparitinase digestion, were from Seikagaku Corp. and rat monoclonal anti-mouse CD44 (IM7) were from BD Bioscience (San Jose). FITC-labelled anti-goat IgG, FITC-labelled anti-mouse IgG, Alexa488-labelled anti-goat IgG, Alexa594-labelled anti-mouse IgG and horseradish peroxidase-conjugated rabbit anti-mouse and goat anti-rat IgG were from Invitrogen (Carlsbad).

Cells—LuM1 and NM11 clones with high and low metastatic potentials, respectively, which were selected from murine colon adenocarcinoma, colon 26, cells on the basis of spontaneous metastatic potentials (*13*, *14*), were maintained in RPMI 1640 containing 10% foetal calf serum (FCS) (Invitrogen), streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37°C under a humidified 5% CO_2 atmosphere as described previously (*14*).

Metastasis Assay and Tumour Tissue Histology—LuM1 or NM11 cells (2×10^5) were transplanted subcutaneously into the abdominal flanks of 6-week-old female syngeneic BALB/c mice (Japan SLC Inc., Shizuoka). After 25 days, the animals were sacrificed and lungs were fixed with Bouin's solution, and the number of visible metastases was determined under a binocular. For histological examination, the tissues were fixed with 3.7% paraformaldehyde and embedded in paraffin. Thin sections were stained with haematoxylin–eosin. All animal experiments were performed according to the approved protocol following the guidelines for animal care at Kyoto Sangyo University.

RT–PCR—The expression of MMPs and TIMPs was detected by RT–PCR using a OneStep RT–PCR Kit (QIAGEN Inc., Valencia) and 1 μg poly(A)+RNA according to the manufacturer's instructions. The primers (Hokkaido System Science, Sapporo) used were 5′-ACCCTGGGAGAAGGACAAGT-3′ and 5′-CGATGCCA TCAAAGACAATG-3′ for MMP-2; 5′ACTACTCTGAAGAC TTGCCG-3′) and 5′-GGTACAGGAAGAGTACTGCT-3′ for MMP-9; 5′-GGGCCCAACATCTGTGAC-3′ and 5′-GCAG CCCATCCAGTCC-3′ for MT1-MMP; 5′-CGGAATTCATG ATGGCCCCCTTTGCATC-3′ and 5′-GAAGATCTTCGGG CCCCAAGGGATCTCC-3′ for TIMP-1; 5′-CGGAATTCAT GGGCGCCGCGGCCCGCAG-3′ and 5′-GAAGATCTCGG GTCCTCGATGTCAAGAA-3′ for TIMP-2; 5′-AACCTGCC GCTACGCAGGTGT-3′ and 5′-TCCATCGAAGGAATTGG GTAG-3′ for CD44 and 5′-GTGGGGCGCCCCAGGCAC CA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′ for β-actin. The PCR conditions were 27 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C and extension for 90 s at 72°C. The reaction product, separated by agarose gel electrophoresis and visualized with ethidium bromide, was quantified using public domain software ImageJ in the 256-grey scale mode.

Analysis of Gelatinases by Gelatin Zymography—Gelatinases (MMP-2 and MMP-9) in conditioned media or cell lysates of 1×10^4 cells were detected by zymography using 10% polyacrylamide gels with 1.0 mg/ml gelatin as a substrate (*15*). Cells were cultured for 48 h in RPMI 1640 without FCS to prepare conditioned media. To prepare cell lysates, 1×10^8 cells suspended in 10 ml of Tris-buffered saline (TBS; 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35) containing 1% Triton X-100 and proteinase inhibitors comprising 1 mM phenylmethylsulphonyl fluoride, 0.8 mM aprotinin, 0.015 mM E-64, 0.05 mM bestatin and 0.01 mM pepstatin A, were lysed by ultra-sonication for 15 s three times, and a supernatant was obtained by centrifugation at 10,000*g* for 30 min. Gelatinases in the supernatant were purified on a Gelatin–Sepharose column (1.5 × 2.5 cm) (GE Healthcare Bio-Science Corp., Piscataway). The eluate from the column with TBS containing 5% dimethyl sulphoxide was concentrated with a Centricon YM-30 (Millipore Corp., Billerica). Activation of proMMPs with 1 mM *p*-aminophenylmercuric acetate (APMA) (EMD Biosciences Inc., San Diego) was performed according to the method of Okada *et al.* (*16*).

Cell Migration Assay—Cells (3.5×10^5) suspended in PBS containing 0.2% bovine serum albumin (BSA) were inoculated onto 24-well plates coated with human fibronectin (IWAKI, Tokyo), and cultured at 37°C for 1 h, and then the cell layer was wounded with a micropipette tip. The wounded cell layers were cultured for 7 h in RPMI 1640 medium without FCS and then the cell migration distance was measured. To examine the effects of inhibitors, 20 μM MMP-2/MMP-9 inhibitor IV (EMD Chemicals Inc.) or 1 mg/ml heparin (Sigma–Aldrich, St Louis) was added to the culture medium.

In Vitro Invasion Assay—The invasive ability of tumour cells was analysed *in vitro* using transwells with an 8.0 μm pore polycarbonate membrane insert (Corning Inc., New York), the upper surface of which was coated with Matrigel (BD Biosciences, San Jose). Cells (5×10^4) were seeded on the upper wells, which were then set on the lower wells filled with PBS containing 50 μg/ml of fibronectin. After 7 h, cells on the reverse surface of the membrane were fixed with methanol and the stained with haematoxylin. The numbers of cells in five randomly selected fields were determined. An MMP inhibitor or heparin, when used, was added to the medium in both the upper and lower wells.

Flow Cytometry—Cells (3×10^5) incubated with or without heparitinase-I (0.1 U/ml) in the presence of the proteinase inhibitors for 30 min at 37°C (*17*), were gently shaken with C-20, F58-10E4 or F69-3G10 in PBS containing 0.2% BSA and 2 mM EDTA for 1 h at 4°C, and then, treated with appropriate fluorescence-labelled second antibodies for 30 min at 4°C in the dark. The intensity of fluorescence was measured with a flow cytometer, FACSort (BD Biosciences).

Immunocytochemical Staining—Cells cultured for 7 h on a cover glass coated with fibronectin were fixed with 3.7% paraformaldehyde, and then treated with C-20 and/or F58-10E4 in PBS containing 0.2% BSA and 10 μM phosphoramidon (Sigma–Aldrich) for 1 h at room temperature. After washing three times with PBS, specimens were treated with Alexa488 and/or Alexa594-labelled second antibodies for 30 min in the dark and then counterstained with 300 nM DAPI (Invitrogen).

Binding Ability of MMP-9 to Heparin—Conditioned medium derived from a culture of LuM1 cells (1×10^7 cells) was applied to a column (1×7.6 cm) of Heparin–Sepharose CL-6B (GE Healthcare Bio-Science Corp.) equilibrated with 10 mM phosphate buffer (pH 7.3). After washing the column with the buffer, bound materials were eluted with a linear concentration gradient formed by 15 ml each of 0 M and 1 M NaCl in the same buffer. Fractions of 1 ml were collected. Aliquots of eluted fractions were subjected to gelatin zymography, and the profile of elution of MMP-9 from the column was densitometrically monitored.

Preparation of Cellular Proteoglycans and Analysis of their Core Proteins—A cellular proteoglycan fraction containing syndecans and glypicans was prepared from LuM1 cells in the presence of proteinase inhibitors as described previously (*17*). To prepare core proteins of proteoglycans, proteoglycan samples were digested by heparitinase-I and chondroitinase ABC in the presence of proteinase inhibitors as described previously (*18*). To analyse core proteins, the digests were subjected to SDS–PAGE, transferred to Hybond-P membranes (GE Healthcare Bio-Science Corp.), and then processed for immunostaining with F69-3G10, followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG. Staining was visualized with ECL-plus (GE Healthcare Bio-Science Corp.).

Co-immunoprecipitation of Proteoglycans with MMP-9—LuM1 cells (1×10^8) were solubilized with 10 ml of TBS containing 1.0% Triton X-100 and the proteinase inhibitors described above with stirring overnight on ice. The supernatant obtained by centrifugation at 10,000*g* for 10 min was incubated with anti-mouse MMP-9 goat antibodies or with non-immune goat IgG overnight with gently shaking, and then with Protein G–Sepharose (GE Healthcare Bio-Science Corp.) for 4 h at 4°C. The immunocomplex bound to Protein G was eluted with 100 mM glycine–HCl (pH 3.0) containing 0.5% Triton X-100. By gelatin zymography, it was confirmed that almost all of the MMP-9 in the supernatant was recovered in the eluate. Core proteins of heparan sulphate proteoglycans co-precipitated with MMP-9 were prepared by digestion of the eluate with heparitinase-I and chondroitinase ABC, and analysed by SDS–PAGE, followed by western blotting with F69-3G10 antibodies.

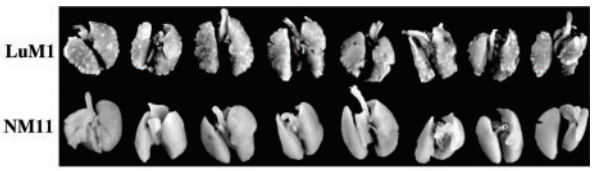
Statistical Analysis—The statistical significance of differences was determined by means of the *t*-test.

RESULTS

Metastatic and Invasive Properties In Vivo of Colon 26-Derived Clones—LuM1 cells cloned from murine colon adenocarcinoma, colon 26, cells formed metastatic nodules in the lungs when the cells were transplanted subcutaneously into the abdominal flanks of syngeneic BALB/c mice (Fig. 1A). The frequency of metastases was very high, and all of the transplanted mice developed metastases (Fig. 1B). In contrast, NM11 cells hardly metastasized (Fig. 1A and B). As shown in Fig. 1C, microscopic examination of the primary tumours revealed that highly metastatic LuM1 cells had actively invaded into the muscle tissues facing the tumour tissues (Fig. 1C, a and c). In contrast, in the case of the NM11 cells, no such invasion was observed (Fig. 1C, b and d). Intravasation of the tumour cells was often observed in LuM1 tumour tissues, but not in NM11 ones, as previously reported (*14*). Thus, as compared with NM11 cells, LuM1 cells proved to be metastatic cells with a highly invasive property.

Expression of MMPs and TIMPs in LuM1 and NM11 Clones—To analyse the metastatic and invasive properties, the expression of gelatinases (MMP-2 and MMP-9), MT1-MMP, TIMP-1 and -2 was compared between the two clones. The expression of MMP-2, MT1-MMP and TIMP-2 was very similar in the two clones as to the mRNA levels detected on RT–PCR (Fig. 2A). However, as to MMP-9, high expression was observed in LuM1 cells and negligibly low expression in NM11 cells. TIMP-1 was expressed more highly in NM11 cells than in LuM1 ones. Gelatin zymography showed that bands corresponding to proMMP-2 (72 kDa) and active MMP-2 (62 kDa) were

A



B

Cell line	Lung metastasis		
	Incidence	Number of foci	
		Range	Mean
LuM1	8/8	72-103	85
NM11	1/8	0-2	0

C

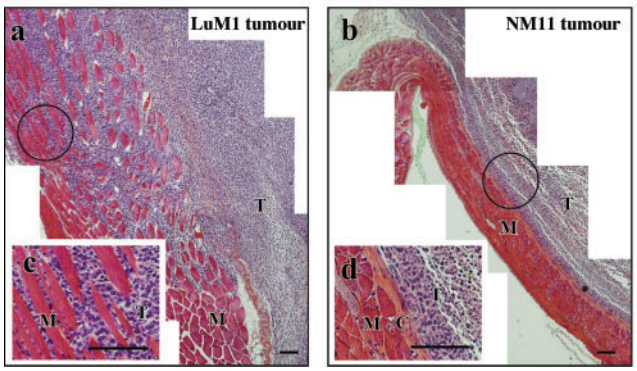


Fig. 1. **Metastatic and invasive properties of LuM1 and NM11 cells.** Cells were transplanted subcutaneously into the abdominal flanks of mice, and after 25 days, lungs and primary tumours were dissected out. (A) Lungs were fixed with Bouin's solution and (B) visible nodules were counted. (C) Primary tumours were fixed and thin sections were stained with haematoxylin/eosin (a and b). Circles show boundary areas between tumour and adjacent muscle tissues, and inserts are enlargements of the circled areas (c and d). T, tumour; M, muscle; C, connective tissue. Bars, 100 μm.

detected for both the media and cell lysates of LuM1 and NM11 cells (Fig. 2B), the densities of the bands being similar for the two clones. On the contrary, as was expected from the RT–PCR results, MMP-9 was highly detected in the conditioned medium and also in the cell lysate of LuM1 cells but not significantly those of NM11 cells (Fig. 2B). Both the pro- and active-forms, 95 and 82 kDa, respectively, of MMP-9 were detected in the medium and also the cell lysate of LuM1 cells, and molecular attribution of the latter form was ascertained from its involvement in the chemical activation of proMMP-9 by APMA. An intermediate form (86 kDa) was significantly detected in the LuM1 cell lysate (Fig. 2B, right panel). On treatment of the cell lysate with APMA, 75 kDa fragment appeared in addition to the active 82 kDa form, as shown previously by others (*19*). Both the bands were reactive with the anti-MMP-9 antibodies used (data not shown). Interestingly, the ratio of the intensities of the active plus intermediate bands, particularly of the intermediate band, to that of the proform band was much greater for the cell lysates than for the media, suggesting the anchorage of the active and intermediate forms on the cell surface. These results suggest that MMP-9 could have some roles in the invasive and metastatic properties of LuM1 cells.

MMP-9-Mediated In Vitro Migration and Invasion of LuM1 Cells—As an *in vitro* model of the invasiveness of tumour cells, we took advantage of the ability of cell migration on fibronectin or invasion through Matrigel (reconstituted basement membrane). The wound assay showed that LuM1 cells exhibited greater migration ability on fibronectin-coated dishes as compared to NM11 cells, and LuM1 cells migrated about five times faster than NM11 cells (Fig. 3A, left panels). MMP-2/MMP-9 Inhibitor IV suppressed LuM1 cell migration to the level in the case of NM11 cells (Fig. 3A, right panel). LuM1 cells also exhibited a higher ability to invade through Matrigel. That is, the number of LuM1 cells crossing a membrane coated with Matrigel was 30 times greater than that of NM11 cells (Fig. 3B, left panel). This invasion of LuM1 cells was also suppressed by the MMP inhibitor to the level in the case of NM11 cells (Fig. 3B, right panel). As the expression of MMP-2, TIMP-2 and MT1-MMP was similar in the two clones, suppression of the two types of cell motility by the MMP inhibitor can be mainly attributed to its effect on MMP-9 activity. This means that MMP-9 is required for the *in vitro* cell migration and invasion of LuM1 cells, and also suggests that MMP-9 functions in the vicinity of cells. Therefore, we next examined the cell surface association of MMP-9.

Localization of MMP-9 on the Surface of LuM1 Cells—The association of MMP-9 on the surface of LuM1 cells but not of NM11 cells, was proved by flow cytometry (Fig. 4A) and confirmed by immunocytochemistry (Fig. 4B) with anti-MMP-9 antibodies. Local condensation of the staining was observed in adherent LuM1 cells, but NM11 cells were not stained at all (Fig. 4B). As the cells were not permeabilized on fixation, the localization of MMP-9 could be supposed to be on the cell surface.

Association of MMP-9 with Cell Surface Heparan Sulphates, and Its Participation in Cell Migration and Invasion In Vitro—To identify the MMP-9-associating component(s) on the cell surface, the possibility of cell surface heparan sulphates was examined. This idea came from our previous study demonstrating that heparin derivatives widely suppressed experimental metastasis of tumour cells including LuM1 clone (*20*). First of all, the binding activity of MMP-9 to heparin was examined using Heparin–Sepharose CL-6B as an alternative receptor (Fig. 5). Zymography of small aliquot of each fraction showed that both pro- and active-MMP-9 bound to heparin (Fig. 5A), and were eluted with relatively high concentrations of NaCl. Interestingly, the active form was eluted with a higher concentration of NaCl (0.6 M) as compared with in the case of proMMP-9 (0.4 M) (Fig. 5B), suggesting that the binding affinity to heparin of the active form was stronger than that of the proform. We next examined whether MMP-9 actually bound to cell surface through heparan sulphates or not. For this purpose, LuM1 cells were treated with heparitinase-I, and then the fate of MMP-9 on the cell surface was followed. The results of flow cytometrical analyses showed that F58-10E4 antibodies specific to heparan sulphates strongly reacted with LuM1 cells, and this reactivity was abolished on digestion with heparitinase-I

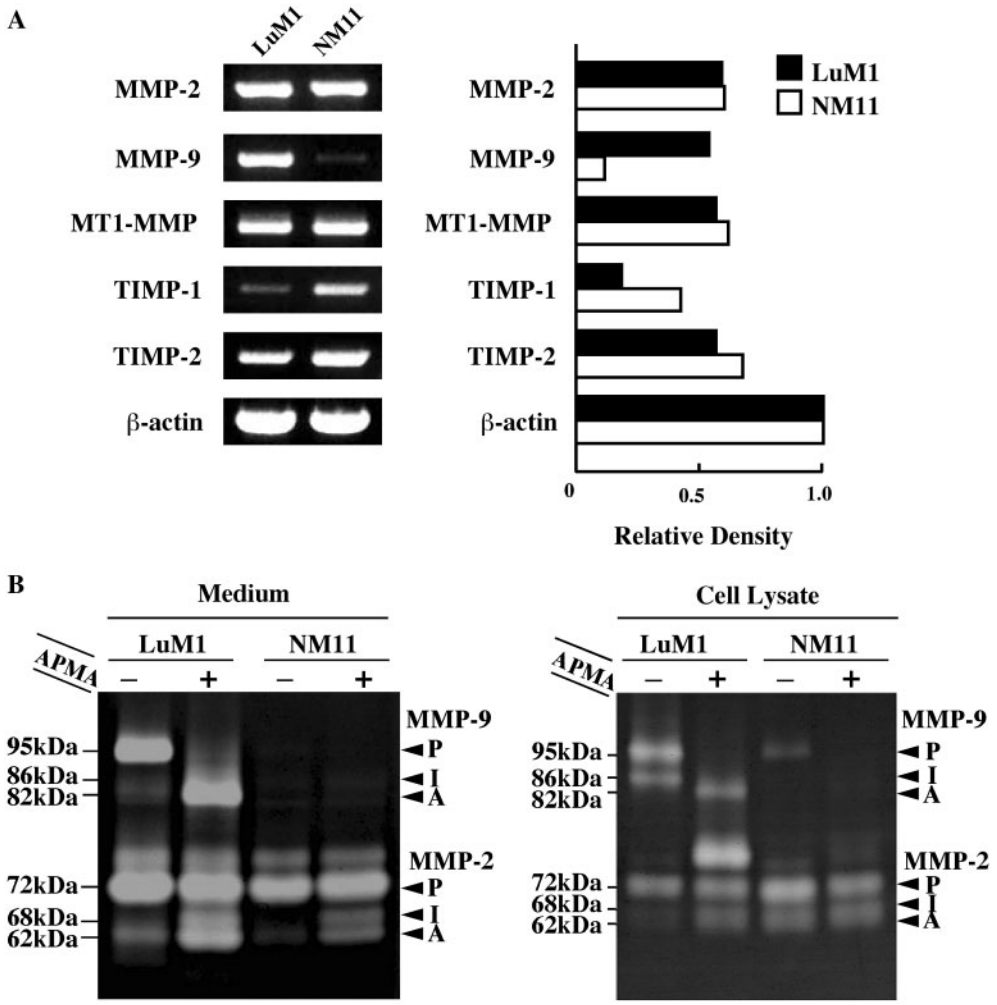


Fig. 2. **Expression and production of MMPs and TIMPs in LuM1 and NM11 cells.** (A) Expression of mRNAs of MMP-2, MMP-9, MT1-MMP, TIMP-1, TIMP-2 and β-actin in LuM1 and NM11 cells was analysed by RT–PCR (left panel). The relative densities of the bands were quantified with Image J (right panel). (B) Gelatin zymography of conditioned media and cell lysates of LuM1 and NM11 cells. Samples were treated with (+) or without (−) APMA before electrophoresis. P, proform; I, intermediate form; A, active form.

(Fig. 6A, left panel), indicating that heparan sulphates were present on the LuM1 cell surface. This was confirmed by the fact that the antigenic epitope for F69-3G10 antibodies, which recognize unsaturated sugar moieties of heparan sulphate, generated by heparitinase-I digestion of the cells (data not shown). Concomitantly with the removal of heparan sulphates from the cell surface by this enzyme treatment, the reactivity of the cells with anti-MMP-9 antibodies was almost completely abolished (Fig. 6A, right panel), suggesting that MMP-9 is associated with heparan sulphate chains on the cell surface. This was supported by the finding that exogenously added heparin caused the release of MMP-9 from a LuM1 cell layer and the cell surfaces (Fig. 6B), that is, heparin acted competitively against the cell surface heparan sulphates. The migration on fibronectin (Fig. 6C, left panel) and also invasion through Matrigel (Fig. 6C, right panel) of LuM1 cells were greatly inhibited by exogenously added heparin, suggesting that functional MMP-9 is tethered by heparan sulphates to the cell surface. In relation to regulation of MMPs by heparin, there is a report demonstrating that heparin inhibits the induction of several MMPs including MMP-9 (*21*). However, under the condition adopted in this study, addition of heparin to LuM1 cells did not affect an expression of MMP-9 in the cells at all (data not shown). Furthermore, it was confirmed that cell proliferation did not contribute to this analysis because in the presence of aphidicolin, an inhibitor of DNA synthesis, exogenously added heparin also suppressed migration of LuM1 cells (data not shown). Double immunostaining of adherent LuM1 cells on a fibronectin substratum revealed that MMP-9 concentrated at the leading edges of migrating cells with heparan sulphate (Fig. 7A and C), though heparan sulphates existed all over the cell surface (Fig. 7B). These results strongly suggested that MMP-9 is co-localized with particular heparan sulphate proteoglycan(s) on LuM1 cell surface.

Identification of Cell Surface Proteoglycans Associating with MMP-9—In order to characterize the heparan

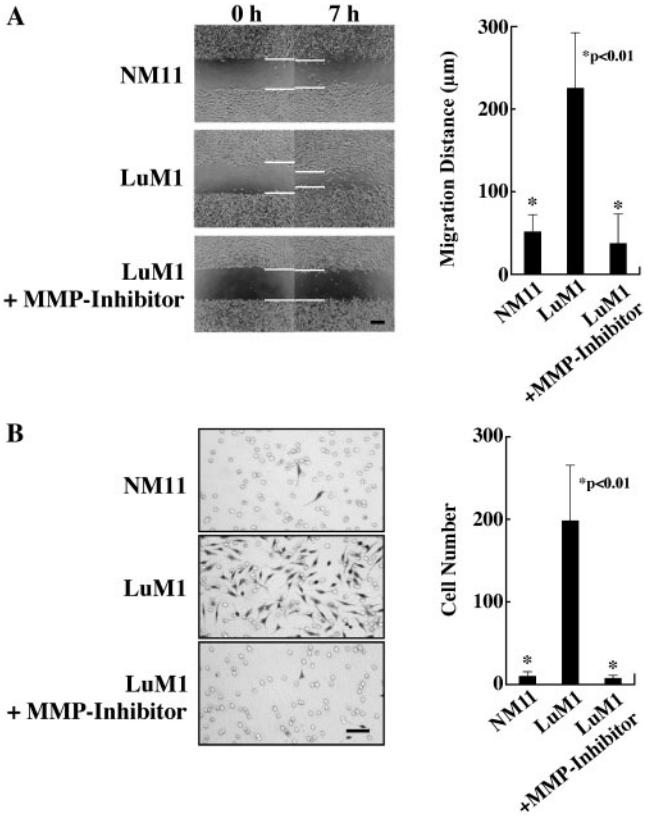


Fig. 3. ***In vitro* migration and invasion of LuM1 and NM11 cells.** (A) Cell migration was determined by the wound assay. Cells inoculated on a fibronectin substratum were incubated at 37°C for 1 h. The cell layer was wounded with a micropipette tip (0 h) and incubated for 7 h in the presence or absence of 20 μM MMP-2/MMP-9 inhibitor IV (left panel). The right panel shows the mean migration distance for 7 h after the wounding, calculated from the width of five randomly chosen cell-free areas. (B) Cell invasion was determined using a transwell chamber with an 8 μm pore membrane of which the upper surface was coated with Matrigel. At 7 h after the incubation of cells seeded on the upper well, the membrane was fixed, and cells on the reverse surface of the membrane were stained with haematoxylin (left panel). The right panel shows the number of cells per five randomly selected fields. Bars, 100 μm.

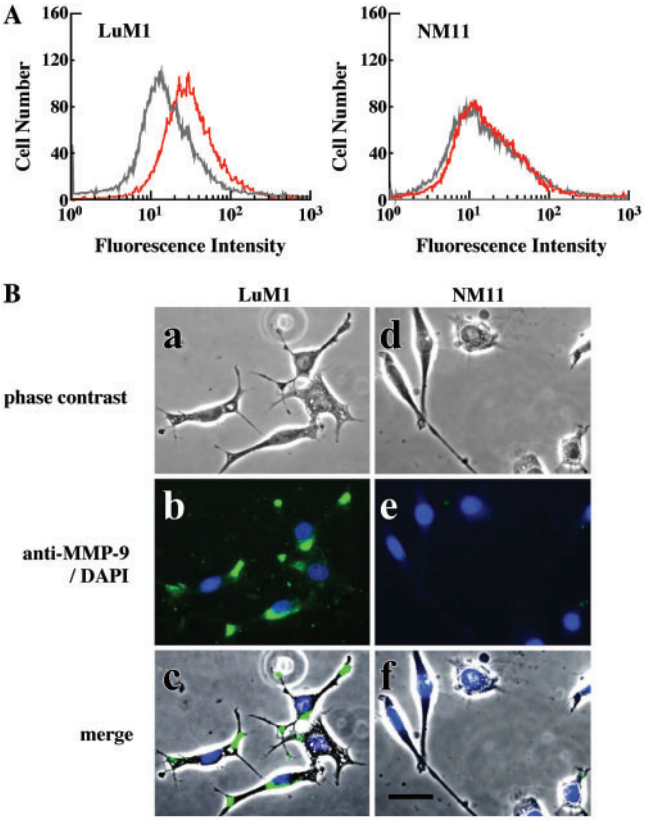


Fig. 4. **Cell surface localization of MMP-9.** (A) Flow cytometrical analysis of MMP-9 on LuM1 and NM11 cells with anti-MMP-9 antibodies (red line) and non-immune IgG (grey line). (B) Immunostaining of adherent LuM1 and NM11 cells on a fibronectin substratum with anti-MMP-9 antibodies (green) and DAPI (blue) for nuclei (b and e). The panels show phase contrast image (a and d) and the merged phase-contrast and immunostaining images (c and f). Bar, 25 μm.

sulphate proteoglycan(s) associating with MMP-9, first, heparan sulphate proteoglycans expressed by LuM1 cells were analysed. The proteoglycan fraction obtained by anionic exchange chromatography of a LuM1 cell extract was treated with or without heparitinase-I and chondroitinase ABC in the presence of proteinase inhibitors and then subjected to SDS–PAGE, followed by western blotting with F69-3G10 antibodies (Fig. 8A). Because the antibodies recognize unsaturated sugar moieties generated on heparitinase-I digestion, the non-digested sample did not contain any materials reacting with the antibodies (Fig. 8A, left lane). On the other hand, in the enzyme-treated sample, five core proteins of heparan sulphate proteoglycans with molecular sizes of 85, 66, 64, 44 and 30 kDa were detected (Fig. 8A, right lane). Being consistent with these results, RT–PCR analyses of expressions of the cell surface heparan sulphate proteoglycans showed that LuM1 cells expressed five cell

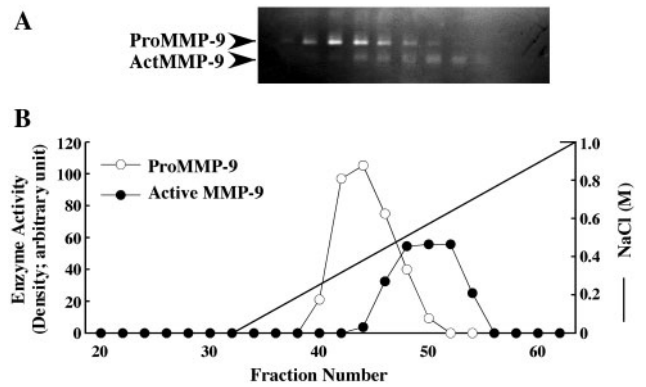


Fig. 5. **Binding ability of MMP-9 to heparin.** The conditioned medium of LuM1 cells was applied to a Heparin–Sepharose CL-6B column equilibrated with 10 mM phosphate buffer (pH 7.3). After washing the column with the buffer, bound materials were eluted with a linear concentration gradient, 0–1.0 M, of NaCl in the same buffer. (A) An aliquot of each fraction eluted was analysed by gelatin zymography. (B) The densities of bands of proform (ProMMP-9) and active form (Active MMP-9) of each fraction were quantified using the ImageJ program and the individual densities were plotted for elution patterns.

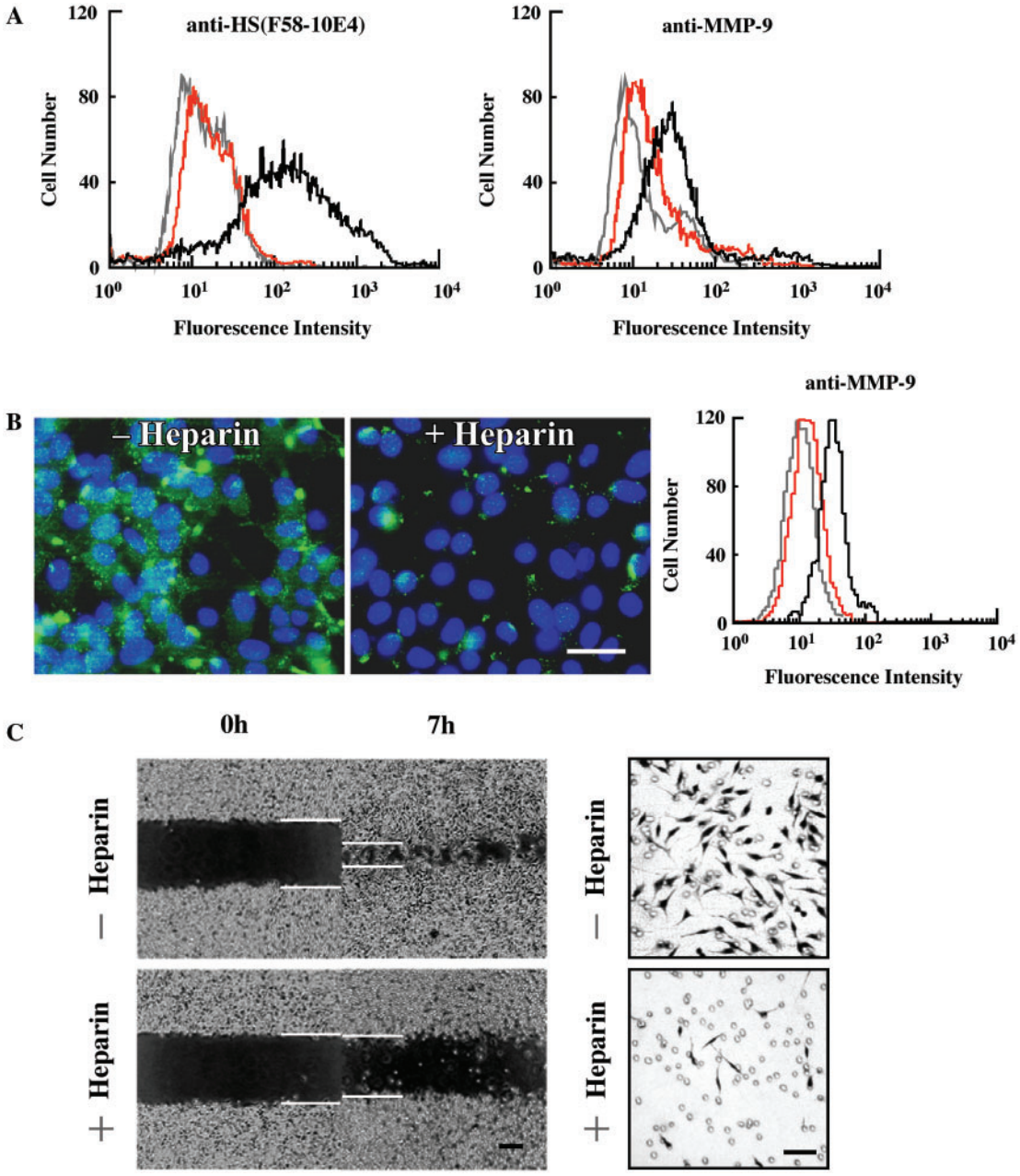


Fig. 6. **Involvement of heparan sulphate in cell surface localization of MMP-9 and its effect on cell motility.** (A) Flow cytometrical analysis of LuM1 cells which were treated with (red line) or without (black line) heparitinase-I. Heparitinase-I digestion removed the cell surface heparan sulphate from the cells [left panel, using anti-heparan sulphate (HS) antibodies, F58-10E4] and also removed MMP-9 (right panel, using anti-MMP-9 antibodies). The grey lines show the controls using non-immune IgG. (B) Immunostaining of adherent LuM1 cells on a fibronectin substratum with anti-MMP-9 antibodies (green) and DAPI (blue) in the presence (middle panel) or absence (left panel) of heparin. Bar, 25 μm. The right panel shows flow cytometrical analysis of the cells which were treated with (red line) or without (black line) heparin (1 mg/ml) and stained with anti-MMP-9. The grey line shows the control using non-immune IgG (A). (C) Effect of exogenously added heparin on *in vitro* migration (left panel) and invasion (right panel) of LuM1 cells. The experiments were performed as described in Fig. 3, in the absence (–) or presence (+) of heparin. Bars, 100 μm.

surface heparan sulphate proteoglycans, that is, three transmembrane type proteoglycans (syndecan-1, -2 and -4) and two GPI-anchored proteoglycans (glypican-1 and -6) (data not shown). Among them, only the 66 and 64 kDa core proteins were detected in the immunoprecipitate derived from LuM1 cell lysate with anti-MMP-9 antibodies (Fig. 8B), indicating that the heparan sulphate proteoglycans having the core proteins of these two molecular sizes were co-precipitated with MMP-9. Interestingly, these heparan sulphate proteoglycans were released from the cell surface by PI-PLC digestion (Fig. 8C), indicating that these proteoglycans were

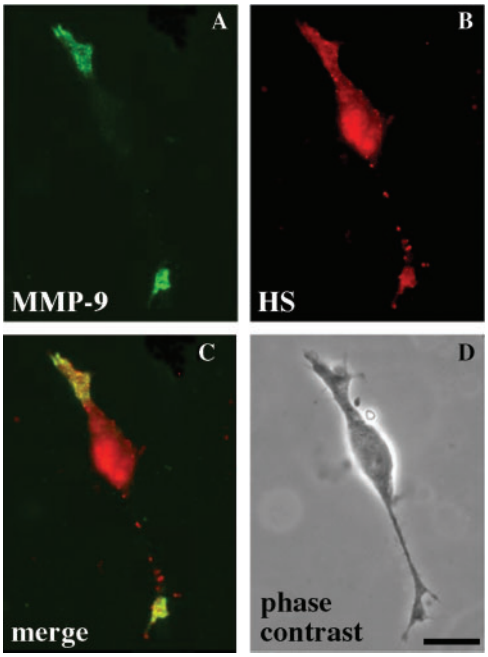


Fig. 7. **Co-localization of MMP-9 with heparan sulphate on the LuM1 cell surface.** Double immunostaining of LuM1 cells on a fibronectin substratum with anti-MMP-9 (A) and anti-heparan sulphate antibodies, F58-10E4 (B). MMP-9 co-localized with a particular heparan sulphate on the leading edges of migrating cells (C). (D) shows a phase-contrast image of the cell. Bar, 25 μm.

anchored to the cell surface through phosphatidylinositol linkages. These results strongly suggest that glypican-like heparan sulphate proteoglycans tether MMP-9 to the surface of LuM1 cells through their heparan sulphate chains. Two faint bands corresponding to 66, and 64 kDa core proteins were observed without treatment of the cells by PI-PLC (Fig. 8C), suggesting that these molecules might metabolically shed from the cells during the incubation period.

Because there have been several reports which showed co-localization of MMP-9 with CD44 (*22*, *23*), and CD44 is so-called part-time proteoglycan which occasionally has chondroitin sulphate or heparan sulphate and has a multitude of isoforms with diverse functions (*24*), an involvement of CD44 in the present phenomena was examined. RT–PCR analysis showed that LuM1 cells expressed CD44 (data not shown). We therefore examined the molecular characteristics of CD44 produced by the cells, and a possibility of its binding to MMP-9. Western blot analysis with anti-CD44 antibodies before and after digestion with heparitinase-I and/or chondroitinase ABC of the LuM1 cell lysate showed that all the samples gave a antibody-reactive band at the position of 75 kDa regardless of the enzyme treatments (Fig. 8D), indicating that CD44 produced by LuM1 cells has 75 kDa molecular size and has neither heparan sulphate nor chondroitin sulphate. Therefore, CD44 of LuM1-cells could not be involved in localization of MMP-9 on the cell surface through heparan sulphate. This was confirmed by immunocoprecipitation analysis using anti-MMP-9 antibodies. CD44 did not precipitate with anti-MMP-9 antibodies (Fig. 8E, IP) under the condition that MMP-9 was completely recovered in the immunoprecipitates (Fig. 8E, zymography). These results consistently indicate that the 75 kDa CD44 molecule has no heparan sulphate and chondroitin sulphate chains, and has no ability to bind to MMP-9 in the case of LuM1 cells.

DISCUSSION

In the present study, we attempted to clarify the molecular background of the malignant behaviour of tumour cells by using colon adenocarcinoma-derived LuM1 and NM11 clones with high and low metastatic potentials, respectively, which were established previously on the basis of their spontaneous metastatic potential (*13*, *14*). When these clones were transplanted subcutaneously to obtain primary tumours, the LuM1 clone exhibited a great ability to invade the muscle tissues adjacent to tumour tissues. On the contrary, the NM11 clone did not show such invasion at all. Being consistent with these *in vivo* properties, LuM1 cells exhibited much higher abilities to migrate on a fibronectin substratum and also to invade through Matrigel than NM11 cells. In order to determine the molecular backgrounds underlying these cell behaviours, the expression and activities of gelatinases and related molecules were compared between these two clones. The results showed that in spite of the similar expression levels of the mRNAs of MMP-2, MT1-MMP and TIMP-2 in the two clones, the expression of MMP-9 was much higher and that of TIMP-1 was relatively lower in LuM1 than NM11 cells. These results strongly suggest that MMP-9 could have a crucial role(s) in the malignant behaviour of LuM1 cells, such as invasion and metastasis. Indeed, both the *in vitro* cell migration on a fibronectin substratum and invasion through Matrigel of LuM1 cells were suppressed by an MMP inhibitor to the levels in the case of NM11 cells.

The results described earlier in regard to the correlation between the expression of MMP-9 and the cell motilities are well consistent with those obtained by others using various systems such as *in vitro* invasion of human microvascular endothelial cells (*25*, *26*), and migration of mouse myoblasts (*27*), keratinocytes (*28*, *29*) airway epithelial cells (*30*) and tumour cells (*31–36*). In these cases, augmentation or inhibition of MMP-9 expression results consistently in the increase or decrease, respectively, of the cell motilities. Many of these studies have been concerned with cellular phenomena in association with expression of MMP-9, and few have studied the functional mechanism and/or microlocalization of MMP-9. In order to elucidate the mechanism underlying the participation of MMP-9 in cell migration and invasion of LuM1 cells, we postulated that MMP-9 would be localized on the surface of LuM1 cells and be activated there. The results obtained showed that MMP-9 is associated with cell surface heparan sulphate chains and that this association is critical for the migration and invasion of LuM1 cells.

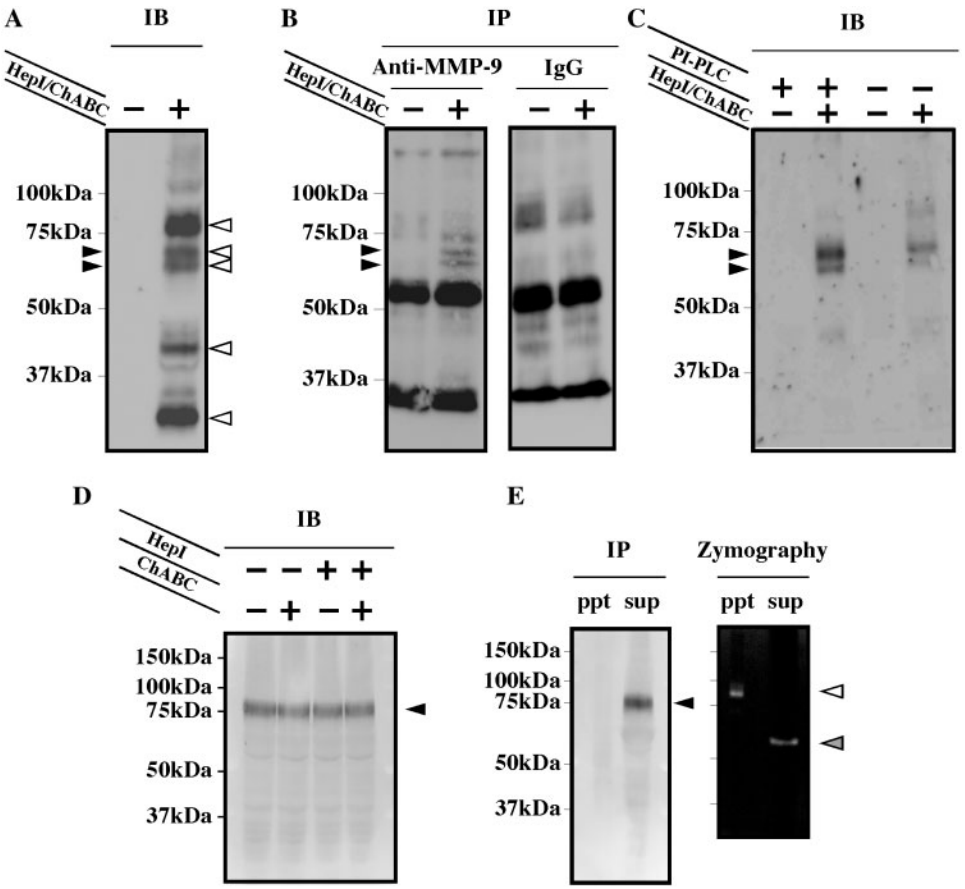


Fig. 8. **Characterization of heparan sulphate proteoglycans tethering MMP-9 to the surface of LuM1 cells.** (A) Immunoblotting (IB) of heparan sulphate proteoglycans extracted from LuM1 cells. Proteoglycans were treated with (+) or without (−) heparitinase-I (HepI) and chondroitinase ABC (ChABC) in the presence of the proteinase inhibitors, and then subjected to SDS–PAGE, followed by immunoblotting with F69-3G10 antibodies. Five core proteins of heparan sulphate proteoglycans were detected after the enzyme-digestion (white arrowheads). Black arrowheads indicate the positions of 66 and 64 kDa core proteins. (B) Immunoprecipitation (IP) of the supernatant of LuM1 cell lysate with anti-MMP-9 antibodies or with non-immune IgG was performed. The precipitates were treated with or without HepI and ChABC, and then subjected to SDS–PAGE, followed by immunoblotting with F69-3G10. Heparan sulphate proteoglycans having the 66 and 64 kDa core proteins (black arrowheads) were co-precipitated with MMP-9. The faint band at a position of 75 kDa and the intense ones at 55 and 35 kDa were deduced to be derived from the goat immunoglobulin samples used in the immunoprecipitation as they were detected in the controls. (C) Immunoblotting of GPI-anchored heparan sulphate proteoglycans of LuM1 cells. The supernatants of LuM1 cells treated with or without PI-PLC were further treated with or without HepI and ChABC, and then subjected to SDS–PAGE, followed by immunoblotting with F69-3G10. Large amounts of the 66 and 64 kDa core proteins of heparan sulphate proteoglycans were contained in the supernatant obtained by treatment of LuM1 cells with PI-PLC. (D) Immunoblotting of CD44 of LuM1 cells. The supernatants of the LuM1 cell lysate were treated with or without HepI or ChABC or both, and subjected to SDS–PAGE, followed by immunoblotting with anti-CD44 antibodies. (E) Immunoprecipitation of the supernatant of LuM1 cell lysate with anti-MMP-9 antibodies was performed under the same condition as described under (B). The precipitates and supernatants were subjected to western blot (left panel) and gelatin zymography (right panel). CD44 (black arrowhead) was recovered in the supernatant whereas almost all of the activity of MMP-9 (white arrowhead) was recovered in the precipitates. Under this condition, MMP-2 (grey arrowhead) contained in the cell lysate was properly recovered in the supernatant.

Interestingly, regarding to MMP-9 contained in the LuM1 cell lysate, the ratio of the active and intermediate forms to the proform was significantly higher than that in the conditioned medium. In particular, a significantly larger amount of the 86 kDa intermediate form was detected in the cell lysate. These findings strongly suggest the possibility that MMP-9 binds to the cell surface and is activated there. Actually, the presence of MMP-9 on the LuM1 cell surface was proved by flow cytometry. Moreover, immunostaining of the cells revealed that MMP-9 was concentrated on the surface of podia of the moving LuM1 cells, leading us to consider that the enzyme participates in cell migration and invasion *in vitro*.

The processes of cell migration have been proposed to comprise the following steps: (i) expansion of lamellipodia, (ii) adhesion to the extracellular matrix at the migration front and (iii) detachment from the matrix at the rear portion (*37*, *38*). At the edges of migrating cells, extracellular matrix proteins could be reorganized to permit the cells to pass through the matrix. Thus, the localization and concentration of MMP-9 on the leading

edges of moving LuM1 cells are supposed to be of great significance as to the migration and invasion of the cells.

In order to determine the molecule(s) tethering MMP-9 to the cell surface, we postulated a cell surface heparan sulphate as a candidate. This idea came from our previous finding that the administration of heparin or heparin derivatives to mice suppressed the experimental lung metastasis of several tumour clones such as lung carcinomas, colon carcinomas, melanomas and fibrosarcomas (*20*). There have been many reports showing suppression of tumour metastases by heparinoids, which is supposed to be due to inhibition of heparanase, an enzyme degrading heparan sulphates, which are one of the major constituents of basement membranes with a barrier property against tumour cell invasion (*39–44*). However, in our recent studies, no correlation was observed between metastatic potential, and the expression and activity of heparanase in some of the cell lines examined earlier. For example, Lewis lung carcinoma cells did not express heparanase at all regardless of the differences in their metastatic potentials. In addition, both the LuM1 and NM11 cells used in this study expressed large amounts of heparanase at similar levels, but NM11 cells exhibited a low metastatic potential (Yoshitomi, Y. *et al.*, unpublished data). That is, despite that there is no correlation between the expression of heparanase and both the invasive and metastatic potentials in these clones, heparin suppressed their metastases to similar levels, as shown for highly metastatic B16F10 melanoma cells (*20*). Thus, the suppressive effects of heparin or heparin derivatives on metastases of these tumour cells have to be attributed to other function(s) than inhibition of heparanase.

Since cell surface heparan sulphates have been shown to be receptors for extracellular heparin-binding molecules, such as matrix components, growth factors and cytokines (*45–48*), the inhibitory effects of heparin or heparin derivatives on tumour metastases may be related to interference with the receptor functions of the cell surface heparan sulphates. Supporting this idea, we demonstrated in this study that both the latent and active forms of MMP-9 showed binding affinity to heparin, suggesting that the cell surface heparan sulphates function as a receptor(s) for MMP-9. Interestingly, the active form exhibited stronger interaction with heparin than the latent form, suggesting that the activation of proMMP-9 is associated with a conformational change that is favourable for its concentration on particular cell surface heparan sulphate chains. MMP-7 (matrilysin) has also been reported to be anchored to heparan sulphates of proteoglycans on the cell surface and/or perlecan in the basement membranes in uterine glandular epithelial tissue (*49*).

The present results obtained by double immunostaining with anti-heparan sulphate and anti-MMP-9 antibodies clearly showed that heparan sulphates existed diffusely all over the surface of LuM1 cells, but that only the heparan sulphates on tips of cell podia were co-localized with MMP-9. Treatment of the cells with heparitinase-I or exogenous addition of heparin to the cells resulted in the release of MMP-9 from the cell surface, indicating that MMP-9 is associated with the cell surface through particular heparan sulphate chains. Concomitantly with this release of the enzyme from the cell surface, the abilities of migration and invasion of LuM1 cells were almost completely abolished, indicating the importance of cellular association of MMP-9 for such cell motility.

Immunoprecipitation of the LuM1 cell lysate with anti-MMP-9 antibodies caused the co-precipitation of glypican-like proteoglycans having 66 and 64 kDa core proteins with MMP-9. Western blot analysis with F69-3G10 antibodies showed that LuM1 cells produced five molecular species of cell surface heparan sulphate proteoglycans probably including glypicans and syndecans. It was confirmed by RT–PCR analysis that the cells expressed syndecan-1, -2, -4 and glypican-1 and -6 (data not shown). It is noteworthy that although LuM1 cells express at least five species of cell surface heparan sulphate proteoglycans, only the GPI-anchored heparan sulphate proteoglycans exhibited specific binding to MMP-9 through their heparan sulphate chains.

The association of secreted MMPs with the cell surface has been reported in several cases, such as MMP-1 (*50*), MMP-2, MMP-7 (*49*) and MMP-13 (*51*). Among them, MMP-2 is well documented to form a complex with MT1-MMP and TIMP-2 on the cell surface, and to be activated by TIMP-2-free MT1-MMP (*9*). As to MMP-9, its cell surface association has been reported on normal and tumour cells (*30*, *34*, *52*). However, how MMP-9 can bind to the cell surface has not yet been elucidated. α2(IV) chain of type IV collagen in breast epithelial cells (*53*), the β2 integrin subunit in leucocytes (*54*) or CD44 in tumour cells (*22*, *55*, *56*) has been reported to be a candidate cell surface receptor for MMP-9. In the case of human colon cancer cells, association of MMP-9 with integrins and CD44 was mediated by dentin matrix protein 1 that bridged MMP-9 to these receptors (*56*). Although some isoforms of CD44 are known to have heparan sulphate side chains (*57*), CD44 produced by LuM1 cells was shown not to have heparan sulphate chains. Consistent with this finding, the CD44 molecule was not detected in the immunoprecipitate with anti-MMP-9 antibodies. Instead, GPI-anchored heparan sulphate proteoglycans having 66 and 64 kDa core proteins were found to associate with MMP-9. The results thus obtained clearly demonstrated that among the five cell surface heparan sulphate proteoglycans which expressed in LuM1 cells, the two glypican-like proteoglycans could act as receptors for MMP-9 through their heparan sulphate side chains. However, in the present study, we could not determine whether the 66 and 64 kDa core proteins are different gene products or the same gene product with different post-translational modifications such as glycosylation. We are now attempting to identify the molecular species of these glypican-like proteoglycans.

In summary, we demonstrate that in highly metastatic colon adenocarcinoma cells, secreted MMP-9 is tethered to the cell surface by a glypican-like proteoglycan(s) through its heparan sulphate chains, condensed on the podia of the leading edges and activated there somehow, and thereby contributes to cell migration and invasion *in vitro*. This was supported by the finding that exogenously added heparin or heparitinase-I digestion greatly inhibited cell migration and invasion,

concomitantly with the release of MMP-9 from the cell surface. Our previous study demonstrated that the administration of heparin to mice suppressed experimental metastases of several tumour cells to similar levels regardless of the levels of heparanase activity of the cells (*20*, Yoshitomi, Y. *et al.*, unpublished data). Taken together, it is reasonable to consider that the suppressive effect of heparin on metastasis is due to the competitive effect of heparin against the cell surface heparan sulphates of glypican-like proteoglycans, which are proposed in the present study to act as a cell surface receptor to MMP-9.

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