A Novel Function of Syndecan-2, Suppression of Matrix Metalloproteinase-2 Activation, Which Causes Suppression of Metastasis*

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The syndecans comprise a family of cell surface heparan sulfate proteoglycans exhibiting complex biological functions involving the interaction of heparan sulfate side chains with a variety of soluble and insoluble heparin-binding extracellular ligands. Here we demonstrate an inverse correlation between the expression level of syndecan-2 and the metastatic potential of three clones derived from Lewis lung carcinoma 3LL. This correlation was proved to be a causal relationship, because transfection of syndecan-2 into the higher metastatic clone resulted in the suppression of both spontaneous and experimental metastases to the lung. Although the expression levels of matrix metalloproteinase-2 (MMP-2) and its cell surface activators, such as membrane-type 1 matrix metalloproteinase and tissue inhibitor of metalloproteinase-2, were similar regardless of the metastatic potentials of the clones, elevated activation of MMP-2 was observed in the higher metastatic clone. Removal of heparan sulfate from the cell surface of low metastatic cells by treatment with heparitinase-I promoted MMP-2 activation, and transfection of syndecan-2 into highly metastatic cells suppressed MMP-2 activation. Furthermore, transfection of mutated syndecan-2 lacking glycosaminoglycan attachment sites into highly metastatic cells did not have any suppressive effect on MMP-2 activation, suggesting that this suppression was mediated by the heparan sulfate side chains of syndecan-2. Actually, MMP-2 was found to exhibit a strong binding ability to heparin, the dissociation constant value being 62 nM. These results indicate a novel function of syndecan-2, which acts as a suppressor for MMP-2 activation, causing suppression of metastasis in at least the metastatic system used in the present study.

Tumor metastasis is accomplished through a multistep process in which individual tumor cells disseminate from a primary tumor to distant secondary sites. In the process of metastasis, tumor cells are involved in numerous interactions with the extracellular matrix (ECM) providing information that controls the behavior of tumor cells. The information inscribed in the ECM is transmitted to tumor cells through interaction between individual ECM ligands and the respective cell surface receptors.

One class of cell surface receptors with such functions is cell surface heparan sulfate proteoglycans, including the transmembrane-type syndecan family and the glycosulfophtatidylinositol-anched-type glypican family (1–3). However, cell surface heparan sulfate proteoglycans are unique and are different from proteinous cell surface receptors in terms of the binding redundancy for ligands. This is because the many ligand-binding sites reside in the polysaccharide moiety (i.e. heparan sulfate side chains). Therefore, theoretically, they can be receptors for all heparin-binding molecules. Actually, a large number of reports have demonstrated that cell surface heparan sulfate proteoglycans function as receptors for soluble heparin-binding ligands, such as cell growth factors and insoluble molecules such as ECM constituents. In many cases, it seems that they cooperate with intrinsic high affinity receptors for heparin-binding molecules as low affinity receptors and thereby regulate their signal transduction. For example, they act as coreceptors for heparin-binding growth factors, such as fibroblast growth factor, hepatocyte growth factor, and vascular endothelial growth factor, and control the strength of the signals (4–11).

Moreover, it is well known that syndecans cooperate with integrins as co-receptors for fibronectin, which constitutes various types of ECM, and participate in cell adhesion and/or migration through regulation of focal contact and stress fiber formation (12–16). Furthermore, besides the function regarding this signal transduction, cell surface proteoglycans provide initial docking sites for the binding to various viruses (17, 18). In such cases, viruses also recognize other proteinous receptors expressed simultaneously on the cell surface. Overall, it can be...
considered that the common function of cell surface heparan sulfate proteoglycans is regulation of the functions of other proteinous molecules on the cell surface. Therefore, heparan sulfate proteoglycans on tumor cells have various functions in the process of metastasis that force the tumor cells to always interact in non-self circumstances.

During metastasis, tumor cells must respond to and adapt to the host ECM, which in general, acts defensively against the tumor cells. First of all, tumor cells have to degrade the host ECM in order to disseminate. A family of ECM degradation enzymes called matrix metalloproteinases (MMPs) has been implicated in not only physiological processes of tissue remodeling but also pathological conditions, such as cancer (19). Although various types of MMPs are involved in metastasis, a great deal of emphasis has been placed on type IV collagenases (20, 21), and, in particular, activation of MMP-2 appears to be closely correlated with tumor metastasis (22). In common with all MMPs, MMP-2 is synthesized as a latent form, requiring proteolytic removal of the propeptide for activation. Therefore, regulation of MMP-2 occurs through three steps (i.e. alteration of gene expression, activation of latentzymogens, and inhibition by tissue inhibitors of metalloproteinases).

The mechanism of activation of MMP-2 on the cell surface has been well documented. This event is triggered by the formation of a tertiary complex comprising (or including) secreted pro-MMP-2, tissue inhibitor of metalloproteinase-2 (TIMP-2), and membrane-type 1 MMP (MT1-MMP) on the cell surface and is followed by the cleavage of the peptide of the pro-MMP-2 by TIMP-2-free MT1-MMP (see Fig. 10A) (23–26). However, it was shown recently that an additional member, a glycosylphophatidylinositol-anchored glycoprotein, RECK (reversion-inducing-cysteine-rich protein with Kazal motifs), suppressively regulates this process on the cell surface, suggesting that the regulation of MMP-2 activation might be more complicated than previously expected (27). The fact that both MT1-MMP and RECK are membrane-anchored proteins strongly suggests the possibility that other cell surface molecules may be involved in this regulation.

Previously, we found an inverse correlation between the expression level of syndecan-2 and the metastatic potential of clones established from Lewis lung carcinoma 3LL (14–16). In our study, we further demonstrated the causal relationship in this inverse correlation and the possible mechanism underlying this relation. The results obtained indicate a novel function of syndecan-2, of which the heparan sulfate side chains suppress the activation of MMP-2 on the cell surface.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—P29, LM12-3, and LM66-H11 cells exhibiting low, intermediate, and high metastatic potential, respectively, were cloned from Lewis lung carcinoma 3LL on the basis of spontaneous metastatic potential (28, 29) (where “P” represents “parent” and LM represents “lung metastasis”; i.e. the LM12 and LM66 series were cloned from the populations undergoing 12 and 66 cycles of spontaneous metastasis from a subcutaneous primary tumor to the lung). Cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), streptomycin (100 μg/ml), and penicillin (100 units/ml) at 37 °C under a humidified 5% CO₂ atmosphere. The cells were harvested after incubation with 2 mM EDTA in phosphate-buffered saline (EDTA/PBS) for 10 min at 37 °C, followed by gentle flushing with a pipette, and subcultured twice a week. After transfection of the expression plasmid for each protein using Tfx-50 reagent (Promega Corp.) according to the manufacturer’s instructions, cells were cultured in the presence of G418 (400 μg/ml), and the G418-resistant colonies were picked up 2 weeks later.

**Construction of Syndecan-2 Core Protein and a Mutant of IIα—cDNA of mouse syndecan-2 core protein containing the entire coding region was generated as described previously (15). The mutant core protein of syndecan-2, of which the glycosaminoglycan-attachable serine residues were changed to alanine residues (S41A, S53A, S55A, and S57A), was generated using a Takara Mutan-Super Express Km Kit (Takara BIO Inc.). The four mutagenic primers that changed thymine to guanine were primer A (5’-GAG GAA GCT GCA GGA GTA TA-3’), primer B (5’-TGA GTA TTT TTC TGC TGC CTC AG-3’), primer C (5’-TAT TCT CCT GCC GCA GCA T C-3’), and primer D (5’-AGG CGG AGG GAC TGA TGA AT-3’). The double underlining in the sequences indicates the altered residues. First, primer A was used to mutate codon 41 of syndecan-2 cDNA cloned into the pkF19k vector (Takara BIO Inc.) by the oligonucleotide-directed dual amber-long and accurate (ODA-LA) PCR method. After confirmation of the sequence of the mutant cDNA, primers B, C, and D were used similarly to mutate codons 53, 55, and 57 of the cDNA in that order. Finally, the mutant syndecan-2/pkF19k was inserted into pcDNA3 (Promega Corp.) at a site downstream of the cytomegalovirus promoter. The nucleotide sequence of the cloned mutant syndecan-2 cDNA was verified with an ABI PRISM 310 sequencer (Applied Biosystems).

**Treatment of Cells with Antisense Oligonucleotides of Syndecan-2**—Treatments of P29 cells with antisense or sense oligonucleotides of syndecan-2 were performed as described previously (14). Briefly, antisense phosphorothioate oligonucleotides complementary to the region around the initiation codon of mouse syndecan-2 mRNA (5’-CAC GCG CGC TGC ATA TT-3’) and the corresponding sense (5’-ATA ATG CAG CGC GGC CGG TG-3’) and scrambled antisense (5’-ACGT CCC TGA GCG ATC T-3’) phosphorothioate oligonucleotides were synthesized using a model 392 DNA synthesizer (Applied Biosystems Inc.). The oligodeoxyribonucleotides were purified by two cycles of reverse-phase high pressure liquid chromatography. P29 cells were cultured in 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium containing 10 μM oligonucleotides for 4 days and then cultured for another 1 day in the medium without fetal bovine serum. The conditioned media were concentrated for gelatin zymography.

**Antibodies**—Rabbit polyclonal antibodies, SN1Ab, SN2Ab, SN3Ab, and SN4Ab, specific to ectodomain recombinants of the mouse syndecan-1, -2, -3, and -4 core proteins, respectively, were prepared as described previously (14, 16). The commercially available antibodies used were as follows: goat anti-human integrin α5β1 antibodies (Chemicon International); mouse monoclonal antibodies F58-10E4, specific to heparan sulfate, and F69-3G10, specific to the unsaturated nonreducing
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end of heparan sulfate chain, generated upon heparitinase digestion (Seikagaku Corp.); and mouse monoclonal antibody F-68 anti-human MMP-2 reactive to mouse MMP-2 (DAIICHI Fine Chemical Co., Ltd.).

Flow Cytometric Assay—Subconfluent cell layers were washed with Dulbecco’s modified Eagle’s medium and then digested with or without 0.1 unit/ml of heparitinase-I (EC 4.2.2.8; Seikagaku) for 24 h at 37 °C. Cells were harvested with EDTA/PBS, suspended in 0.2% bovine serum albumin/Dulbecco’s modified Eagle’s medium (3 × 10^5 cells/50 μl), and then incubated with antibodies or the respective nonimmune serum or Ig for 1 h at 4 °C with gentle agitation. After washing three times with PBS, they were exposed to an fluorescein isothiocyanate-conjugated second antibody for 30 min. The labeled cells were washed, and the intensity of fluorescence was measured with a flow cytometer, FACSort (BD Biosciences).

Metastasis Assay—The metastasis assay was performed as described previously (28). Briefly, cells (2 × 10^6 cells) suspended in 0.2 ml of PBS were injected into the tail veins of 6-week-old male C57BL/6 mice for experimental metastasis or subcutaneously into the right abdominal flank for spontaneous metastasis. The animals were sacrificed 4 weeks later, and the numbers of visible nodules in lungs fixed in Bouin’s solution were determined.

Northern Blot Analysis—Poly(A)^+ RNA was isolated from 1 × 10^7 cells of each clone, using a QuickPrep mRNA Purification Kit (GE Healthcare Bio-Sciences Corp.). Northern blot analysis was performed as described previously (15). In brief, 2 μg of each poly(A)^+ RNA from cells was electrophoresed on a 1.0% agarose gel containing 1.1M formaldehyde, transferred to a Hybond N (nucleotides 1115–1696), TIMP-2 (nucleotides 227–691), tides 92–534), MMP-2 (nucleotides 683–1484), MT1-MMP 1175), syndecan-3 (nucleotides 370–931), syndecan-4 (nucleotides 487–1175), syndecan-1 (nucleotides 409–967), syndecan-2 (nucleotides 487–1175), syndecan-3 (nucleotides 370–931), syndecan-4 (nucleotides 92–534), MMP-2 (nucleotides 683–1484), MT1-MMP (nucleotides 1115–1696), TIMP-2 (nucleotides 227–691), β-actin (nucleotides 183–722), or glyceraldehyde-3-phosphate dehydrogenase (nucleotides 319–1047), which had been labeled with [α-32P]dCTP by the random labeling method. After hybridization for 14 h at 42 °C, the membranes were exposed to x-ray films, and the films were scanned with a CanoScan 600 (Canon). Quantification of the bands was performed with the public domain NIH Image program in the 256-grayscale mode.

Western Blot Analysis—Cell surface proteoglycans were extracted from cell layers with 2% Triton X-100, 25 mM KCl, 50 mM Tris-HCl, pH 7.3, containing 10 mM EDTA, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 0.036 mM pepstatin A as proteinase inhibitors for 12 h on ice and then purified as described previously (14). Samples were digested with heparitinase-I plus chondroitinase ABC to remove glycosaminoglycan side chains (29) and then subjected to SDS-PAGE, followed by transfer to Hybond-P membranes (GE Healthcare Bio-Sciences Corp.). The membranes were blocked with 3% bovine serum albumin in PBST for 1 h and then reacted with antibodies for 1 h. After washing with PBST, the membranes were reacted with horseradish peroxidase-conjugated second antibodies for 1 h and then stained with Immunostain (Konica) or ECL detection reagent (GE Healthcare Bio-Sciences Corp.).

Gelatin Zymography—Gelatin zymography was conducted on an SDS-polyacrylamide gel containing gelatin (1 mg/ml) as described previously (23). Briefly, conditioned media in which cells were cultured for 15 h without fetal bovine serum were concentrated with a Microcon YM-30 centrifugal filter device (Millipore), adjusted to the SDS-PAGE loading buffer without the reducing agent, and then subjected to electrophoresis through a gel containing gelatin at room temperature. After electrophoresis, the gels were washed twice for 30 min at 37 °C with 2.5% Triton X-100, 10 mM Tris-HCl, pH 8.0, and then incubated in 1 μM ZnCl₂, 10 mM CaCl₂, 0.2 mM NaCl, 50 mM Tris-HCl, pH 8.0, for 18 h at 37 °C. Enzyme activity was visualized as negative staining with Coomassie Brilliant Blue R250. The band density ratios, active form to latent form, were calculated according to Ixland et al. (30). If necessary, the following polypeptides were used as a substratum; human plasma fibronectin (Iwaki Glass) and its recombinant polypeptides (i.e. the RGD-containing cell-binding domain C-274, the C-terminal heparin-binding domain H-271, and CH-271, a fusion peptide of C-274 and H-271) (TakaRa Bio Inc.) (14).

Affinity Chromatography of MMP-2 on a Heparin-Sepharose CL-6B Column—The conditioned medium of H11 cells (1 × 10⁶ cells) was applied to a column (1 × 9 cm) of Heparin-Sepharose CL-6B (GE Healthcare Bio-Sciences Corp.) equilibrated with 10 mM phosphate buffer, pH 7.3. The column was then washed extensively with the equilibrating buffer. The bound material was eluted with a linear gradient of 0–1.0 M NaCl in the same buffer, 1-ml fractions being collected. The conductivity of each fraction was measured by CD-35MII (M & S Instruments Inc.). Each fraction was concentrated and then subjected to gelatin zymography as described above.

Surface Plasmon Resonance Binding Analysis—Surface plasmon resonance measurements were carried out with a Biacore X system (Biacore AB). A C1 biosensor chip was activated according to the manufacturer’s recommendation (Biacore AB). 1340 response units of streptavidin (Sigma) was coupled to the surface of the C1 biosensor chip. Then 150 response units of biotinylated heparin (Seikagaku), produced as described previously (31), was captured by streptavidin on the chip. The amount of biotinylated heparin coupled to the sensor surface was limited to avoid mass transfer limitation. Kinetic binding analysis was performed in 10 mM HEPES buffer containing 150 mM NaCl and 5 mM CaCl₂, pH 7.4, at a constant flow rate of 5 μl/min, the instrument being equilibrated at 25 °C. Mouse calvaria-derived pro-MMP-2 (Calbiochem) was perfused at various concentrations. At least two different replicated experiments were performed. Response curves were generated by subtraction of the background signal generated simultaneously in the control flow cell. Kinetic parameters were obtained by fitting of the sensorgrams to a 1:1 (Langmuir) binding model using the BIAevaluation 3.1 software (Biacore AB).

RESULTS

Inverse Correlation between Metastatic Potential and Syndecan-2 Expression Level in Clones Derived from Lewis Lung Carcinoma 3LL—Our previous paper (16) showed that Lewis lung carcinoma 3LL-derived low metastatic P29 and highly metastatic LM66-H11 (abbreviated as H11 hereafter) clones
expressed three members of the syndecan family (i.e. syndecan-1, -2, and -4). Among them the expression level of only syndecan-2, a key molecule for regulation of actin cytoskeletal organization in cooperation with integrin α5β1, was different between the clones, although the expression levels of the other two syndecans were very similar in the two clones (14, 15). In this study, to verify the correlation between the metastatic potential and the expression level of syndecan-2, another clone, LM12-3, with intermediate metastatic potential (28), was used. As shown in Table 1, both the experimental and spontaneous metastatic potentials of the three clones used gradually increased in the order of P29 < LM12-3 < H11. Northern blot analysis clearly showed that the expression levels of mRNA of syndecan-1 (2.6 kbp) and -4 (5.6 kbp) were similar among the three clones, but that of only syndecan-2 was different (Fig. 1A, left). Quantification of the densities of the bands followed by normalization as to the respective β-actin bands revealed that syndecan-2 mRNA linearly decreased as the metastatic potential of the clones increased (Fig. 1A, right). These results were very consistent with the results of flow cytometric analysis showing cell surface expression of the syndecan family in the clones (Fig. 1B). Syndecan-3 expression was hardly detected on any of these clones, and the expression levels of syndecan-1 and -4 were not significantly different among the clones. In contrast, syndecan-2 expression decreased inversely with the metastatic potentials of the clones. Since it became clear that there is an inverse correlation between the syndecan-2 expression level and the metastatic potential in these clones, next the causality underlying this inverse correlation was investigated.

Causal Relationship of the Inverse Correlation between the Metastatic Potential and Syndecan-2 Expression—In order to examine the causality, a stable transfectant, the H11-SN2 clone, which was obtained upon transfection of syndecan-2 core protein cDNA into H11 cells, and a mock transfectant, H11-Vec (15), were used. Northern blot analysis confirmed that the expression of syndecan-2 mRNA in the H11-SN2 clone was elevated, although the level of H11-Vec was similar to that in the parent H11 cells (Fig. 2A). Western blot analysis of the core proteins obtained upon digestion of samples with heparitinase-I plus chondroitinase ABC revealed that the three clones produced four species of heparan sulfate proteoglycans of which the molecular masses of the core proteins are 85, 61, 48, and 38 kDa (Fig. 2B, left). These were the core proteins of syndecan-1, glypican-1, syndecan-2, and syndecan-4, respectively (16). Among them, the expression level of the core protein of syndecan-2 was elevated in only the H11-SN2 clone. These results were confirmed using anti-syndecan-2 core protein antibodies, SN2Ab (Fig. 2B, right). Moreover, the elevated expression of syndecan-2 on the surface of H11-SN2 cells was directly proved by flow cytometric analysis (Fig. 2C). The expression level of syndecan-2 in H11-SN2 cells was similar to that in P29 cells. Furthermore, this transfection did not affect the expression levels of other syndecans, syndecan-1 and -4 (Fig. 2C) and syndecan-3 (data not shown).

Then the metastatic potentials of the transfectants were examined. Although the experimental metastatic potential of the mock transfectant was not different compared with that of the parent cells, the potential of the H11-SN2 clone was dramatically suppressed to the level in P29 cells (Fig. 3 and Table 2). Similar results were obtained upon assaying of spontaneous metastatic potentials (Table 2), indicating that, irrespective of the injection site, overexpression of syndecan-2 caused a striking reduction of metastasis. Thus, the causal relationship of the inverse correlation between the syndecan-2 expression and the metastatic potential was proved. To clarify the mechanism

#### TABLE 1

| Injection and clone | Incidence | Number of lung foci |
|--------------------|-----------|---------------------|
| **Intravenous**    |           |                     |
| P29                | 3/7       | 0–1                 |
| LM12-3             | 7/7       | 19–92               |
| LM66-H11           | 7/7       | 467–744             |
| **Subcutaneous**   |           |                     |
| P29                | 0/7       | 0                   |
| LM12-3             | 6/7       | 0–14                |
| LM66-H11           | 7/7       | 23–56               |

#### FIGURE 1. Comparison of expression of syndecan family members among three clones with different metastatic potentials. A, Northern blot analysis of syndecan family members and β-actin in P29, LM-12-3, and H11 clones, with low, intermediate, and highly metastatic potentials, respectively (left). The appearance of three mRNA bands corresponding to different sizes (arrows; 3.5, 2.3, and 1.2 kbp) of syndecan-2 depends on the different lengths of poly(A) attached (40). The densities of individual bands (in the case of syndecan-2, the sum of the three bands) were quantified using the NIH Image program and normalized by each β-actin band (right). B, flow cytometric analysis of syndecans in the three clones. Cells were reacted with antibodies against each syndecan core protein (black line and red line) or with IgG of nonimmune serum (gray line) and then stained with fluorescein isothiocyanate-conjugated secondary antibodies. Syn-1, -2, -3, and -4, syndecan-1, -2, -3, and -4, respectively.
underlying this relationship, gelatinases (MMP-2 and MMP-9) known to participate directly in tumor metastasis were analyzed.

Expression and Activation of MMP-2—Gelatin zymography revealed that the culture media of the four clones contained MMP-2 but hardly contained MMP-9 (Fig. 4A). Every clone showed the presence of a large amount of a latent form of MMP-2 (pro-MMP-2) of 72 kDa (Fig. 4A). Interestingly, the levels of the active forms of MMP-2 (intermediate MMP-2 and active MMP-2 with molecular masses of 68 and 62 kDa, respectively) present in the conditioned media of only the highly metastatic clones, H11 and H11-Vec, were significantly higher than those in the medium of the H11-SN2 clone, of which the level was similar to that of the P29 clone. The results showed that proteolytic processing of pro-MMP-2 was promoted in the highly metastatic clones. MMP-2 is secreted as an inactive proenzyme and activated through proteolysis on the cell surface (25, 26). The activation process for MMP-2 has been proposed to be as follows (see Fig. 8A). TIMP-2 bound to the catalytic domain of MT1-MMP on the cell surface acts as a receptor complex for pro-MMP-2, and pro-MMP-2 binds to the C terminus of the TIMP-2 through its own C terminus. Subsequently, adjacent TIMP-2-free MT1-MMP clips the propeptide of the pro-MMP-2 in the ternary complex to activate MMP-2. Therefore, we compared the expression levels of the constituents of the ternary complex among the four clones. The expression levels of mRNAs not only of MMP-2 but also of MT1-MMP and TIMP-2 were not significantly different among the four clones (Fig. 4B). However, upon Western blot analysis with anti-MMP2 antibodies, it was shown that the amounts of the proforms of MMP-2 in the medium of H11-SN2 cells were significantly higher than in those of the parent H11 cells and the mock transfectant and similar to that of P29 cells (Fig. 4C).

Interaction of MMP-2 with Heparin—Affinity chromatography on a Heparin-Sepharose CL-6B column followed by gelatin zymography revealed that both the latent and active
forms of MMP-2 could bind to heparin and were eluted with ~0.6 M NaCl from the column (Fig. 5A). Thus, MMP-2 exhibited a considerably high ability to bind to heparin. Next we analyzed a dissociation constant between these two materials by surface plasmon resonance analysis with mouse calvaria-derived pro-MMP-2 as an analyte and heparin as an immobilized substrate (Fig. 5B). MMP-2 bound to heparin in a concentration-dependent manner. From the association and dissociation curves, the dissociation constant ($K_D$) of MMP-2 for heparin was calculated to be 61.7 nM ($k_a = 2.72 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, and $k_d = 1.68 \times 10^{-3} \text{s}^{-1}$).

Involvement of Heparan Sulfate Chains of Syndecan-2 in Regulation of MMP-2 Activation—The results obtained above suggested that cell surface heparan sulfate might act as a suppressor for MMP-2 activation. Thus, MMP-2 activation of P29 cells was examined after cell surface heparan sulfates had been removed. The flow cytometric analysis verified that almost all of the cell surface heparan sulfate was removed upon digestion with heparitinase-I (Fig. 6A). Under these conditions, the quantity of active MMP-2 in the medium increased about 2-fold (Fig. 6B), indicating that the cell surface heparan sulfate was involved in the suppression of MMP-2 activation.

To clarify a molecule bearing this cell surface heparan sulfate, the expression of syndecan-2 on P29 cells was suppressed by antisense oligonucleotide of mouse syndecan-2 mRNA, because the expression level of syndecan-2 related inversely to a metastatic potential of each clone. The antisense oligonucleotide treatment suppressed the expression of syndecan-2 in P29 cells, a level similar to that of non-treated H11 cells, whereas the treatments of sense or scramble antisense (data not shown) oligonucleotide did not affect its expression (Fig. 7A). Furthermore, these treatments did not affect at all the expression levels of other syndecans (Fig. 7A). Flow cytometric analyses using SN2Ab showed clearly that cell surface expression of syndecan-2 was also suppressed by the antisense oligonucleotide treatment (Fig. 7B). The suppression of syndecan-2 expression resulted in the elevated activation of pro-MMP-2 (Fig. 7C), suggesting strongly that syndecan-2 was responsible for carrying the active heparan sulfates.

To confirm the participation of the heparan sulfate side chains of syndecan-2 in this suppression, H11 cells were transfected with the mutated syndecan-2 core protein cDNA to produce a core protein of which the four serine residues assumed to elongate glycosaminoglycan chains had been changed to alanine. The stable transfectant was named H11-SN2GAG. Flow cytometric analysis with SN2Ab, antibodies specific for syndecan-2 core protein, showed that H11-SN2GAG cells expressed syndecan-2 core protein on the cell surface at a sim-
ilar level to H11-SN2 cells (Fig. 8A), and, as expected, it was proved that the major syndecan-2 produced by H11-SN2/H9004GAG cells had obviously no heparan sulfate side chains, because the core protein of 48 kDa could be detected without heparitinase-I digestion upon Western blot analysis with anti-syndecan-2 core protein antibodies (Fig. 8B, top). In contrast, the 48-kDa core protein of the other two clones was generated only after digestion with glycosaminoglycan degradation enzymes. Confirmation of the presence of endogenous syndecan-2 on H11-SN2ΔGAG cells was performed using F69-3G10, which recognizes the unsaturated disaccharides of the nonreduc-
The expression level of the transfectant was similar to those in the parent H11 cells (Fig. 8B, middle) and the mock transfectant (data not shown). It was confirmed that this transfection did not affect the expression of other syndecans by flow cytometric analysis (data not shown).

The gelatin zymography clearly showed that the high expression of syndecan-2 suppressed the generation of the active form of MMP-2, but the corresponding expression of syndecan-2 without heparan sulfate side chains did not affect this suppression (Fig. 8C). Moreover, the metastatic potential of H11-SN2ΔGAG cells was almost the same as that of the parent H11 cells (Fig. 8D). These findings indicated that syndecan-2 on the cell surface suppresses the activation of MMP-2 through its heparan sulfate side chains with concomitant suppression of metastasis of the tumor cells.

**Influence of the Signal from a Substratum through Syndecan-2 upon MMP-2 Activation**—We have previously demonstrated that P29 and H11 cells used here adhere to a fibronectin substratum through syndecan-2 and that the binding signal affects the signal through integrin α5β1 in the formation of actin cytoskeleton (14–16). Therefore, we examined an influence of stimulation by extracellular matrix on the suppression of MMP-2 activation by syndecan-2. The activation ratios of MMP-2 of both P29 and H11 cells did not significantly change on fibronectin and the recombinant peptides, such as the RGD-containing cell-binding domain (C-241), the heparin-binding domain (H-271), and the fusion form of C-241 and H-271 (CH-271) (Fig. 9A). This suggested that the function of syndecan-2 upon suppression of MMP-2 activation was independent from these signal transductions. Furthermore, the activation ratios of MMP-2 of the cells stimulated by anti-syndecan-2 antibody as a substratum did not change to those on fibronectin (Fig. 9B). Although H11-SN2 and H11-SN2ΔGAG cells expressed syndecan-2 suppressions of MMP-2 activation.
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can-2 core protein in similar levels (Fig. 8A), the suppression of MMP-2 activation occurred in only H11-SN2 having syndecan-2 possessing heparan sulfate chains. These results showed that simple clustering of the core protein of syndecan-2 did not contribute to suppression of MMP-2 activation and indicated that heparan sulfate of syndecan-2 was essential for the suppression function.

DISCUSSION
Here we clearly demonstrated that there is an inverse correlation between the metastatic potential and the expression level of syndecan-2, using three Lewis lung carcinoma 3LL-derived clones with different metastatic potentials, and also showed the causality of this inverse correlation; i.e. transfection of syndecan-2 into the higher metastatic H11 clone exhibiting low syndecan-2 expression suppressed both spontaneous and experimental metastatic potentials of the clone. Many types of cells express more than one species of syndecans. The three clones used in this study, like other cells, expressed syndecan-1 and syndecan-4 in addition to syndecan-2, and the expression levels of the former two syndecans were very similar among these clones. The transfection of syndecan-2 into H11 cells did not affect the expression of other syndecans, suggesting that the suppression of metastasis of H11-SN2 cells is due to an increase in syndecan-2 expression.

As a possible mechanism by which syndecan-2 suppresses the metastatic potential of tumor cells in an expression level-dependent manner, we assumed the interaction of syndecan-2 with MMP-2 in the immediate vicinity on the cell surface for the following reasons. First, many studies have indicated pivotal roles of gelatinases (MMP-2 and MMP-9) derived from both tumor cells and stromal cells in tumor metastasis (20, 21, 32). Second, the three clones expressed only MMP-2 as a gelatinase under the conditions used. Finally, it is well known that the regulation of MMP-2 activation occurs on the cell surface in the complex systems involving TIMP-2, MT1-MMP, and pro-MMP-2 (Fig. 10A) (33). Moreover, RECK, a glycosylphosphatidylinositol-anchored glycoprotein, was reported to be involved in this activation process (27), suggesting the possibility that other cell surface molecules (syndecan-2 in this case) might also be involved. This idea was further supported by the previous work that indicated that MT1-MMP had a heparin-binding domain and exogenously added heparin potentiated the rate of MMP-2 activation by soluble MT1-MMP in a cell-free system (25).

First of all, we found that activation of pro-MMP-2 was promoted in the highly metastatic clones, despite the similar expression levels of pro-MMP-2, TIMP-2, and MT1-MMP (i.e. regardless of their metastatic potentials). The finding that both the latent and active forms of MMP-2 exhibited higher binding avidity to heparin ($K_d = 61.7$ nM) strongly suggested the interaction of cell surface heparan sulfates with the ternary complex on the cell surface. Actually, it was proved that cell surface heparan sulfates participated suppressively in MMP-2 activation, since removal of cell surface heparan sulfates from the low metastatic P29 cells by digestion with heparitinase-I enhanced the generation of the active form of MMP-2. Moreover, the fact that the suppression of syndecan-2 expression by the antisense oligonucleotide treatment promoted an activation of MMP-2 suggested strongly that the particular heparan sulfates were carried by syndecan-2. Additional evidence that heparan sulfate of syndecan-2 contributes to regulation of MMP-2 activation was obtained in the experiment upon transfection of the mutated syndecan-2. Although the transfection of syndecan-2 into the highly metastatic H11 cells greatly suppressed the activation of MMP-2 with concomitant suppression of their hematogenous metastasis, the transfection of the mutated syndecan-2 lacking glycosaminoglycan side chains did not affect...
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either the activation of MMP-2 or the metastasis at all. This strongly suggested that syndecan-2 regulated suppressively the activation of MMP-2 on the cell surface through its heparan sulfate side chains. Moreover, the fact that clustering of the core protein of syndecan-2 without heparan sulfate chains on H11-SN2ΔGAG did not cause the suppression of MMP-2 activation further supported the direct involvement of heparan sulfate of syndecan-2 in the suppression.

It is well documented that the activation of MMP-2 on the cell surface takes place in the ternary complex composed of MT1-MMP, TIMP-2, and pro-MMP-2 (Fig. 10A). Possible mechanisms of the involvement of syndecan-2 in suppression of this activation process are illustrated in Fig. 10B. Syndecan-2 might act as a competitor to the activation receptor composed of TIMP-2 and MT1-MMP by binding MMP-2 through heparan sulfates, and/or syndecan-2 might interact with the heparin-binding domain of MT1-MMP (25) and perturb the formation of the ternary complex or directly inhibit the proteolytic activity of MT1-MMP. Although we have no direct evidence, TIMP-2 may also participate in this interaction through its heparin binding ability that is not proved yet. In any case, it is reasonable to consider that the control of the proteolytic activity on the cell surface has an influence on tumor cell invasion and metastasis.

Friedl and co-workers (34) reported a cocluster of β1 integrin and MT1-MMP at the leading edge of tumor cells in a three-dimensional collagen matrix, and the generation of tubelike proteolytic degradation tracks in the matrix upon cell migration. Such clusters disappeared in a short time, and new ones were always reconstructed at the leading edge of cell movement. For tumor cells responding to and migrating through a host microenvironment, it is reasonable that cell adhesion to ECM through ECM component-specific receptors, such as integrins, not only reorganizes the cytoskeleton of the cells but also triggers the formation of a (or the) molecular cluster on the cell surface involving MMPs. It is noteworthy that syndecan-2 of the tumor cells used in this study cooperated with integrin α5β1 on the fibronectin substratum to regulate organization of the cytoskeleton (14, 15). In cell surface molecular clusters formed in response to ECM, the regulation of degradation enzymes such as MMP-2 in addition to MT1-MMP might occur through the heparan sulfate side chains of syndecan.

On the basis of the present results, it is interesting to consider the antimetastatic effect of heparin, of which the mechanism is not fully understood, although the phenomenon is well known (35). Several mechanisms by which heparin can inhibit metastasis have been proposed. Because of elevated levels of heparanase in the sera of metastatic tumor-bearing animals and malignant melanoma patients and its increased expression in various human malignant tumors, the suppressive effect of heparin on metastasis has been attributed to the inhibition of heparanases produced by tumor cells (36–38). Another supposition concerns the anticoagulant effect of heparin, which prevents the formation of emboli of platelets and tumor cells through inhibition of platelet aggregation, resulting in suppression of hematogenous metastasis. Our previous study demonstrated that the administration of chemically modified heparin with low anticoagulant activity resulted in great suppression of experimental lung metastasis by various types of tumor cell clones, including Lewis lung carcinoma, B16F10 melanoma, colon 26 carcinoma, and FBJ osteosarcoma (39). Because the heparin used was a chemically modified one with low anticoagulant activity, the possibility of the prevention of platelet aggregation could be excluded. Interestingly, although B16F10 and FBJ cells expressed higher levels of heparanase, both the low and high metastatic clones (P29 and H11 clones) derived from Lewis lung carcinoma 3LL used in this study did not express the enzyme. Moreover, a colon 26-derived low metastatic clone (NM11) expressed a little bit more heparanase than the highly metastatic LuM1 clone.3 Regardless of such great differences in the expression levels of heparanase, heparin suppressed the metastasis of these clones to similar degrees, suggesting strongly that the major effect of heparin to suppress metastasis was not an inhibition of heparanase. The present results, taken together with our previous ones (39), strongly suggest that the administered heparin might function to suppress MMP-2 activation in the vicinity of tumor cells and suppress metastasis, as syndecan-2 does naturally on the surface of tumor cells.

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