Involvement of Highly Sulfated Chondroitin Sulfate in the Metastasis of the Lewis Lung Carcinoma Cells*5

Fuchuan Li†1,2, Gerdy B. ten Dam†1,3, Sengottuvelan Murugan2, Shuhei Yamada15, Taishi Hashiguchi1, Shuji Mizumoto1, Kayoko Oguri1, Minoru Okayama**, Toin H. van Kuppevelt4, and Kazuyuki Sugahara†3,4

From the 4Graduate School of Life Science, Hokkaido University, Sapporo 001-0021, Japan, the 6Department of Biochemistry, Kobe Pharmaceutical University, Kobe 658-8558, Japan, the Clinical Research Center, National Hospital Organization Nagoya Medical Center, Nagoya 460-0001, Japan, the 5*Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University, Kyoto 603-8555, Japan, and the 6Department of Biochemistry, Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Center, 6500 HB Nijmegen, The Netherlands

Received for publication, August 5, 2008, and in revised form, October 8, 2008. Published, JBC Papers in Press, October 16, 2008, DOI 10.1074/jbc.M806015200

The altered expression of cell surface chondroitin sulfate (CS) and dermatan sulfate (DS) in cancer cells has been demonstrated to play a key role in malignant transformation and tumor metastasis. However, the functional highly sulfated structures in CS/DS chains and their involvement in the process have not been well documented. In the present study, a structural analysis of CS/DS from two mouse Lewis lung carcinoma (3LL)-derived cell lines with different metastatic potentials revealed a higher proportion of $\Delta^{4,6}$HexUA-GalNAc(4,6-O-sulfate) generated from E-units (GlcUA-GalNAc(4,6-O-sulfate)) in highly metastatic LM66-H11 cells than in low metastatic P29 cells, although much less CS/DS is expressed by LM66-H11 than P29 cells. This key finding prompted us to study the role of CS-E-like structures in experimental lung metastasis. The metastasis of LM66-H11 cells to lungs was effectively inhibited by enzymatic removal of tumor cell surface CS or by preadministration of CS-E rich in E-units in a dose-dependent manner. In addition, immunocytochemical analysis showed that LM66-H11 rather than P29 cells expressed more strongly the CS-E epitope, which was specifically recognized by the phage display antibody GD3G7. More importantly, this antibody and a CS-E decasaccharide fraction, the minimal structure recognized by GD3G7, strongly inhibited the metastasis of LM66-H11 cells probably by modifying the proliferative and invading behavior of the metastatic tumor cells. These results suggest that the E-unit-containing epitopes are involved in the metastatic process and a potential target for the diagnosis and treatment of malignant tumors.

The poor prognosis of cancer is mainly due to the metastasis of malignant cells from the primary neoplasm. Metastasis is a selective process involving invasion, embolization, survival in the circulation, arrest in distant capillary beds, and extravasation into and multiplication within the target organ parenchyma (1, 2). In the process of metastasis, tumor cells are involved in a series of interactions with surrounding extracellular matrix (ECM)5 components and nontumor cells such as platelets and endothelial cells. Proteoglycans (PGs), which bear heparan sulfate (HS) or chondroitin sulfate (CS)/dermatan sulfate (DS) side chains and are widely expressed on the cell surface and in the ECM, are important in modulating these interactions (3–5). It has been well documented that PGs with HS side chains play important roles in metastasis (6–8). In addition to HS-PGs, CS/DS-PGs have also been implicated in normal biological processes such as neuronal development, morphogenesis, growth factor binding, and cell signaling (9, 10) but also play a crucial role in the metastatic process (4).

Human melanoma CS/DS-PG and its homologue NG2 in rats are involved in matrix adhesion, migration, and invasion, a role that is abolished by treatment with antibodies against CS/DS-PG (11–13). Moreover, CD44-related CS/DS-PG on the cell surface is essential in the invasion/migration of normal endothelial and melanoma cells (14, 15). The interaction between CS/DS-PG and membrane type 3 matrix metalloproteinase is important for the invasion of melanoma cells (16). In addition, treatment with chondroitinase (CSase) AC (specifically cleaving CS) or CSase B (specifically cleaving DS) signifi-

* This work was supported in part by Grant-in-aid 16390026 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT), funds from the New Energy and Industrial Technology Development Organization (to K. S.) and the Core Research for Evolutional Science and Technology Program of the Japan Science and Technology Agency, Human Frontier Science Program Grants RGP62/2004 (to T. H. V. K.) and RGP18/2003 (to K. S.), Dutch Cancer Society Grant 2008-4058 (to G. T. D.), the “Academic Frontier” Project for Private Universities matching funds subvention to K. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

‡ These authors contributed equally to this work.

§ Supported by a postdoctoral fellowship from the Japan Society for the Promotion of Science.

¶ To whom correspondence may be addressed. Present address: Dept. of Biochemistry, Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen Medical Center, 6500 HB Nijmegen, The Netherlands. Tel.: 31-24-3610759/14270; Fax: 31-24-3540339; E-mail: g.tendam@ncmls.ru.nl.

5 The abbreviations used are: ECM, extracellular matrix; PG, proteoglycan; DS, dermatan sulfate; CS, chondroitin sulfate; GAG, glycosaminoglycan; CSase, chondroitinase; 2AB, 2-aminobenzamide; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high performance liquid chromatography; GlcUA, $\alpha$-glucuronic acid; PBS, phosphate-buffered saline; HexUA, hexuronic acid; $\Delta^{4,6}$HexUA, 4,5-unaturated hexuronic acid; E-unit, GlcUA$\beta$1–3GalNAc(4S,6S); 25, 2-O-sulfate; 4S, 4-O-sulfate; 6S, 6-O-sulfate.
cantly inhibited the proliferation and invasion of melanoma cells (17). Most recently, cell surface CS/DS was shown to participate in basic fibroblast growth factor-induced proliferation of human metastatic melanoma cell lines (18), the membrane type 3 matrix metalloproteinase-mediated activation of pro-matrix metalloproteinase-2 (19), and P-selectin-mediated metastasis of breast cancer cell lines (20). These studies point to the fact that CS/DS side chains play crucial roles through binding to various ligands, although the core protein also has ligand binding capabilities (21).

CS chains are composed of repeating disaccharide units of GlcUA-GalNAc, where GlcUA and GalNAc represent D-glucuronic acid and N-acetyl-D-galactosamine, respectively, whereas DS is a stereoisomer of CS chains formed from precursor CS chains through the action of glucuronyl C5 epimerase (22, 23). CS and DS chains are often found as co-polymERIC structures (CS/DS) that tend to exhibit a periodic distribution of GlcUA-containing disaccharide repeats and L-iduronic acid-containing disaccharide repeats in a cell/tissue-specific manner (9, 24). CS/DS chains are further modified by the differential sulfation pattern of specific sulfotransferases at C-2 of GlcUA/L-iduronic acid and/or C-4 and/or C-6 of GalNAc to yield enormous structural diversity (25). Hence, functional structures of CS/DS chains may be generated in tumor cells because of the differential expression of the individual modification enzymes during tumor progression, which may have a direct/indirect link with metastatic potential. Thus, identification of these altered functional structures would be a significant step in understanding the mechanism of involvement of CS/DS-PGs in metastasis and enable us to address the diagnosis and treatment of malignant tumors.

Highly sulfated disaccharide units like E-unit, GlcUAβ1–3GalNAc(4S,6S) (26), where 4S and 6S stand for 4-O- and 6-O-sulfate, respectively, are rare, and E-unit-rich CS preparations show remarkable biological activities like the promotion of neurite outgrowth and high affinity binding to growth factors (9, 10, 27). Increasing evidence suggests that the expression pattern of cell surface CS/DS-PGs is related to metastatic potential (3, 12, 13). However, the involvement of highly sulfated structures of CS/DS chains in the metastatic process remains obscure. In this study, E-unit-containing epitopes specifically recognized by the antibody GD3G7 (28, 29) were identified as crucial for the highly metastatic potential of the Lewis lung carcinoma-derived LM66-H11 clone.

**EXPERIMENTAL PROCEDURES**

**Materials**—CSase ABC (EC 4.2.2.4), highly purified CSase ABC (protease-free CSase ABC), standard unsaturated disaccharides, CS-A from whale cartilage, CS-B from porcine skin, CS-C and CS-D from shark cartilage, and CS-E from squid cartilage were purchased from Seikagaku Corp. (Tokyo, Japan). The single chain antibody GD3G7 was selected for reactivity with rat embryo-derived GAGs by the phage display technique (28). The monoclonal anti-vesicular stomatitis virus tag antibody P5D4 and porcine intestinal mucosal heparin were from Sigma. Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) was obtained from Invitrogen. Actinase E was from Kaken Pharmaceutical Co. (Tokyo, Japan). The Diff-Quick solution was purchased from International Reagent Corp. (Kobe, Japan). 2-Aminobenzamide (2AB) was purchased from Nacalai Tesque (Kyoto, Japan). Sodium cyanoborohydride (NaBH₃CN) was from Aldrich. All other chemicals and reagents were of the highest quality available. Size-defined even-numbered CS-E oligosaccharides were prepared by enzymatic fragmentation of a commercial squid cartilage CS-E with sheep testicular hyaluronidase (Sigma), followed by fractionation using gel filtration column chromatography on Bio-Gel P-10 as described previously (30). For RNA extraction, a QuickPrep™ total RNA extraction kit was purchased from GE Healthcare. RNA-qualified RNase-free DNase, RNasin® ribonuclease inhibitor, and Moloney murine leukemia virus reverse transcriptase were obtained from Promega (Madison, WI). A Platinum® SYBR® Green qPCR Supermix-UDG kit was purchased from Invitrogen.

**Animals and Cell Lines**—Six-week-old male C57BL/6 mice and 6-week-old female C3H/HeN mice were obtained from Japan SLC (Hamamatsu, Japan) and kept in standard housing. All of the experiments were performed under the experimental protocol approved by the local animal care committee of Hokkaido University. The low metastatic P29 and high metastatic LM66-H11 cells cloned from a murine Lewis lung carcinoma 3LL were prepared as reported (31) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Thermo Trace, Melbourne, Australia), streptomycin (100 µg/ml), and penicillin (100 units/ml) at 37 °C in 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 by gentle flushing with a pipette and subcultured twice a week.

**Extraction of GAGs from P29 and LM66-H11 Cells**—The cells were dehydrated and depididated by digestion with acetone, air-dried, and used for extraction of GAGs essentially as described previously (32) with some modifications. Briefly, the dried acetone powder was digested with heat-activated (60 °C, 30 min) actinase E in 200 µl of 0.1 M sodium borate, pH 8.0, containing 10 mM calcium acetate at 60 °C for 48 h. Following incubation, each sample was treated with 5% trichloroacetic acid, and the resultant precipitate was removed by centrifugation. The supernatant was extracted with ether to remove trichloroacetic acid. After neutralization with 1.0 M sodium carbonate, the aqueous phase was adjusted to contain 80% ethanol and 1% sodium acetate and kept at 4 °C overnight. The precipitated crude GAGs were recovered by centrifugation, desalted on a PD-10 column (GE Healthcare) using 50 mM pyridine acetate, pH 5.0, as an eluent, and evaporated dry.

**Disaccharide Composition Analysis of CS Chains**—The disaccharide composition of the GAG preparations from both Lewis lung carcinoma cell lines was determined as previously described (32). Briefly, the sample was dissolved in water, and an aliquot was digested with CSase ABC (33), the digest was labeled with 2AB (34), and excess 2AB was removed by extraction with chloroform (35). The 2AB-labeled digest was analyzed by anion exchange HPLC on a PA-03 silica column (YMC-Pack PA, Kyoto, Japan). Identification and quantification of the resulting disaccharides were achieved by compari-
son with the elution positions of CS-derived authentic unsaturated disaccharides (Ref. 34 and Table 1).

**Immunocytochemistry**—E-unit-containing epitopes on the cell surface of P29 and LM66-H11 cells were stabilized using a single chain phage display antibody, GD3G7 (28, 29). Briefly, the carcinoma cells (5 × 10^4 cells/well) were plated on 8-well Lab-Tech chamber slides (Nalge Nunc International), cultured in DMEM supplemented with 10% fetal bovine serum for 24 h, and fixed with the Diff-Quick reagent A. After being blocked in DMEM supplemented with 10% fetal bovine serum for 24 h, the carcinoma cells (5 × 10^4 cells/well) were stained using a primary antibody GD3G7 (diluted 1:100 (10^5/g/ml), or GD3G7 (2 × 10^4/g/ml) or the irrelevant antibody MPB49V (0.5 g/ml) for 30 min at 37 °C and used for metastasis experiments in both C57BL/6 and C3H/HeN mice. Aliquots of the cell suspension were assessed for cell viability before the injection.

**Dynamic Monitoring of LM66-H11 Cell Proliferation Using the RT-CES™ System**—The cell proliferation assay was done using the RT-CES™ system (ACEA Biosciences, San Diego, CA). Briefly, ACEA 96X microtiter plates (e-plate™) were coated with laminin (25 μg/ml) at 37 °C for 30 min, and LM66-H11 cells (2 × 10^4/well) were seeded in 100 μl of medium. In the first experiment, cell attachment was monitored up to 20 min, at which point various inhibitors, CS-A (100 μg/ml), CS-E (100 μg/ml), or GD3G7 (2 μg/ml) in 150 μl of DMEM were individually added. The spreading of the cells was continuously monitored up to 60 min using the RT-CES™ system. In other instances, ~24 h after seeding, when the cells were in a log growth phase, serially diluted GD3G7 (2, 0.5, and 0.2 μg/ml) in 150 μl of DMEM was added into the corresponding wells. The proliferation was monitored for a period of 64 h and expressed as a cell index as per the manufacturer’s instructions. The cell index is a quantitative measure of the spreading and/or proliferative status of the cells in an electrode-containing well.

**Cell Migration and Invasion Assays in Vitro**—The ability of LM66-H11 and P29 cells to migrate and invade was assessed using the BD BioCoat™ chamber with or without Matrigel (BD Biosciences) in vitro. In some instances, single cell suspensions of LM66-H11 (5 × 10^4 cells/ml) were prepared by detaching and resuspending in serum-free DMEM. Before being added to the upper chamber (8 μm PET pores), LM66-H11 cells were preincubated with CS-A (100 μg/ml), CS-E (100 μg/ml), or GD3G7 (2 μg/ml) in 500 μl DMEM for 30 min at 37 °C in a CO₂ incubator. The lower chambers were filled with DMEM containing 10% fetal bovine serum. After incubation for 24 h, the cells that had migrated and invaded through the membrane alone or the Matrigel-coated membrane remained bound to the underside of the membranes. These cells were stained
TABLE 2
Primer sequences utilized for quantitative reverse transcription-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (bp)</th>
<th>Forward</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GalNAc4S-6ST</td>
<td>153</td>
<td>Forward</td>
<td>5′-TATGCAAAGACGACACAGCGG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-TGCAATGGTGGATGACAGCT-3′</td>
</tr>
<tr>
<td>UST</td>
<td>151</td>
<td>Forward</td>
<td>5′-TATGCAAGACGACACACCTTTA-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-TGAAATGTCAGTGGACCAA-3′</td>
</tr>
<tr>
<td>C4ST-1</td>
<td>141</td>
<td>Forward</td>
<td>5′-ACCTCGGGCAAGATAGAAGGACG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-CTTTACGACCAGTGCTGCTC-3′</td>
</tr>
<tr>
<td>C4ST-2</td>
<td>102</td>
<td>Forward</td>
<td>5′-CAGACCAAGGTAATGAAAGGAC-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-CATGAGGAGGCACGCAGGTAAG-3′</td>
</tr>
<tr>
<td>D4ST-1</td>
<td>175</td>
<td>Forward</td>
<td>5′-GGCTTCCTGAAACACAGGGTT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-CTCTGACACCCGTTACACGG-3′</td>
</tr>
<tr>
<td>CSE</td>
<td>139</td>
<td>Forward</td>
<td>5′-AGCGCTTGGGTCCGTCACCTAC-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-CAGGTTGCTGAGCAGCTCAGAC-3′</td>
</tr>
<tr>
<td>G3PDH</td>
<td>204</td>
<td>Forward</td>
<td>5′-CATTGAAAGGGGACCAGG-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse</td>
<td>5′-GAAGGACATTGACGCGCTG-3′</td>
</tr>
</tbody>
</table>

with the Diff-Quik staining kit and counted in five random microscopic fields/filter.

Biodistribution of Intravenously Injected Radiolabeled CS-E—

3H-Labeled CS-E (4 × 10^6 cpm) was prepared as described previously (27) and injected into C3H/HeN mice through a lateral tail vein. At 1, 1.5, and 2.5 h post-injection, the blood samples were collected from anesthetized animals via cardiac puncture. The animals were then sacrificed, and the organs including liver, lungs, kidneys, spleen, and brain were dissected out, quickly rinsed, and weighed. The blood and each tissue from all the animals were pooled and homogenized with 1% Triton X-100/PBS in a Polytron homogenizer while being cooled on ice. Aliquots of the tissue homogenates and the plasma were freeze-thawed (five times) using liquid nitrogen bath and treated with 5% trichloroacetic acid. The resultant supernatants were recovered by centrifugation at 15,000 rpm for 10 min, and the radioactivity was measured using a multi-purpose liquid scintillation counter (Beckman coulter LS6500). The counts were converted to disintegrations on the basis of a standard quench correction curve, and the distribution of 3H-CS-E in the plasma and tissues was expressed as dpm/ml and dpm/g, respectively.

Relative Quantification of Gene Expression of Sulfotransferases and Epimerase—Total RNA was extracted from P29 and LM66-H11 cells in 100-mm culture plates using a QuickPrep total RNA extraction kit according to the manufacturer’s instructions, and then each extract was treated with RNase-free DNase for 30 min at 37 °C. The cDNA was synthesized from instructions, and then each extract was treated with RNase-free DNase for 30 min at 37 °C. The cDNA was synthesized from instructions, and then each extract was treated with RNase-free DNase for 30 min at 37 °C. The cDNA was synthesized from instructions, and then each extract was treated with RNase-free DNase for 30 min at 37 °C.

RESULTS

Comparison of Amount and Composition of CS/DS between P29 and LM66-H11 Cells—To investigate the possible alteration in the expression of CS/DS in the carcinoma clones with different metastatic potentials, GAGs were extracted from P29 and LM66-H11 cells, as described under “Experimental Procedures,” and the amount and composition of CS/DS in both GAG preparations were determined by digestion with CSase ABC followed by anion exchange HPLC. The results are detailed in Table 1. The amount of CS/DS expressed in the low metastatic P29 cells was 10 times that in the LM66-H11 cells with a high metastatic potential (Table 1). CS/DS chains expressed by both cell lines contained Δ^4,5^HexUAα1–3GalNAc (ΔO-unit), Δ^4,5^HexUAα1–3GalNAc(6S) (ΔC-unit), Δ^4,5^HexUAα1–3GalNAc(4S) (ΔA-unit), Δ^4,5^HexUAα2(S)α1–3GalNAc(4S) (ΔB-unit), and Δ^4,5^HexUAα1–3GalNAc(4S,6S) (ΔE-unit) in varying proportions. CS/DS from LM66-H11 cells was relatively low sulfated because of a significant increase in the proportion of nonsulfated disaccharides (27.4%), with a sulfat/disaccharide unit (S/unit) ratio of 0.79, compared with 0.98 in P29 cells (Table 1). In addition, the proportion of Δ^4,5^HexUAα1–3GalNAc(4S) was significantly decreased from 94.5% in CS/DS from P29 cells to 65.6% in CS/DS from LM66-H11 cells. However, it is interesting to note that both the amount and proportion of Δ^4,5^HexUAα1–3GalNAc(4S,6S) were substantially increased in highly metastatic LM66-H11 cells.

The unique highly sulfated structure GlcUAβ1–3GalNAc(4S,6S) in CS/DS chains has been shown to be very important for the interactions of the chains with various functional proteins (27, 35). The increasing proportion of Δ^4,5^HexUAα1–3GalNAc(4S,6S) units in the CS/DS preparation from LM66-H11 cells may be a key factor in metastasis. To confirm the high expression of E-units on the surface of LM66-H11 cells, a phage display antibody, GD3G7, which specifically recognizes E-unit-containing CS/DS (28, 29), was used for immunostaining both the cell lines. The results showed that LM66-H11 cells were more strongly stained by GD3G7 than P29 cells (Fig. 1, A and C), and the staining was completely abolished by pretreatment with CSase ABC (Fig. 1, B and D). Preincubation of GD3G7 with CS-E significantly suppressed the staining of LM66-H11 cells by GD3G7, supporting the specificity of the antibody (supplemental Fig. S1). Taken together, these results support the high expression of E-units on the surface of LM66-H11 cells.

Involvement of Cell Surface CS/DS in the Metastasis of LM66-H11 Cells—To examine the possible functions of CS/DS in metastasis, LM66-H11 cells were treated with protease-free CSase ABC to remove the cell surface CS/DS before being injected into mice. After 3 weeks, the mice were sacrificed, and pulmonary metastasis was analyzed by counting tumor foci on the lung surface. The treatment with CSase ABC significantly reduced the metastasis of LM66-H11 cells compared with a control treated with DMEM (Fig. 2), suggesting an important role for cell surface CS/DS in the metastasis of LM66-H11 cells.

Chondroitin Sulfate in Metastasis
face CS/DS in the metastatic process. The involvement of the cell surface CS/DS in the metastasis prompted us to explore the possibility that the administration of CS/DS preparations inhibits the metastatic process, as in the case of heparin/HS in the previous work by us (36) and others (37).

**Characterization of Anti-metastatic Activity of CS-E**—To investigate this possibility, various commercial CS/DS preparations (100 μg/mouse) were individually preinjected into the mice 30 min before the injection of LM66-H11 cells. All of the CS/DS preparations tested showed anti-metastatic activity to some degree, except CS-C from shark cartilage (Fig. 3). Remarkably, CS-E from squid cartilage, characterized by a high proportion (62%) of E-units (30), was not only the strongest inhibitor among the CS/DS preparations but also more potent than heparin, well known for its anti-metastatic activity (37), suggesting the importance of E-units in the metastasis. In the next experiments, dose-dependent inhibition was observed for CS-E against the LM66-H11 cell metastasis in the low dose range (10–100 μg/mouse), whereas the inhibition gradually decreased at higher doses (>100 μg/mouse) (Fig. 4A). The seemingly conflicting results at low and high doses may be due to interactions with various proteins at different concentrations and remain to be investigated. The anti-metastatic activity of CS-E was almost completely abolished by digestion with CSase ABC (Fig. 4B), suggesting that the inhibitory activity of the CS-E preparation is due to CS-E itself rather than impurities.

**Anti-metastatic Activity of Antibody GD3G7**—Higher expression of E-units on the surface of LM66-H11 cells and the potent anti-metastatic activity of CS-E led us to hypothesize that the E-unit-containing CS/DS chains on the tumor cell surface may be involved in the metastatic process. To investigate this assumption, the antibody GD3G7, which recognizes E-unit-containing CS/DS chains, was used for anti-metastasis assays. The preincubation of LM66-H11 cells with GD3G7 (0.5, 0.25, and 0.125 μg/ml) for 30 min of preinjection strongly reduced their lung metastasis in a dose-dependent manner, in contrast to preincubation with the irrelevant antibody MPB49V (negative control single-chain antibody) (Fig. 5), suggesting that the epitopes of the antigen for the antibody GD3G7 play a key role in the metastasis of LM66-H11 cells to the lung.

**The Minimal Anti-metastatic Structure of CS-E Recognized by GD3G7**—The strong anti-metastatic activity of the antibody GD3G7 suggests that the epitope sequences of antigen act as functional domains in metastasis. Most recently, we found that a CS-E decasaccharide fraction was the critical minimal structure needed for recognition by GD3G7, and at least four structurally defined decasaccharides from this fraction were identified as epitopic sequences (29). In the present study, we aimed...
to determine the minimal size of the functional domains of CS/DS chains involved in the metastasis. The decasaccharide fraction and three other size-defined oligosaccharide fractions, all of which were prepared by partial digestion of CS-E with sheep testicular hyaluronidase, were used for inhibition assays. The results showed that the deca- and dodecasaccharides were substantial inhibitors of the metastasis of LM66-H11 cells compared with the hexa- and octasaccharides (Fig. 6), suggesting that E-unit-containing decasaccharides recognized by the antibody GD3G7 act as minimal sized functional domains of CS/DS chains in the metastatic process.

**Effects of CS-E and GD3G7 on the Proliferation, Migration and Invasion of LM66-H11 Cells**—Although our findings clearly show CS-E and GD3G7 to have anti-metastatic properties, the mechanism involved is unknown. We further extended our study to include cell proliferation. Inhibitory effects of CS-A (100 μg/ml), CS-E (100 μg/ml), MPB49V, and GD3G7 (2 μg/ml) on the proliferation of LM66-H11 cells on laminin-coated plates were tested using the RT-CESTM system. As shown in Fig. 7A, CS-E and GD3G7 strongly suppressed the spreading, whereas CS-A did not. No cytotoxicity of CS-E and GD3G7 was observed, as verified by staining for viability using trypan blue. Therefore, in subsequent experiments, LM66-H11 cells were treated with various dilutions of GD3G7 (2, 0.5, and 0.2 μg/ml), which showed that the 2 μg/ml was the most effective for the inhibition of the proliferation of LM66-H11 cells (Fig. 7B). The migration and invasion studies clearly showed that both the migration and invasion of highly metastatic LM66-H11 cells were greater than those of low metastatic P29 cells (Fig. 8A). The effects of CS-A (100 μg/ml), CS-E (100 μg/ml), or GD3G7 (2 μg/ml) on the migration and invasion of LM66-H11 cells were examined next. Although the migration was not significantly diminished by any of the inhibitors tested when compared with the control (Fig. 8B), the invasion of LM66-H11 cells through Matrigel was significantly suppressed by CS-E and GD3G7 as compared with the control (Fig. 8C), suggesting that CS-E and GD3G7 interfere with the metastasis via anti-proliferative and anti-invasive means.

**Efficient Accumulation of CS-E in the Lungs**—Cell/organ-specific accumulation of anti-cancer drugs is essential for the success of drug targeting in vivo. To examine the biodistribution of CS-E, radiolabeled CS-E (3H-CS-E) was administered intravenously, and the distribution of the radioactivity was
determined after 1, 1.5, and 2.5 h. As shown in Fig. 9, $^3$H-CS-E accumulated preferentially in the lungs, where LM66-H11 cells form tumor nodules. $^3$H-CS-E accumulated quickly in the lungs and may explain the lower levels of this compound in the blood and other organs. We also noted an increase in the level of $^3$H-CS-E in the kidneys, which could be another indication of the rapid clearance.

Expression of the N-Acetyl-D-galactosamine-4-O-sulfate 6-O-Sulfotransferase Gene in Highly Metastatic LM66-H11 Cells—Quantitative real time reverse transcription-PCR revealed that the expression level of the $N$-acetyl-D-galactosamine-4-O-sulfate 6-O-sulfotransferase gene, which encodes the enzyme essential for the biosynthesis of E-units, was 5.5-fold higher in LM66-H11 cells than in P29 cells (Fig. 10). This observation was consistent with the higher proportion of E-units in the disaccharide analysis of CS chains (Table 1). In contrast, the expression level of the chondroitin 4-O-sulfotransferase-1 gene, which is involved in the biosynthesis of GlcUA$_1$$1$–3GalNAc(4S) units, a precursor for an E-unit (10), was lower and may explain the lower levels of this compound in the blood and other organs. We also noted an increase in the level of $^3$H-CS-E in the kidneys, which could be another indication of the rapid clearance.

Expression of the N-Acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-Sulfotransferase Gene in Highly Metastatic LM66-H11 Cells—Quantitative real time reverse transcription-PCR revealed that the expression level of the N-acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-sulfotransferase gene, which encodes the enzyme essential for the biosynthesis of E-units, was 5.5-fold higher in LM66-H11 cells than in P29 cells (Fig. 10). This observation was consistent with the higher proportion of E-units in the disaccharide analysis of CS chains (Table 1). In contrast, the expression level of the chondroitin 4-O-sulfotransferase-1 gene, which is involved in the biosynthesis of GlcUA$_1$$1$–3GalNAc(4S) units, a precursor for an E-unit (10), was lower and may explain the lower levels of this compound in the blood and other organs. We also noted an increase in the level of $^3$H-CS-E in the kidneys, which could be another indication of the rapid clearance.

Expression of the N-Acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-Sulfotransferase Gene in Highly Metastatic LM66-H11 Cells—Quantitative real time reverse transcription-PCR revealed that the expression level of the N-acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-sulfotransferase gene, which encodes the enzyme essential for the biosynthesis of E-units, was 5.5-fold higher in LM66-H11 cells than in P29 cells (Fig. 10). This observation was consistent with the higher proportion of E-units in the disaccharide analysis of CS chains (Table 1). In contrast, the expression level of the chondroitin 4-O-sulfotransferase-1 gene, which is involved in the biosynthesis of GlcUA$_1$$1$–3GalNAc(4S) units, a precursor for an E-unit (10), was lower and may explain the lower levels of this compound in the blood and other organs. We also noted an increase in the level of $^3$H-CS-E in the kidneys, which could be another indication of the rapid clearance.

Expression of the N-Acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-Sulfotransferase Gene in Highly Metastatic LM66-H11 Cells—Quantitative real time reverse transcription-PCR revealed that the expression level of the N-acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-sulfotransferase gene, which encodes the enzyme essential for the biosynthesis of E-units, was 5.5-fold higher in LM66-H11 cells than in P29 cells (Fig. 10). This observation was consistent with the higher proportion of E-units in the disaccharide analysis of CS chains (Table 1). In contrast, the expression level of the chondroitin 4-O-sulfotransferase-1 gene, which is involved in the biosynthesis of GlcUA$_1$$1$–3GalNAc(4S) units, a precursor for an E-unit (10), was lower and may explain the lower levels of this compound in the blood and other organs. We also noted an increase in the level of $^3$H-CS-E in the kidneys, which could be another indication of the rapid clearance.

Expression of the N-Acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-Sulfotransferase Gene in Highly Metastatic LM66-H11 Cells—Quantitative real time reverse transcription-PCR revealed that the expression level of the N-acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-sulfotransferase gene, which encodes the enzyme essential for the biosynthesis of E-units, was 5.5-fold higher in LM66-H11 cells than in P29 cells (Fig. 10). This observation was consistent with the higher proportion of E-units in the disaccharide analysis of CS chains (Table 1). In contrast, the expression level of the chondroitin 4-O-sulfotransferase-1 gene, which is involved in the biosynthesis of GlcUA$_1$$1$–3GalNAc(4S) units, a precursor for an E-unit (10), was lower and may explain the lower levels of this compound in the blood and other organs. We also noted an increase in the level of $^3$H-CS-E in the kidneys, which could be another indication of the rapid clearance.

Expression of the N-Acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-Sulfotransferase Gene in Highly Metastatic LM66-H11 Cells—Quantitative real time reverse transcription-PCR revealed that the expression level of the N-acetyl-$\beta$-galactosamine-4-O-sulfate 6-O-sulfotransferase gene, which encodes the enzyme essential for the biosynthesis of E-units, was 5.5-fold higher in LM66-H11 cells than in P29 cells (Fig. 10). This observation was consistent with the higher proportion of E-units in the disaccharide analysis of CS chains (Table 1). In contrast, the expression level of the chondroitin 4-O-sulfotransferase-1 gene, which is involved in the biosynthesis of GlcUA$_1$$1$–3GalNAc(4S) units, a precursor for an E-unit (10), was lower and may explain the lower levels of this compound in the blood and other organs. We also noted an increase in the level of $^3$H-CS-E in the kidneys, which could be another indication of the rapid clearance.
(one-fourth) in LM66-H11 cells than in P29 cells and coincided with the proportion of GlcUA/H9252 1–3 GalNAc(4S) units (66 and 95%, respectively) as shown in Table 1. The expression of the glucuronyl C5 epimerase, dermatan 4-0-sulfotransferase-1, and chondroitin 4-0-sulfotransferase-2 genes, which are involved in DS biosynthesis, was stronger in LM66-H11 cells than in P29 cells (Fig. 10). No significant difference was observed in the expression of the uronyl 2-0-sulfotransferase gene or B-unit between LM66-H11 and P29 cells (Table 1 and Fig. 10).

**DISCUSSION**

In this study, we demonstrated that administered CS-E accumulated in the mouse lungs, where LM66-H11 cells highly expressing E-units colonized to form tumor nodules. This accumulation of CS-E in the lungs of mice may be important for the inhibition of metastasis, particularly the proliferation and invasion of tumor cells. The therapeutic index of a drug would be increased if it enables better transport to the target cell/organ. We also showed that a single administration of CS-E or pretreatment of LM66-H11 cells with the phage display antibody GD3G7, which specifically recognizes E-unit-containing epitopes, suppressed the metastasis.

During tumor progression, the cell surface expression of PGs changes as shown at genetic and structural levels and is thought to play a Janus-faced role (3). PGs and GAGs are often overexpressed in tumor stroma and tumor fibrotic tissues compared with surrounding normal tissues (3). However, accumulating
Chondroitin Sulfate in Metastasis

evidence suggests that cell surface PGs inhibit metastasis by promoting tight cell-cell and cell-extracellular matrix adhesion (6). Low levels of cell surface HS correlate with high metastatic activity of many tumors (38–41), although there are some exceptions (42). Increasing evidence shows that the expression of some HS-PGs such as syndecan-1 on various tumor cells decreases with increasing metastatic potential (43–48). Our recent studies revealed that the expression of syndecan-2 in highly metastatic LM66-H11 cells was significantly lower than that in low metastatic P29 cells from the same parental line, leading to a failure of LM66-H11 cells to form stress fibers on the fibronectin substratum (49).

In the present study, LM66-H11 cells were shown to have a much smaller amount of CS/DS on the cell surface than low metastatic P29 cells, suggesting the concomitant expression of certain particular CS/DS-PGs, which may be involved in cell adhesion and facilitate metastasis, together with decreased syndecan-2. Therefore, identification of specific structures of cell surface CS/DS-PGs is important for understanding details of the malignant progression of Lewis lung carcinoma in further studies. Other than a decrease in the overall amount of CS/DS, the disaccharide composition of CS/DS of LM66-H11 cells was significantly different from that of P29 cells (Table 1). $\Delta^{4,5}$HexUA$\alpha$1–3GalNAc(4S) was the major disaccharide in both cases, but its relative proportion was markedly decreased in the CS/DS preparation from LM66-H11 cells, and correspondingly the proportion of nonsulfated disaccharide was dramatically increased up to 27.4% from 3.7% in P29 cells, resulting in undersulfation (S/unit: 0.79) of CS/DS in the highly metastatic LM66-H11 cells. Similarly, the disaccharide composition of perlecan HS has been shown to be altered specifically during the transition to malignancy (8), especially from colon adenoma to carcinoma, and in transformed mammary epithelial cells (50, 51), and undersulfation of HS is another substantial change that facilitates metastasis by weakening tumor cell adhesion to extracellular matrix molecules (52, 53). Taken together, the lower content and sulfation of CS/DS appear to endow LM66-H11 cells with a greater capacity to escape from the primary tumor surrounded by various extracellular matrices. Moreover, the changes may mimic the CS/DS level on the normal cell surface to avoid monitoring by the immune system.

An intriguing finding of this study was a substantial increase in the amount of $\Delta^{4,5}$HexUA$\alpha$1–3GalNAc(4S,6S) in CS/DS from LM66-H11 cells compared with that from P29 cells. CS/DS chains rich in GlcUA$\beta$1–3GalNAc(4S,6S) (E-unit), a parental structure of $\Delta^{4,5}$HexUA$\alpha$1–3GalNAc(4S,6S), have been implicated in various biological events, such as the development of the central nervous system (10) and viral attachment (54–56). In particular, E-units are present in the CS/DS moiety of versican, a key component for the specific interaction with L- and P-selectin, as well as various chemokines (35). Most recently, CS chains on metastatic breast cancer cell lines have been demonstrated to be a major P-selectin ligand involved in the prometastatic heterotypic adhesion to platelets and endothelium (20). Therefore, we speculate that up-regulated expression of E-units further endows LM66-H11 cells with metastatic potential through the specific interaction of the E-unit-containing CS/DS chains with adhesion molecules such as selectins expressed on the surfaces of platelets and the vascular endothelium. This speculation was supported by our observations that experimental lung metastasis of LM66-H11 cells was strongly inhibited by the pretreatment of cells with CSase ABC or the preadministration of CS-E to mice. A putative receptor in the lung for CS-E remains to be identified.

Our recent studies showed that a single chain antibody, GD3G7, strongly reacts with E-unit-containing CS/DS, and CS- E-derived decasaccharides with three consecutive E-units were identified as possible minimal epitope structures for GD3G7 (29). The expression of the epitope recognized by GD3G7 is strongly up-regulated in ovarian carcinomas, and its expression is undetectable in normal ovary. Moreover, expression of this epitope is found in fenestrated and tumor blood vessels, both of which are vascular endothelial growth factor-dependent (28). Consistent with these findings, LM66-H11 cells rather than P29 cells are specifically immunostained by GD3G7, suggesting that the expression of such E-unit-containing epitopes is also up-regulated with the increase in metastatic potential. The strong inhibitory activity of the antibody GD3G7 against experimental lung metastasis of LM66-H11 cells provides powerful evidence for the involvement of the GD3G7 epitope in the metastasis. Furthermore, our results showed that CS-E decasaccharides were minimal oligosaccharides with substantial inhibitory activity against the metastasis of LM66-H11 cells, which is consistent with the notion that CS-E-derived decasaccharides were the minimal size required for the binding of the antibody GD3G7 (29). A few studies have addressed that CS-PGs play a major role in cancer cell behavior (57) and that alterations in their expression and function during transformation facilitate tumor progression (57). However, the expression of specific CS/DS structures has not been correlated to tumor aggressiveness.

Several studies on blood-borne metastasis have shown that tumor cells in the circulation generally get arrested in the microcirculation of secondary organs and may extravagate with high efficiency (58). Because tumor cell migration, invasion, and survival are also crucial components of metastasis, here we asked whether the up-regulated expression of E-units plays a role in the metastasis of Lewis lung carcinoma cells. Therefore, we compared the migratory and invasive activities of LM66-H11 cells highly expressing E-units versus P29 cells expressing fewer E-units. As expected, the migration and invasion were significantly higher for LM66-H11 cells than for the low metastatic P29 cells (Fig. 8A), which may be correlated with the ECM-degradation process. These phenomena prompted us to see at which step of the metastatic cascade these inhibitors (CS-E and GD3G7) might interfere in vivo. Our studies showed that none of the CS variants or GD3G7 affected the migration of these cells in vitro. In contrast, CS-E and GD3G7 markedly suppressed the invasion of LM66-H11 cells. Next, we tested the anti-proliferative effect of these inhibitors, because intravascular proliferation of attached tumor cells is also essential to the metastatic process (59). CS-E and GD3G7 significantly inhibited the LM66-H11 cell proliferation on laminin-coated plates. Other major findings of this study are structural changes of CS chains at the surface of highly metastatic LM66-H11 cells and differences in the invasion and proliferation of LM66-H11 cells
as compared with those of low metastatic P29 cells. These changes may modulate the behavior of these tumor cells.

In support of our disaccharide and immunocytotochemical analyses showing that LM66-H11 cells highly express E-unit-containing structures, gene expression studies confirmed the up-regulated expression of N-acetyl-d-galactosamine-4-O-sulfate 6-O-sulfotransferase-2 involved in the synthesis of DS corroborated the results from the disaccharide analysis (Table 1) and is consistent with tumor cells may provide a new therapeutic approach for general biological processes of E-unit-rich CS/DS on the surface of highly metastatic lung carcinoma cells to be involved in cell and/or activation of matrix metalloproteinase-2 and thereby and invasion of at least Lewis lung carcinoma cells during phage display antibody GD3G7 interfere with the proliferation and invasion of at least Lewis lung carcinoma cells during metastasis. These inhibitors may also suppress the expression and/or activation of matrix metalloproteinase-2 and thereby prevent invasion and metastasis as in the case of HS (7). This study shows the cell surface E-unit-rich CS/DS produced by highly metastatic lung carcinoma cells to be involved in cell proliferation, survival, and invasion, and so attenuating the several biological processes of E-unit-rich CS/DS on the surface of tumor cells may provide a new therapeutic approach for tumors.

REFERENCES
Chondroitin Sulfate in Metastasis