

Heparan Sulfate Enhances Invasion by Human Colon Carcinoma Cell Lines through Expression of CD44 Variant Exon 3

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ABSTRACT

CD44 variant exon (CD44v) 3 is a heparan sulfate-binding isoform of CD44. The role of CD44v3 in invasion and metastasis associated with heparan sulfate in colon cancer cell lines and cases of colon cancer was examined. Expression of CD44v3 mRNA and protein was observed in five of six human colorectal cancer cell lines. Colo320 and WiDr cells expressed CD44v3 at high levels. Heparan sulfate treatment increased the invasive activity of Colo320 and WiDr cells to rates 14.3 and 12.6 times higher, respectively, than that of untreated cells. However, heparan sulfate treatment did not affect cell growth. Repression of CD44v3 protein production by antisense S-oligodeoxynucleotide treatment reduced the binding affinities and capacities for heparan sulfate by Colo320 and WiDr cells in comparison with that of control cells, and it also reduced the invasiveness of both cell lines to one-fifth that of control cells. In heparan sulfate-treated Colo320 cells, the levels of CD44v3 protein in the Triton X-100-insoluble fraction and moesin-precipitated fraction were increased, suggesting that heparan sulfate treatment facilitates association of CD44 molecules with the cytoskeleton. Immunohistochemical analysis showed CD44v3 to be expressed in 21 of 37 (57%) colorectal cancer cases. Positive CD44v3 expression was associated with more advanced pathological stage and poorer prognosis than negative CD44v3 expression. These data support a role for CD44v3 in invasion and metastasis by colorectal carcinoma cells.

INTRODUCTION

CD44 comprises a family of heterogenous integral membrane proteoglycans derived from a single gene (1). The CD44

gene is composed of 20 exons, 10 of which are variably expressed due to alternative splicing (2). CD44 functions primarily in the immune system in processes such as lymphocyte homing (3), lymphohemopoiesis (4), and T-cell activation (5). Recent research in the field has focused on the specificity of CD44 expression in human malignancies. In many types of human malignancy, CD44 acts as a metastasis-related molecule with multiple functions, such as cell-cell and cell-extracellular matrix adhesions, cell migration, and presentation of growth factors and cytokines (6–8). CD44 also plays roles in disease progression and metastasis of colorectal cancer (9). In particular, CD44v6² and CD44v5 are markers of disease prognosis and early transformation of colon cancer, respectively (9, 10).

Polymorphism of the CD44 molecule depends on the numerous patterns of alternative splicing of the extracellular variant domains, glycosylation sites, and glycosaminoglycan attachment sites (11). The hyaluronan-binding sites are located on exons 2 and 5, and the chondroitin sulfate attachment sites are located on exons 5, 15, 16, and 17. The heparan sulfate attachment site is located in exon 8 (CD44v3; Ref. 11). Heparan sulfate is responsible for the presentation of several growth factors, such as heparin-binding epidermal growth factor, basic fibroblast growth factor, VEGF, hepatocyte growth factor, KGF, and some cytokines (12–16). Thus, CD44v3 is thought to contribute to malignant behavior through heparan sulfate.

In the present study, we found expression of CD44v3 in human colon cancer cells to be associated with increased cell invasion after heparan sulfate treatment. Moreover, patients with CD44v3-positive colon cancer have a poorer prognosis than those with CD44v3-negative colon cancer. Human colon cancer cells that express CD44v3 are stimulated by free heparan sulfate to invade, which is also correlated with poor prognosis.

MATERIALS AND METHODS

Cell Culture. Six colorectal carcinoma cell lines were studied. Colo320, DLD1, WiDr, Colo201, and LoVo were obtained from the Japanese Cancer Research Resources Bank. TCO was established in our laboratory (17). All cell lines were routinely maintained in RPMI 1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Whittaker M.A. Bioproducts Inc., Walkersville, MD) under conditions of 5% CO₂ in air at 37°C.

RT-PCR. CD44v3 mRNA transcription was assessed with RT-PCR using 0.5 µg of total RNA extracted by using the RNeasy kit (Qiagen, Hilden, Germany). A primer set for CD44v3 amplification is upper 5'-GTACGTCTTCAAT-

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² The abbreviations used are: CD44v, CD44 variant exon; KGF, keratinocyte growth factor; RT-PCR, reverse transcription-PCR; PMSF, phenylmethylsulfonyl fluoride; ERM, ezrin, radixin, and moesin; VEGF, vascular endothelial growth factor.

ACCA-3' and lower 5'-GTGGTTGAAATGGTGC-3' (with reference to GenBank accession number AJ251595). PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. The experiment was repeated twice.

Cell Growth. Cultured colorectal carcinoma cells were harvested from 80% confluent monolayer cultures by a brief trypsinization with 0.1% trypsin and 0.1% EDTA. The cells were seeded at a density of 2000 cells/well in 96-well tissue culture plates and cultured for 12 h in regular medium. The cells were washed twice with PBS and treated under the conditions mentioned in "Results." Cell growth was monitored after 24 and 48 h by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

In Vitro Invasion Assay. A modified Boyden chamber assay was performed to examine the *in vitro* invasion of heparan sulfate-treated CoLo320 and WiDr colon cancer cell lines. Polycarbonate filters (pore size, 3 μ m; diameter, 5 mm) were glued to collagen type IV inserts (Becton Dickinson Labware, Bedford, MA), which were placed in the wells of 24-well tissue culture plates. The lower part and/or upper part of the chamber were filled with heparan sulfate (1 or 10 μ g/ml). Colo320 and WiDr cells were suspended in 500 μ l of regular medium and placed in the upper part of the chamber. After a 12- or 24-h incubation at 37°C, the filters were carefully removed from the inserts, stained with hematoxylin for 10 min, and mounted on microscopic slides. The number of stained cells was counted in whole inserts at $\times 100$ magnification. Invasion activity was quantified by the average count of cells/well.

Antisense Phosphorothioate Oligodeoxynucleotide Assay. The 15-mer phosphorothioate oligodeoxynucleotide antisense sequence of the first 15 nucleotides of CD44v3 was synthesized and purified by reverse-phase high-performance liquid chromatography (Espec Oligo Service, Tsukuba, Japan). The sequence was 5'-TATTTGAAGACGTAC-3' (with reference to GenBank accession number AJ251595), and the random 15-mer for negative control was a random mixture of oligonucleotides with all four nucleotides at every position. Cells were pretreated with 4 μ M antisense or random S-oligodeoxynucleotide for 6 days, with medium exchange and addition of antisense or random S-oligodeoxynucleotide every 2 days before being used for experiments. When CD44v3 antisense or random S-oligodeoxynucleotide (2, 4, and 8 μ M) was added to the culture medium of Colo320 and WiDr cells, inhibition of growth due to cytotoxicity from the antisense or random S-oligodeoxynucleotide was not observed after 6 days of culture with each treatment.

Cell Lysate. Cells were seeded on 3.5-cm cell culture plates and cultured in RPMI 1640 with 10% fetal bovine serum. The cultured cells were washed three times with PBS. Cells were lysed in boiling SDS-lysis buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 2% SDS, 10 μ g/ml leupeptin, and 50 μ g/ml PMSF] for 10 min. The extract was centrifuged, and the supernatant was used for immunoblotting.

For extraction of ionizing detergent-soluble and -insoluble fractions, cultured cells were washed with PBS containing 1 mM CaCl₂, 1 mM Na₃VO₄, 10 μ g/ml leupeptin and 50 μ g/ml PMSF. Cells were lysed in lysis buffer A [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 1% Triton X-100, 10

μ g/ml leupeptin, and 50 μ g/ml PMSF] for 30 min on ice. The crude extract was centrifuged, and supernatant constituted the Triton-soluble fraction. Pellets were extracted with SDS-lysis buffer, boiled for 10 min, and centrifuged. Supernatant constituted the Triton-insoluble fraction.

Immunoblotting. Fifty μ g of lysate were subjected to immunoblot analysis after 12.5% SDS-PAGE followed by electrotransfer onto nitrocellulose filters. The filters were first incubated with a primary antibody, anti-CD44v3 (Chemicon International, Inc., Temecula, CA), and then incubated with peroxidase-conjugated antigoat IgG antibody (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) in the secondary reaction. The immune complex was visualized using the enhanced chemiluminescence Western blot detection system (Amersham, Aylesbury, United Kingdom).

Immunoprecipitation. For immunoprecipitation, the lysates were precleaned in lysis buffer with protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C and subsequently centrifuged. The supernatants were incubated with anti-moesin antibody (Santa Cruz Biotechnology) and protein A/G-agarose for 16 h at 4°C. Precipitates were collected by centrifugation and washed five times with lysis buffer for SDS-PAGE. For loading control, 5 μ l of each preimmunoprecipitated sample (lysate diluted with buffer) were slot-blotted onto nitrocellulose membrane and stained with Coomassie Blue.

[³H]Heparan Sulfate Binding Assay. Heparan sulfate oligosaccharide (Seikagaku Corp., Tokyo, Japan; mean molecular weight, 10,000) was nonenzymatically labeled with [³H]acetic anhydride (Amersham) in sodium acetate to a specific activity range of 30,000–31,000 mCi/ μ mol (18). Cells were seeded in 6-well culture plates at 1×10^5 cells/well. After 24 h, the cells were washed with the binding medium (RPMI 1640 containing 1% BSA) and treated with various dilutions of [³H]heparan sulfate ranging from 10 ng to 100 μ g with (non-specific binding) or without (specific binding) unlabeled [³H]heparan sulfate (200 μ g) in 0.5 ml of binding medium. Binding was carried out for 4 h at 4°C. After incubation, the medium was removed, and the dishes were washed four times with PBS. Cells were dissolved in 0.1 N NaOH containing 0.05% SDS, and dishes were rinsed with more water. The radioactivity was measured with a liquid scintillation counter. Data were obtained from duplicate experiments.

Surgical Specimens. Thirty-seven formalin-fixed, paraffin-embedded archival surgical specimens from Hiroshima University Hospital of human primary colon adenocarcinomas that had invaded the subserosal layer were chosen at random. The 37 cases contained 8 nodal metastasis-positive cases (Dukes' C) and 10 liver metastasis cases (Dukes' D), which were confirmed by histopathological examination. The remaining 19 cases showed no lymph node metastasis (Dukes' B). It was confirmed from operational findings and clinical examinations that all Dukes' B and C patients did not have any distant metastases at the primary operation. All cases were also followed-up at the hospital. Prognosis study was based on the clinical database of the patients.

Immunohistochemistry. Consecutive 4- μ m sections were immunostained using the immunoperoxidase technique described previously (19). Antigenicity was retrieved with microwave treatment (1000 W, 10 min) in citrate buffer. Anti-

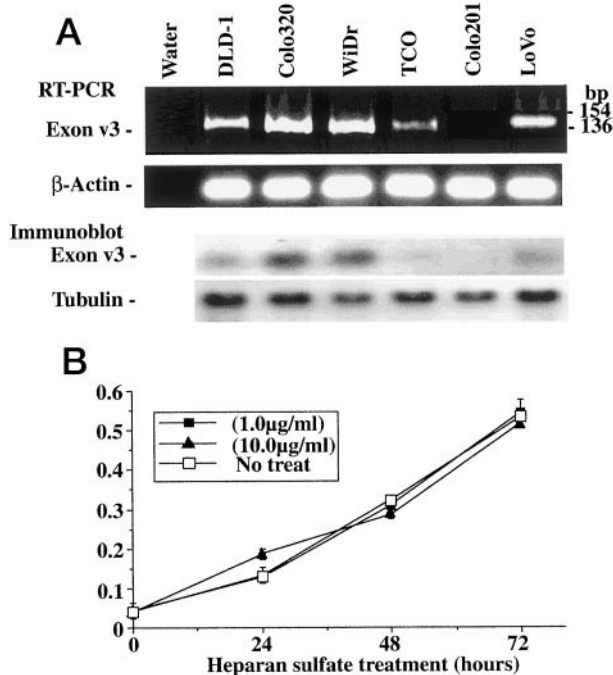


Fig. 1 A, mRNA (top panel) and protein (bottom panel) expression of CD44v3 in human colorectal carcinoma cell lines. RT-PCR products of 136 bp were visualized by ethidium bromide staining. Water, a negative control of the PCR technique using no template. β-Actin was also amplified as an internal control. The CD44v3 protein was detected by immunoblotting using anti-CD44v3 antibody. Tubulin was examined as an internal control. B, effect of heparan sulfate on the cell growth of human colorectal carcinoma cell line Colo320. Cells were treated with 0, 1, and 10 μg/ml heparan sulfate. Cell growth was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay 24, 48, and 72 h after treatment. The Y axis represents the optic density at 595 nm light.

CD44v3 antibody (Chemicon International, Inc.) was used at 1:200 for 2 h at room temperature, followed by treatment with peroxidase-conjugated anti-goat IgG antibody (Medical & Biological Laboratories Co. Ltd.) at room temperature. The specimens were color-developed by diaminobenzidine solution (DAKO Corp., Carpinteria, CA) and counterstained with Meyer's hematoxylin (Sigma Chemical Co., St. Louis, MO).

RESULTS

mRNA Transcription and Protein Expression of CD44v3 and Effect of Heparan Sulfate Oligosaccharides on Growth of Colon Cancer Cells. Five of six cell lines expressed both mRNA and protein of CD44v3 at varying levels, but Colo201 cells did not express CD44v3 mRNA and protein (Fig. 1A). Colo320 and WiDr cells, which expressed high levels of CD44v3 mRNA and protein, were used for additional studies. Cell growth was almost similar between heparan sulfate-treated cells and untreated Colo320 cells (Fig. 1B). Different concentrations of heparan sulfate did not affect the growth characteristics of cells.

Effect of Heparan Sulfate Oligosaccharides on Invasiveness of Colon Cancer Cells. After a 24-h treatment with 10 μg/ml heparan sulfate applied in the lower chamber, Colo320

Table 1 Effect of heparan sulfate on the invasive capacity of human colon cancer cells

	Heparan sulfate treatment				
	None	1 μg/ml	None	10 μg/ml	None
Upper chamber ^a	None	1 μg/ml	None	10 μg/ml	None
Lower chamber ^a	None	None	1 μg/ml	None	10 μg/ml
Colo320					
12 h ^b	0	15 ± 2 ^c	43 ± 2	13 ± 1	95 ± 6
24 h	9 ± 1	33 ± 3	65 ± 4	73 ± 8	129 ± 11
WiDr					
12 h	1 ± 0.2	11 ± 2	38 ± 2	18 ± 2	58 ± 6
24 h	8 ± 1	27 ± 3	52 ± 5	66 ± 3	101 ± 14

^a Upper and lower chambers of modified Boyden chamber.

^b Duration of heparan sulfate treatment.

^c Number of invading cells/well, mean ± SD.

and WiDr cells invaded type IV collagen-coated membrane at rates 14.3 and 12.6 times greater, respectively, than that of untreated cells (Table 1). Both cell lines treated with 10 μg/ml heparan sulfate applied in the upper chamber also invaded at rates 8.1 and 8.5 times greater, respectively, than that of untreated cells.

Effect of CD44v3 Antisense S-oligodeoxynucleotide on CD44v3 Protein Expression, Heparan Sulfate-binding Activity, and Invasiveness of Colon Cancer Cells. To confirm the invasion-promoting effect of heparan sulfate mediated by CD44v3, Colo320 and WiDr cells were treated with 4 μM CD44v3 antisense or random S-oligodeoxynucleotide to reduce CD44v3 expression. After treatment, cells were used in three types of experiments: (a) immunoblot analysis (Fig. 2A); (b) [³H]heparan sulfate oligosaccharide binding assay (Fig. 2B); and (c) *in vitro* invasion assay after heparan sulfate treatment (Fig. 2C). In antisense S-oligodeoxynucleotide-treated Colo320 and WiDr cells, the levels of CD44v3 protein production decreased by one-third and one-fifth, respectively, of the level of production in random S-oligodeoxynucleotide-treated cells (Fig. 2A). The binding capacities and affinities for [³H]heparan sulfate were compared between antisense- and random S-oligodeoxynucleotide-treated cells (Fig. 2B). In antisense S-oligodeoxynucleotide-treated Colo320 cells, the binding affinity (dissociation constant, K_d) and binding capacity (maximal binding, B_{max}) for heparan sulfate were reduced to 18% and 27%, respectively, of those of random S-oligodeoxynucleotide-treated cells. In antisense S-oligodeoxynucleotide-treated WiDr cells, the binding affinity and capacity for heparan sulfate were also reduced to 11% and 71%, respectively, of those in random S-oligodeoxynucleotide-treated cells. Reduction of heparan sulfate binding capacity by antisense S-oligodeoxynucleotide treatment diminished the invasive potential of Colo320 and WiDr cells to one-fifth of pretreatment levels (Fig. 2C).

Effect of Heparan Sulfate on Association between CD44v3 Protein and the Cytoskeleton. The intracellular signals that are activated after heparan sulfate oligosaccharide treatment were examined (Fig. 3). Triton X-100-insoluble CD44 is associated with ERM proteins, which are linked to the cytoskeleton (20). Total protein levels of CD44v3 were not altered after heparan sulfate treatment. CD44v3 protein levels were reduced in the soluble fraction and increased in the insoluble fraction of heparan sulfate-treated Colo320 cells. To confirm the

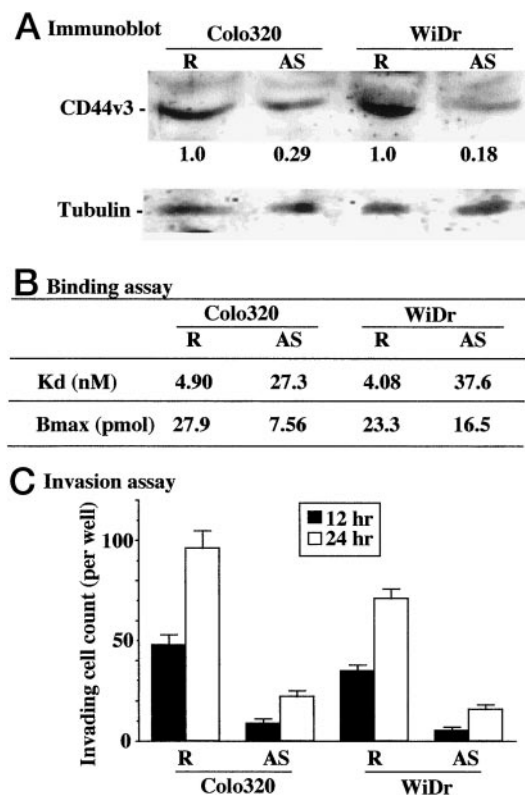


Fig. 2 Effect of CD44v3 antisense phosphorothioate oligonucleotides on CD44v3 protein expression, heparan sulfate binding capacity, and invasion. **A**, CD44v3 protein expression in CD44v3 antisense S-oligodeoxynucleotide-treated Colo320 and WiDr cells was detected by immunoblotting. *Numbers* below the panel are CD44v3 protein levels compared with the protein level of random S-oligodeoxynucleotide-treated Colo320 cells (which was set to equal 1.0). Tubulin was examined as an internal control. **B**, treated cells were subjected to a binding assay with [³H]heparan sulfate. *Kd*, dissociation constant; *Bmax*, maximum binding. **C**, antisense or random S-oligodeoxynucleotide-treated cells were incubated in a modified Boyden chamber with 1.0 or 10.0 μg/ml heparan sulfate. Cells invading the type IV collagen matrix were counted after 12 and 24 h.

physical association between CD44v3 and ERM proteins, immunoprecipitation was performed. Anti-moesin immunoprecipitates were reacted with anti-CD44v3 antibody, and the amount of CD44v3 coimmunoprecipitated with moesin increased after heparan sulfate oligosaccharide treatment. No obvious alterations in phosphotyrosine levels were detected after heparan sulfate treatment (data not shown).

Immunohistochemical Detection of CD44v3 Protein in Surgical Specimens of Colorectal Cancer. We examined CD44v3 production in human colorectal cancer specimens by immunohistochemistry. Immunoreactivity was observed at the cytoplasmic membrane and on the cytoplasm of cancer cells, but not in normal mucosal cells (Fig. 4, *a* and *b*). Twenty-one of 37 (57%) colorectal cancer cases showed CD44v3 immunoreactivity without deviated distribution to the invasive front. CD44v3-positive cases showed a higher frequency of metastasis (Dukes' C and D) than did CD44v3-negative cases (Dukes' B; $P = 0.0006$ by χ^2 test; Table 2). Cancer cells showed strong staining

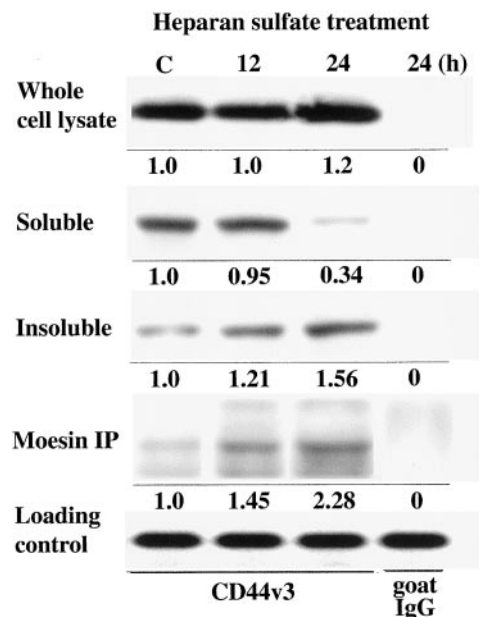


Fig. 3 Effect of heparan sulfate on association between CD44v3 protein and cytoskeleton. Whole cell lysates and Triton X-100-soluble and -insoluble protein fractions were extracted from the heparan sulfate-treated Colo320 cells (rows 1–3). Fifty μg of the proteins were separated with SDS-PAGE and blotted onto nitrocellulose membrane. CD44v3 protein was detected by anti-CD44v3 antibody in each sample. In row 4, immunoprecipitates with anti-moesin antibody were detected with anti-CD44v3 antibody. Detection with goat IgG was performed for a negative control. Coomassie Blue-stained preimmunoprecipitated samples were added as a loading control (row 5). *Numbers* below the panels show CD44v3 protein levels compared with the protein level in untreated Colo320 cells (which was set to equal 1.0).

for CD44v3 at the metastatic foci in the lymph nodes and liver. Study of survival revealed that CD44v3-positive cancer patients showed significantly reduced survival as compared with CD44v3-negative patients ($P = 0.0081$ by a Cox proportional hazards model; Fig. 5). A multivariate statistical examination revealed that CD44v3 expression was the second significant factor, after the final pathological stage (Dukes' classification), for predicting overall survival of the patients (Table 3).

DISCUSSION

We have demonstrated that heparan sulfate enhances the invasive capacity of colon cancer cells by a mechanism mediated by CD44v3 protein. Recent studies have focused on the mechanism of heparan sulfate enhancement of cancer invasion and metastasis (21, 22). Heparan sulfate and a heparan sulfate-modified isoform of CD44, CD44v3, have been implicated in growth factor presentation at sites of inflammation and the cancer host microenvironment. Many heparan sulfate-binding growth factors have been reported, including the insulin-like growth factor family, the fibroblast growth factor family, transforming growth factor β , hepatocyte growth factor (15, 23), KGF (16), interleukin 8 (24), and VEGF (13). Binding to heparan sulfate facilitates the interactions of these growth factors with specific receptors (23). The fact that CD44v3 binds to and presents basic fibroblast growth factor and heparin-binding

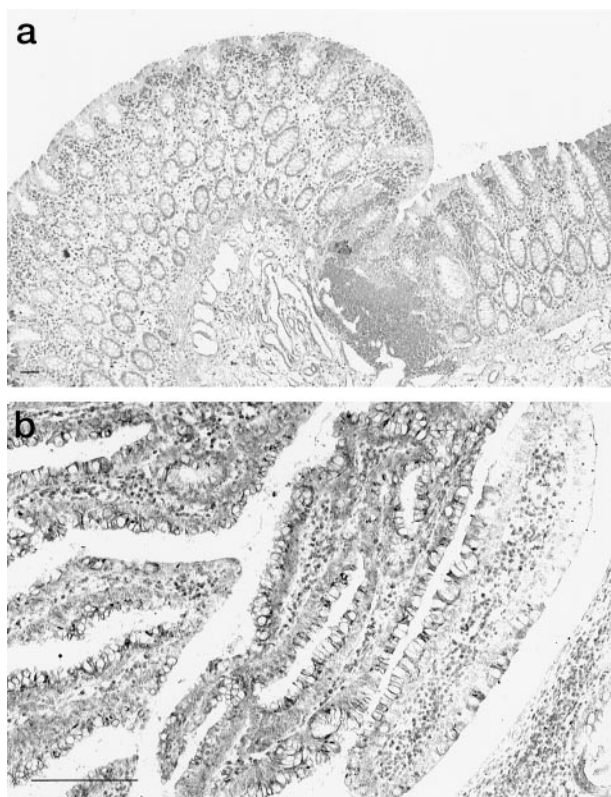


Fig. 4 Immunohistochemistry of anti-CD44v3 in human colon cancer. *a*, CD44v3 immunostaining of colon mucosa. No immunoreactivity was found in nonneoplastic epithelial cells. *b*, CD44v3 immunostaining of a colon carcinoma (Dukes' C) showing immunoreactivity at the cytoplasmic membrane and cytoplasm. Bars, 150 μ m.

Table 2 Number of cases positive for CD44v3 expression in colorectal carcinomas by immunohistochemical analysis

	No. of cases	CD44v3 positive
Total	37	21 (57%)
Dukes' stage ^a		
B	19	5 (26%)
C	8	7 (88%) ^b
D	10	9 (90%)

^a Dukes' B, invasion to or beyond proper muscle layer with no metastasis; Dukes' C, any invasive depth with lymph node metastasis; Dukes' D, distant metastasis.

^b Difference of stage distribution between CD44v3-positive cases was examined by χ^2 test ($P = 0.0006$).

epidermal growth factor (12) suggests that CD44v3 is also responsible for heparan sulfate-associated growth factor presentation.

In the present experiments, heparan sulfate oligosaccharide treatment increased cell invasion activity *in vitro* on type IV collagen. Invasion was inhibited by repression of CD44v3 production by antisense S-oligodeoxynucleotide treatment. One explanation for this result is that Colo320 and WiDr cells express several growth factors, such as KGF (25) and VEGF (26), that bind to heparan sulfate in association with CD44v3

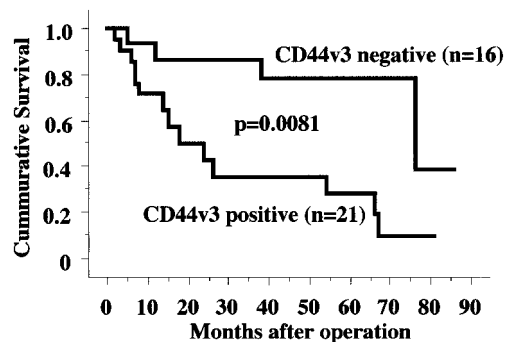


Fig. 5 Survival of colon cancer patients stratified by CD44v3 protein expression. Survival period after surgical resection of 37 cases of human primary colon cancer was calculated by Kaplan-Meier method. The statistical difference in survival curves between CD44v3-positive and -negative cases was tested by the Cox proportional hazards model.

Table 3 Prediction of survival periods of colorectal carcinomas by a multivariate statistical analysis

Parameter	P^a
CD44v3 expression (positive, negative)	0.0235
Dukes' classification (stage B, stage C–D)	0.0011
Histological type (well, moderate-poor)	0.3541
Tumor size (<4 cm, \leq 4 cm)	0.8734

^a P was calculated by Cox proportional hazards model.

molecules, and these growth factors might be displaced by high concentrations of heparan sulfate oligosaccharide. It is also possible that binding of heparan sulfate to CD44v3 generates intracellular signals. Two types of intracellular signals generated by CD44 have been reported. One is tyrosine phosphorylation of non-receptor-type tyrosine kinases of *src* family members including *c-yes*, *c-fgr*, *fyn*, *lyn*, and *lck* (27–29). However, we could not detect phosphotyrosine signals after heparan sulfate treatment (data not shown). The other intracellular signal generated by CD44 is association of CD44 through ERM proteins with actin filaments of the cytoskeleton. This is closely associated with rho activation and hence with acceleration of cell motility (20, 30, 31). In heparan sulfate-treated Colo320 cells, CD44v3 protein was translocated from the Triton X-100-soluble fraction to the Triton X-100-insoluble fraction through the interaction of CD44v3 with moesin. These findings suggest that heparan sulfate treatment induces an association between CD44 protein and the cytoskeleton and that this may enhance the invasion capacity of cells.

In several types of human cancers, CD44v3 expression is associated with the clinical course of the disease. CD44v3 expression is closely correlated with nodal metastasis in breast cancer (32) and with positive surgical margins, a high Gleason score, and the presence of perineural infiltration in prostate cancer (33). Patients with CD44v3-positive vulvar cancer had poorer prognoses for both relapse-free and overall survival (34). However, CD44v3 expression was not predictive for tumor spread or patient survival in cases of gastric cancer (35). Our present results indicate that colon cancers positive for CD44v3 expression show greater disease progression and a poorer prog-

nosis than do CD44v3-negative cancers. Whereas cancer cells producing CD44v3 were a minority within the tumor, CD44v3 expression was frequently found in lymph node metastases, suggesting that cancer cells that express CD44v3 possess a metastatic advantage.

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