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Restoration of CD44H Expression in Colon Carcinomas Reduces Tumorigenicity

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Objective
The functional consequences of reintroduction of the CD44H cell adhesion molecule into colon carcinomas were investigated.

Background
CD44 is a cell surface adhesion molecule that is normally present in numerous isoforms as a result of messenger RNA alternative splicing. Individual CD44 isoforms differ in their ability to enhance tumorigenic or metastatic potential when overexpressed on tumor cells. Reverse transcriptase–polymerase chain reaction analysis demonstrates that CD44H is down-regulated during transformation of normal colon mucosa to carcinoma. The functional consequences of CD44H down-regulation in colon carcinomas has not been clarified.

Methods
Tumor cell lines and fresh tissue specimens were examined for CD44 expression by Western blot analysis. *CD44*H cDNA and site-directed mutants of *CD44*H cDNA were transfected into colon carcinoma cells. Stable transfectants were examined for adhesion to hyaluronate, *in vitro* growth, and *in vivo* growth.

Results
CD44H expression was nearly undetectable in primary colon carcinomas and colon carcinoma cell lines. In contrast, normal mucosa expressed high levels of CD44H. When CD44H was reintroduced into colon carcinoma cells, their *in vitro* and *in vivo* growth was significantly reduced. This CD44H-mediated growth rate reduction required an intact cytoplasmic domain.

Conclusions
Transformation of normal mucosa to colon carcinoma is associated with a down-regulation of CD44H, which consequently may enhance the growth rate and tumorigenicity.

CD44 is a cell surface glycoprotein displayed by a wide variety of normal and malignant tissues.[1–6] Several CD44 isoforms exist and have been demonstrated to arise from mRNA alternative splicing.[7–15] This process allows cells to exclude or include specific segments of mRNA in the final mRNA transcript in a regulated fashion, thereby generating several related proteins from a single gene (Fig. 1)[16] The alternatively spliced exons en-

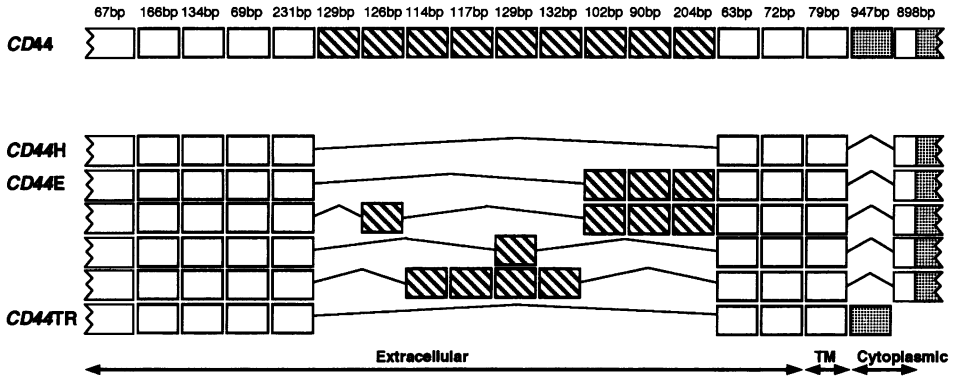

Figure 1. Exon map of the *CD44* gene. The top row is a schematic representation of all known exons in the *CD44* gene, and the lower rows are schematic representations of several *CD44* isoforms. The white boxes represent constitutively spliced exons, and the striped boxes represent alternatively spliced exons in the highly variable membrane proximal domain. The stippled boxes represent 3′ untranslated regions. Many additional *CD44* transcripts with different combinations of exons have been identified and are not depicted in this figure. Several isoforms have no designated name, and no standard nomenclature has been developed for the numerous *CD44* isoforms. TM is the transmembrane region of the corresponding protein.

code a portion of the extracellular domain, suggesting that the inclusion of one or more of these exons may confer changes in ligand specificity or ligand affinity of the resulting CD44 protein. The CD44H isoform serves as the principal cell surface receptor for hyaluronate, a proteoglycan found in abundance in extracellular matrix.[17–19]

In vitro studies of CD44 have indicated that it plays a role in hyaluronate-mediated adhesion,[13,17,20–22] motility,[23,24] hyaluronate degradation,[25] homotypic aggregation,[26] and adhesion to lymphoid tissue.[20,27–30] These properties are among several that are required by invasive and metastatic tumor cells.[31] In several animal models, experimental overexpression of specific CD44 alternative splice variants on tumor cells has resulted in enhanced metastatic potential.[11,24,32,33] Moreover, the stage of disease and overall survival correlates with CD44 expression levels in patients with colon cancer,[6,34,35] breast cancer,[35,36] non-Hodgkin's lymphoma,[37–40] and gastric cancer.[41]

We have previously demonstrated that transformation of normal colonic mucosa is associated with alterations in *CD44* alternative splicing that result in a down-regulation of *CD44*H relative to several other *CD44* transcripts, including *CD44*E.[1] Several investigators have examined the functional consequences of increased expression of several *CD44* alternative splice products in tumors.[1,11,42] In the current study, however, we examined the functional consequences of the CD44H protein down-regulation that occurs during the transformation of normal colonic mucosa to carcinoma. Specifically, we introduced CD44H back into colon carcinoma cell lines by stable transfection and examined its influence on adhesion to hyaluronate, *in vitro* growth, and *in vivo* growth.

MATERIALS AND METHODS

Cell Lines, Tumor Specimens, and Monoclonal Antibodies

The human colon carcinoma cell lines HT29 and LOVO were obtained from the American Type Culture Collection. Human colon carcinoma cell lines KM12L4, KM12C6, and KM20 were generously provided by Dr. Isaiah Fidler (M.D. Anderson Cancer Center, Houston, TX). Human colon carcinoma cell lines SW620 and SNU C2B were provided by Dr. Lee Ellis (M.D. Anderson Cancer Center). Cells were maintained in Dulbecco's modified Eagle's medium with Hamm's F12 supplement and 8% (v/v) fetal calf serum. Human tissue specimens were immediately frozen in liquid nitrogen in the operating room and stored until further processing.

The monoclonal antibodies F10-44-2 (Biodesign International, Kennebunkport, ME) and BU52 (Binding Site, Inc., San Diego, CA) recognize epitopes in the ex-

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tracellular domain common to all previously described CD44 isoforms.

Western Blot and Immunoprecipitation

For Western blot analysis, tumor tissue was homogenized in 50 mM Tris (pH = 8), 150 mM NaCl, 0.2% sodium azide, 100 μg/mL phenylmethylsulfonyl fluoride, 1 mg/mL aprotinin, and 1% Triton X-100. Total protein concentration was measured with the BCA assay (Pierce Chemical Co., Rockford, IL). Lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions and transferred to nitrocellulose filters by electroblotting at 4 C. After being blocked for 1 hour in phosphate buffered saline (PBS) containing 5% dry milk, the filters were incubated with F10-44-2, washed in PBS containing 1% dry milk and 0.2% Tween-20, incubated with horseradish peroxidase-conjugated anti-mouse antibody (Amersham Corp., Arlington Heights, IL), and washed in 150 mM NaCl, 10 mM Tris (pH = 8), 0.05% Tween-20. Specific proteins were detected with an enhanced chemiluminescence system (Amersham Corp.).

For immunoprecipitation, tissue culture cells were harvested in PBS containing 5 mM ethylene diaminetetraacetic acid (EDTA) and washed in PBS. Cell surface proteins were labeled with NHS-LC-biotin (Pierce Chemical) in PBS for 1 hour at 4 C. Excess biotin was then washed away with PBS, and cells were lysed in a buffer containing 0.25% Triton X-100, 10 μg/mL leupeptin, 100 units/mL aprotinin, and 100 μg/mL phenylmethylsulfonyl fluoride. Nuclei and debris were removed by centrifugation, and lysates were precleared with protein G-agarose beads (Oncogene Science, Cambridge, MA). The supernatant was then incubated with protein G-agarose beads coated with monoclonal antibody F10-44-2 for 1 hour at 4 C. The beads were washed and precipitates eluted by boiling. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions and electroblotted described as above. After the filters were blocked, specific proteins were detected with horseradish peroxidase-conjugated streptavidin and an enhanced chemiluminescence system.

CD44H Expression Vectors and Transfection

Previously described cDNA for CD44H[20] was cloned into the multiple cloning site of the vector pRC/CMV (Invitrogen, San Diego, CA), which contains the cytomegalovirus (CMV) promoter for constitutive gene expression as well as a neomycin-resistant gene for selection of clones resistant to the neomycin analogue G418. Site-directed mutagenesis of *CD44*H cDNA to obtain CD44H with only three amino acids in the cytoplasmic domain has been described previously.[23] This cDNA was similarly cloned into pRC/CMV.

Five million colon carcinoma cells were electroporated using 10 μg of purified plasmid DNA linearized with *Bgl* II. The Cell-Porator (Gibco/BRL, Gaithersburg, MD) was set at 800 V/cm and 800 μF; cells were electroporated at 4C in Hepes buffered saline (HBS). After 3 days in complete media, G418 (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 500 μg/mL for selection of drug resistant clones. Clones transfected with cDNA for *CD44*H, *CD44*H with a truncated cytoplasmic region, and vector only (no insert) were designated with the suffices ΔH, ΔTR, and Δneo, respectively.

Adhesion Assay

Ninety-six–well flat bottom plates (Corning, Corning, NY) were coated with chondroitin sulfate A, chondroitin sulfate C, hyaluronate, vitronectin, laminin, or heat denatured bovine serum albumin in PBS overnight at 4 C (all but the bovine serum albumin were obtained from Sigma). After washing with PBS, nonspecific binding sites were blocked with 1 mg/mL bovine serum albumin in PBS for 2 hours at 37 C. Colon carcinoma cells detached from plates with 5 mM EDTA in PBS were resuspended carefully as a single-cell suspension, and 1×10^5 cells were added to each well. Adhesion was allowed to proceed for 1 hour at 4 C, at which time the plates were inverted and centrifuged for 4 minutes at 150 *g*. Unattached cells were aspirated, and the number of adherent cells was measured by methylthiotetrazole (MTT; Sigma Chemical Corp.) labeling (see below). Adhesion was normalized for the number of cells plated as measured by MTT labeling.

In Vitro Growth Analysis

Cells were detached from plates with 5 mM EDTA in PBS, and 2×10^3 cells were added to each well in triplicate. After growth for 1, 3, 5, or 7 days, the cells were placed in 0.5 mg/mL MTT in RPMI-1640 without phenol red for 2 hours at 37 C. The media was removed, and the formazan crystals were solubilized with 50 μL dimethyl sulfoxide. The number of viable cells at each time point was calculated by measuring the optical density on an automatic plate reader using a 550 nm test wavelength and a 650 nm reference wavelength.

In Vivo Growth Analysis

Pathogen-free 4- to 5-week-old male athymic BALB/c nude mice (Steele Laboratory, Boston, MA) were al-

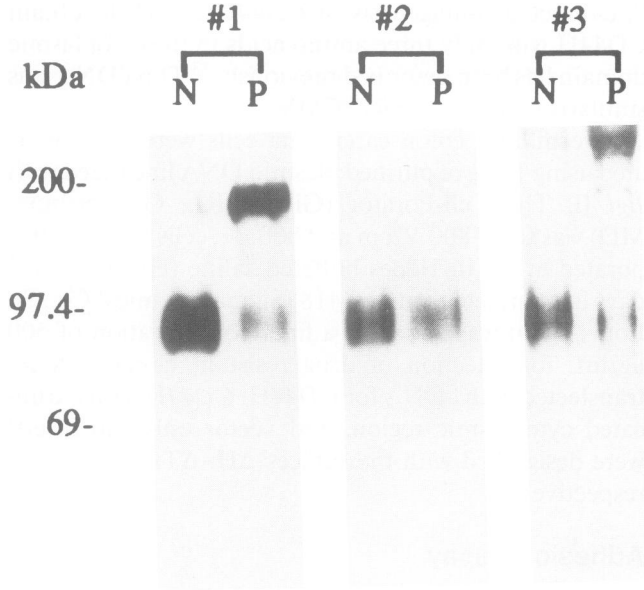

Figure 2. Western blot of CD44 expression in human tissue specimens with use of monoclonal antibody F10-44-2. Tissue specimens representing normal mucosa (N) and primary colon carcinomas (P) from three patients were analyzed for CD44 expression by Western blot. Molecular-weight markers are indicated on the left.

lowed to acclimate for 1 week. Colon carcinoma cells growing in log phase were harvested with 5 mM EDTA in PBS, washed, and resuspended as a single-cell suspension. Cell viability was confirmed to be greater than 90%, as measured by trypan blue dye exclusion. Five million cells in a total volume of 50 μL were implanted subcutaneously. Animals were killed at 16 days for measurement of tumor weight.

RESULTS

CD44 Expression in Human Colon Mucosa and Carcinomas

We examined CD44 expression on primary colon carcinomas and paired normal mucosa from three patients (Fig. 2). The broad band detectable at 80 to 90 kDa represents CD44H, an isoform whose transcript does not contain any of the alternatively spliced exons in the central portion of the transcript. The normal mucosa expressed significantly more CD44H than the primary tumors. Furthermore, normal mucosa did not express the high-molecular-weight CD44 isoforms seen in two of the three primary colon carcinomas. These higher-molecular-weight CD44 isoforms arose from inclusion of additional alternatively spliced exons in the corresponding transcripts (data not shown). These data indicated that CD44H was significantly down-regulated in primary tumors as compared with paired normal mucosa.

CD44 Expression in Colon Carcinoma Cell Lines

We next examined the CD44 alternative splicing pattern in colon carcinoma cell lines to see if it was similar to that seen in primary colon carcinomas. Western blot analysis of CD44 expression in seven colon carcinoma cell lines indicated that most of the cell lines expressed high-molecular-weight CD44 isoforms (Fig. 3). One of the high-molecular-weight CD44 isoforms in HT29 had been cloned previously and was found to be CD44E. More importantly, similar to primary colon carcinomas, none of the colon carcinoma cell lines expressed any significant level of CD44H. Overexposure of the blots detected only a trace quantity of CD44H in KM12C6 and SNU C2B (data not shown).

Reintroduction of CD44H Into Human Colon Carcinomas

We next sought to restore CD44H properties lost by these colon carcinoma cells by restoring cell surface ex-

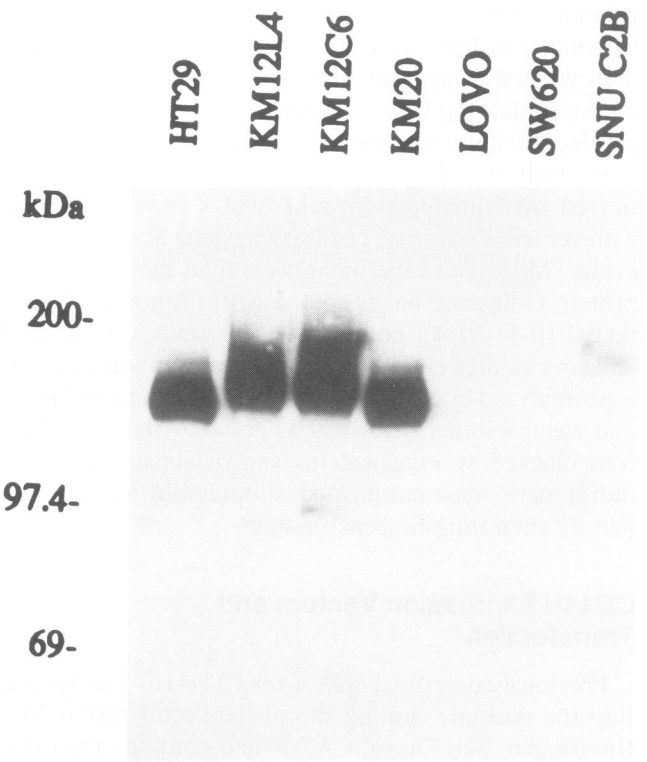

Figure 3. Western blot of CD44 expression by several colon carcinoma cell lines with use of monoclonal antibody F10-44-2. Molecular-weight markers are indicated on the left.

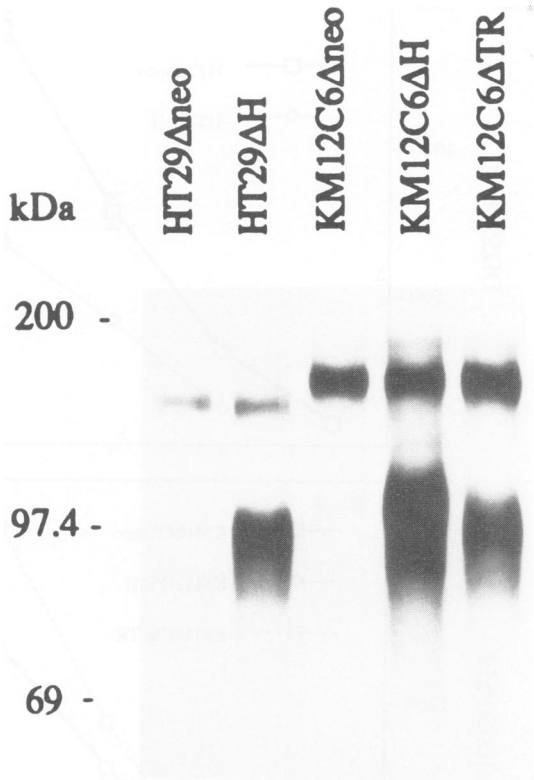

Figure 4. Immunoprecipitation of cell surface labelled CD44 on transfectants with use of monoclonal antibody F10-44-2. HT29 and KM12C6 were transfected with either *CD44*H cDNA (designated with the suffix ΔH), *CD44*TR (designated with the suffix ΔTR), or vector containing no cDNA (designated with the suffix Δneo). Immunoprecipitation of cell lysates from G418-resistant clones with use of monoclonal antibody F10-44-2 was performed, and results of representative clones are shown. Molecular-weight markers are indicated on the left.

pression of CD44H. The HT29 and KM12C6 cell lines were selected and electroporated with *CD44*H cDNA cloned into the pRC/CMV expression vector. G418-resistant colonies expressing cell surface CD44H were identified by immunoprecipitation and retained for further experiments. For analysis of the role of the cytoplasmic domain of the CD44 molecule, *CD44*H cDNA altered to encode only a three-amino-acid cytoplasmic domain was also transfected into the KM12C6 cell line. Immunoprecipitation of CD44 from representative clones is shown in Figure 4.

Adhesion Properties of CD44 on Colon Carcinoma Cells

The HT29 and KM12C6 cells expressed predominantly high-molecular-weight CD44 isoforms. These high-molecular-weight CD44 isoforms demonstrated moderate affinity for hyaluronate in a dose-dependent fashion (Fig. 5A). The small amount of CD44H detected on the surface of KM12C6 cells may account for its greater affinity for hyaluronate than that of HT29 cells. Adhesion to hyaluronate could be abrogated completely

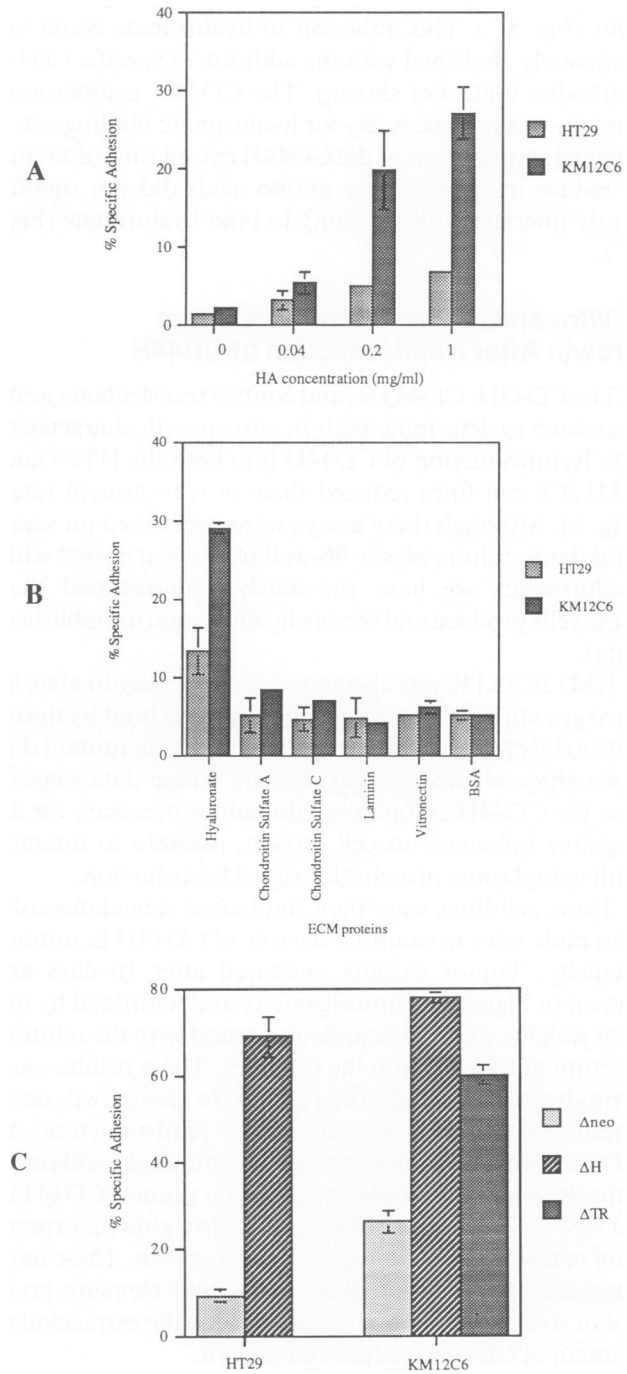

Figure 5. Adhesion to extracellular matrix substances by colon carcinoma cells. Adhesion of untransfected HT29 or KM12C6 cells to (A) hyaluronate and (B) other extracellular matrix components is shown. (C) Cells transfected with *CD44H* cDNA (ΔH), *CD44TR* cDNA (ΔTR), and no cDNA (Δneo) were also tested for adhesion to hyaluronate.

with anti-CD44 blocking antibodies (data not shown). Minimal binding to other extracellular matrix substances was detectable (Fig. 5B). In contrast, HT29 and KM12C6 transfectants expressing high levels of cell surface CD44H demonstrated significantly enhanced affinity for hyaluronate, compared with control transfectants (Fig. 5C). This adhesion to hyaluronate could be completely abolished with the addition of specific CD44 antibodies (data not shown). The CD44H cytoplasmic domain was not necessary for hyaluronate binding; site-directed mutagenesis of the CD44H cytoplasmic domain to reduce it to only three amino acids did not significantly interfere with its ability to bind hyaluronate (Fig. 5C).

In Vitro and *In Vivo* Colon Carcinoma Growth After Reintroduction of CD44H

The CD44H, CD44TR, and control transfectants were examined to determine their *in vitro* growth characteristics. Reintroduction of CD44H into both the HT29 and KM12C6 cell lines reduced their *in vitro* growth rates (Fig. 6). Although these assays were performed on standard tissue culture plastic 96-well plates (not coated with hyaluronate), we have previously demonstrated that these cells produce and secrete hyaluronate (unpublished data).

KM12C6ΔTR was also tested for its ability to alter *in vitro* growth kinetics. Despite its ability to bind hyaluronate avidly (Fig. 5), this cytoplasmic deletion mutant did not reduce *in vitro* growth (Fig. 6). These data suggest that the CD44H cytoplasmic domain is necessary for its negative influence on cell growth, possibly to interact with cytoplasmic proteins for signal transduction.

These cell lines were then implanted subcutaneously into nude mice to examine the role of CD44H in tumorigenicity. Tumor weights measured after 16 days are shown in Figure 7. Tumorigenicity as determined by tumor weights was significantly decreased with the reintroduction of CD44H into the cell lines. These results were reproducible with additional clones. *In vivo* growth rates paralleled *in vitro* growth rates; reintroduction of CD44H reduced tumorigenicity. Interestingly, although expression of the cytoplasmic deletion mutant CD44TR on KM12C6 cells did not reduce *in vitro* growth, expression of this mutant did reduce *in vivo* growth. These data suggest that the additional environmental elements present *in vivo* but not *in vitro* are using only the extracellular domain of CD44 to reduce cell growth.

DISCUSSION

We have previously reported that transformation of colonic mucosa is associated with a relative decrease in

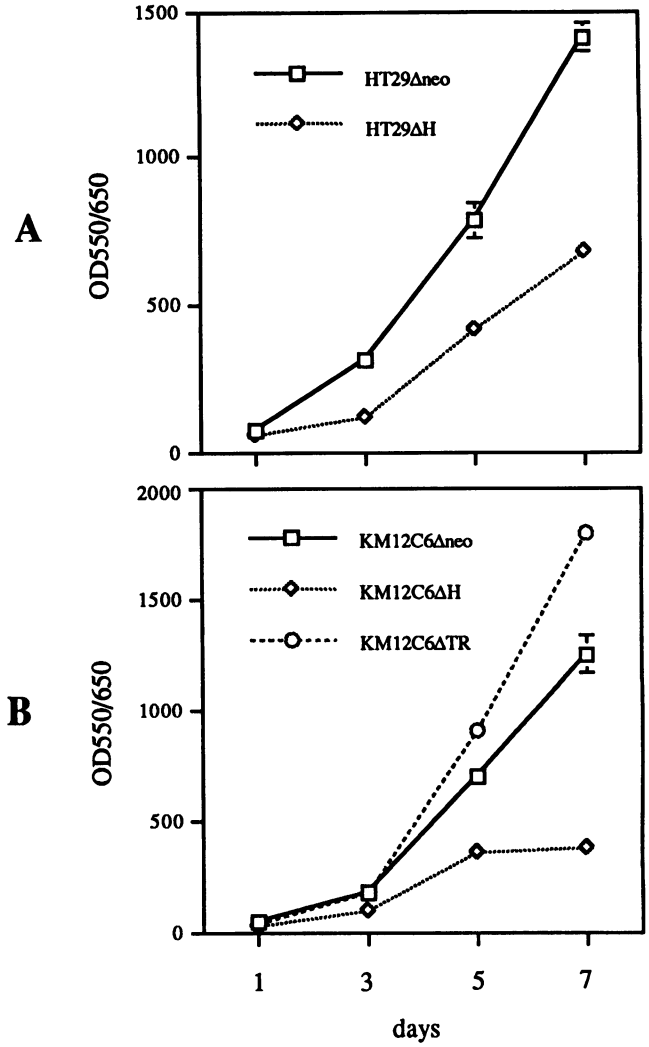

Figure 6. *In vitro* growth analysis of CD44 transfectants. *In vitro* growth of (A) HT29 or (B) KM12C6 cells transfected with either no cDNA (Δneo), CD44H cDNA (ΔH), or CD44TR cDNA (ΔTR) was measured by the MTT assay.

*CD44*H transcript expression.[1] Furthermore, we and others have demonstrated that this down-regulation arises from messenger RNA alternative splicing.[7–15] We have extended these findings in the current study and have demonstrated that these alternative splicing changes result in a decrease in expression of cell surface CD44H on colon carcinoma cells compared with normal mucosa. Furthermore, this decrease in CD44H may provide cells with a growth advantage based on the finding that reintroduction of CD44H into colon carcinoma cells reduces their growth *in vitro* and *in vivo*.

Several studies involving immunohistochemistry have indicated that many high-molecular-weight CD44 isoforms are up-regulated in colon carcinomas relative to normal mucosa.[6,34,35] The expression pattern of these

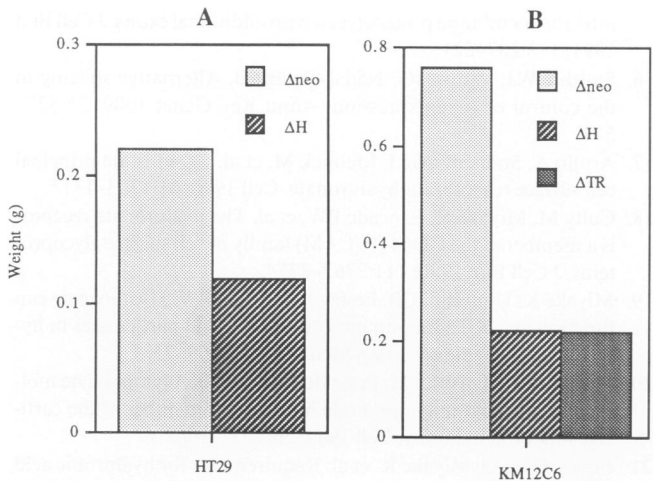

Figure 7. *In vivo* growth analysis of CD44 transfectants. *In vivo* growth of (A) HT29 and (B) KM12C6 transfectants was determined through measurement of tumor weights 16 days after subcutaneous implantation of 5 $\times 10^6$ cells.

isoforms correlates with stage of disease and metastatic potential. However, the absence of antibodies that react with epitopes specific to CD44H has precluded an immunohistochemical analysis of CD44H expression in colon carcinomas. In the current study, Western blot analysis of tissue specimens indicated that CD44H is down-regulated in colon carcinomas compared with normal mucosa. The exact cellular localization of this protein cannot be ascertained by Western blot analysis of heterogeneous tissue specimens. Nonetheless, Western blot analysis of several colon carcinoma cell lines supported the hypothesis that this CD44 isoform is significantly down-regulated in colon carcinomas.

We as well as other investigators have demonstrated functional differences between CD44H and CD44E isoforms in nonepithelial tumors. CD44E binds hyaluronate with significantly less affinity than does CD44H when expressed on melanoma and lymphoma cells.[13,32,33] This pattern was also noted in the colon carcinoma cell lines used in this study. Although posttranslational CD44 modification differs between lymphoma cells and colon carcinoma cells (unpublished observations), these differences do not appear to significantly alter hyaluronate affinity.

Regulation of cell growth involves a complex integration of intracellular signals within the context of the extracellular environment. It is well established that most normal cells require attachment to a substrate to grow, and this *anchorage dependence* is reduced in malignant cells.[43] Several molecules are important in epithelial cell adhesion/anchorage, including cadherins, integrins, selectins, carcinoembryonic antigen, and CD44.[44–46] CD44H displays high affinity for hyaluronate, a glycosaminoglycan that is present in high levels in extracellular matrix. Consequently, it is not surprising that CD44H may play a significant role in transducing signals that influence cell growth based on its interaction with the extracellular environment.

Our finding that reintroduction of CD44H into colon carcinoma cells reduces *in vitro* and *in vivo* growth parallels the results seen with $\alpha_5\beta_1$ integrin (VLA-5) control of cell growth. The $\alpha_5\beta_1$ integrin is a well characterized cell surface receptor for fibronectin.[47,48] Similar to CD44H down-regulation in colon carcinomas, $\alpha_5\beta_1$ integrin levels are reduced in malignant cells compared with those in normal cells.[49] Reintroduction of $\alpha_5\beta_1$ integrin into Chinese hamster ovary cells rendered the cells nontumorigenic when injected subcutaneously into nude mice.[50] The tumorigenicity of these cells is inversely proportional to the level of cell surface $\alpha_5\beta_1$ integrin.[51] Our results demonstrating decreased *in vitro* and *in vivo* growth after reintroduction of CD44H into colon carcinoma cells also support the concept that cellular interaction with extracellular matrix substances directly influences cell growth kinetics.

We can only postulate the normal role for CD44H-mediated growth control. Epithelial cells at the bases of colonic mucosal crypts proliferate more rapidly than cells that have migrated to the tops of these crypts. A delicate balance between cell division in the crypt bases and apoptosis or sloughing in the crypt apexes is required for normal mucosal homeostasis. Cell adhesion molecules, such as CD44H or $\alpha_5\beta_1$ integrin, may an play important role in this homeostasis by signaling cells to reduce their growth rate as the cell/cell and cell/matrix interactions change with migration toward the crypt apexes.

The function of the CD44 cytoplasmic domain requires further study. This region of the protein shares no homologic features with any other cell surface receptors and has no known enzymatic activity.[20] Recently, ezrin, moesin, and radixin have been shown to bind CD44.[52] Our results indicate that the cytoplasmic domain of CD44H is not necessary for hyaluronate binding, but must be intact to reduce *in vitro* growth. These results suggest that intracellular signaling proteins, such as ezrin, radixin, and moesin, may interact with the CD44H cytoplasmic domain to modulate growth control.

Transformation of colon mucosa to carcinoma is associated with a down-regulation of CD44H. Reintroduction of CD44H into these cells by stable transfection results in growth suppression but, moreover, provides a system to analyze the function of this receptor in transformation and tumor progression. Identification of the CD44H signal transduction pathway may provide in-

sights into normal colonic epithelial cell growth control as well as new targets for therapeutic intervention in colon carcinomas.

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Discussion

DR. SAMUEL A. WELLS (St. Louis, Missouri): I very much enjoyed the paper presented by Dr. Tanabe and his associates. This group has played a very important role in defining the biochemistry and molecular oncology of CD44 and its variants. I would like to ask Dr. Tanabe four questions.

Do the various isoforms of CD44 confer different tumorigenic properties to epithelial cells compared with lymphoid cells? For example, as I recall, your group has previously shown that Burkitt lymphoma cells stably transfected with CD44H have both greatly enhanced tumor formation and the propensity to metastasize, whereas cells transfected with CD44E do not. In the present experiment, however, you found that transfection of colon carcinoma cell lines with CD44H reduced their growth both *in vivo* and *in vitro*. What accounts for this difference in cell response and mechanistically how does the presence or absence of any specific CD44 isoform enhance or reduce the malignant potential of a given cell type?

It has been shown that in nonmetastasizing rat pancreatic adenocarcinoma cell lines, metastatic properties are acquired by the cells when they are transfected with a rat homologue of CD44E. In these experiments, the carcinoma cells express the rat homologous CD44H, and supposedly, the enhanced metastatic potential of the transfected cells was in part related to coexpression of both CD44 isoforms. In your experiments, were both the CD44H and CD44E isoforms coexpressed in the CD44 transfected HT29 cells? Did the coexpression or lack thereof have any effect on the growth potential of the transfected cells?

Would you say something about the clinical utility of this abnormal activity of CD44, both in the diagnosis of small tumors and the assessment of their metastatic potential? With current technology it is possible to detect CD44 splice variants in extremely small numbers of tumor cells either in peripheral blood or other body fluids.

In experimental animal studies, and also in humans, if it is known, is there any relationship between the expression of a CD44 isoform and the pattern of metastatic spread of the tumor? For example, there are some reasons that certain tumors preferentially seed the liver, or the lung, or the brain, or the adrenal gland. The CD44 could perhaps in part hold the key to this fascinating clinical problem.

DR. JAMES C. THOMPSON (Galveston, Texas): Anybody who knows me knows that this work is at the limits of or beyond my competence. But rest assured, I have had help.

This is an excellent paper that describes a novel finding regarding CD44, the H variant, a molecule that is responsible in part for the anchorage-dependence of normal cells to their extracellular matrix; that is, it fixes them in place. Normal colonic mucosa expresses high levels of CD44H, but it is down-regulated in colon cancer, as the authors have shown. And in a painstaking set of stable-transfection experiments, they have shown that when CD44H is reintroduced into colon cells by transfection, the cancer cell growth is significantly reduced both *in vivo* and *in vitro*.

The authors have thereby identified a role, possibly unique, for this cell-surface glycoprotein in the regulation of growth of colon cancer. This may provide not only a better understanding of the mechanisms for transformation of normal colonic mucosa to cancer, but it may also provide a new target for novel therapeutic interventions.

I have a few questions. The first two relate to a possible association of this molecule with degrees of malignancy, and they overlap with a couple of questions that Dr. Wells asked.

First, where exactly in the progression from normal to cancerous mucosa does this down-regulation of CD44H occur?