Inhibition of Functional Hyaluronan-CD44 Interactions in CD133-positive Primary Human Ovarian Carcinoma Cells by Small Hyaluronan Oligosaccharides

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Abstract

Purpose: CD44 is one of the most common markers used for identification of highly tumorigenic subpopulations of human carcinoma cells, but little is known about the function of CD44 or its major ligand, hyaluronan, in these cells. The purpose of this study was to investigate the involvement of hyaluronan and its interaction with CD44 in the properties of a tumorigenic subpopulation of primary ovarian carcinoma cells.

Experimental Design: A tumorigenic subpopulation was identified in ascites fluids from ovarian carcinoma patients by expression of high CD133 levels. Treatment with small hyaluronan oligosaccharides, which dissociate constitutive hyaluronan polymer-CD44 interactions, was used to test the importance of hyaluronan-CD44 interaction in assembly of multidrug and monocarboxylate transporters and receptor tyrosine kinases in the plasma membrane of cells with high CD133 levels, and in the tumorigenic capacity of the CD133-high subpopulation.

Results: Although total CD44 levels were similar in cells with high or low CD133 expression, CD44 was present in close association with transporters, receptor tyrosine kinases, and emmprin (CD147) in the plasma membrane of cells with high CD133 levels. Treatment with small hyaluronan oligosaccharides reduced association of the transporters and receptor tyrosine kinases with CD44 in the plasma membrane, diminished drug transporter activity, and inhibited i.p. tumorigenesis in these cells.

Conclusions: We conclude that hyaluronan-CD44 interaction plays an important role in the properties of highly tumorigenic cells by stabilizing oncogenic complexes in their plasma membrane, and that treatment with hyaluronan-CD44 antagonists provides a logical therapeutic approach for abrogating the properties of these cells. (Clin Cancer Res 2009;15(24):7593–601)

Ovarian carcinoma is the most lethal malignancy of the female reproductive system. Most ovarian cancers arise from the surface epithelium of the ovary, but the detailed mechanisms whereby ovarian carcinomas develop and progress are unclear. Most patients are diagnosed with advanced disease that involves accumulation of peritoneal ascites fluid containing tumor and inflammatory cells, implantation and invasion of various adjacent organs by tumor cells, and metastases. Standard therapy of surgery combined with chemotherapy results in complete remission in most patients, but relapse and onset of chemoresistant disease are very common (1, 2). Thus, improved therapeutic strategies, including adjuvant treatments that sensitize recurring tumors to chemotherapeutic agents, are crucial for reducing mortality. Resistance of cancers to chemotherapeutic agents can arise in various ways, but in recent years many studies have focused on the potential role of highly tumorigenic, therapy-resistant subpopulations of cancer cells that are present within many types of tumors and are often termed cancer stem cells or tumor-initiating cells (3, 4). Although the origins and characteristics of these cells are still controversial and may simply reflect tumor heterogeneity, it seems clear that highly malignant, multidrug-resistant subpopulations are present in many tumors (4–6).

One of the most common markers used for the identification of highly tumorigenic subpopulations in carcinomas is CD44 (4), and it was recently shown that CD44 is a useful marker for isolating these subpopulations from patient-derived ovarian carcinomas (7). Hyaluronan, the major ligand for CD44, is a very large polysaccharide that is assembled into pericellular and extracellular matrices in many tissues and serves both structural and signaling functions (8). Hyaluronan...
Translational Relevance

CD44 is a common marker for identification of human carcinoma tumor-initiating cells, but its function in these cells is unknown. We treated a highly tumorigenic subpopulation of primary ovarian carcinoma cells isolated from patient ascites with small hyaluronan oligosaccharides, an established antagonist of interactions between CD44 and its major ligand, hyaluronan. The oligosaccharides reduced association of receptor tyrosine kinases, multidrug transporters, and lactate transporters with CD44 in the plasma membrane, diminished drug transporter activity, and inhibited i.p. tumorigenic capacity in these cells. These and previous results suggest that multivalent hyaluronan-CD44 interactions play an important role in the malignant and chemoresistant properties of tumor-initiating cells by stabilizing oncogenic complexes in their plasma membrane. Treatment with hyaluronan-CD44 antagonists such as small hyaluronan oligosaccharides may provide a logical therapeutic approach for targeting these cells, especially as an adjuvant in the prevention of tumor recurrence as commonly occurs in ovarian cancer.

Materials and Methods

Reagents. Fetal bovine serum was purchased from Atlas Biologicals. RPMI 1640 was purchased from Sigma. The following antibodies were obtained for these studies: breast cancer resistance protein/ABCG2 (BCRP) clone BX-21 (Kamiya Biomedical Company); CD44/HCAM (DF1485) and MCT4 (Santa Cruz Biotechnology); Alexa555-tagged CD44 (Cedarlane Laboratories); P-glycoprotein/ABC1 (Pgp; Calbiochem); β-actin (Sigma); CD133/1(Ac-133)-PE antibody (Mitenyi Biotec GmbH); CD133 and epidermal growth factor receptor (EGFR; Cell Signaling Technology); emmprin/CD147 (BD Biosciences); goat antimouse horseradish peroxidase (HRP) and goat anti-rabbit HRP (Chemicon); and Alexa Fluor 488, 555, 647 (Invitrogen). DRAQ5 nuclear stain was obtained from Biostatus Limited. Western blotting detection reagent (ECL) was purchased from Pierce. Hyaluronan oligosaccharides used in this study were a mixed fraction of average molecular weight ~2.5 × 10^3, composed of 2 to 10 disaccharide units, that were fractionated from testicular hyaluronidase (Sigma, type 1-S) digests of hyaluronan polymer (Sigma, sodium salt); fractionation was by trichloroacetic acid precipitation followed by serial dialysis, using membranes of Amicon Ultra Ultracleat 5,000 MWCO (Millipore) and Spectra/Perf Membrane 1,000 MWCO (Spectrum Laboratories). All other chemicals were of reagent grade or higher.

Primary ovarian carcinoma cell preparation and culture. Use of patient ascites fluids was approved by Medical University of South Carolina Institutional Review Board. Cells were collected from ascites of patients with stage III/IV ovarian carcinoma by centrifugation at 500 g for 10 min at room temperature. The cells were washed twice with RBC lysis buffer (150 mmol/L ammonium chloride, 10 mmol/L potassium bicharbonate, 0.1 mmol/L EDTA, pH 7.4) and once with HBSS, before being plated in RPMI 1640 with 2.38 g/L HEPES, 2 g/L sodium bicarbonate, penicillin/streptomycin, and 10% fetal bovine serum, pH 7.4, at 37°C in a humidified 5% CO2-95% air incubator. After 1 h, nonadherent cells were removed (rapidly adherent cells are mainly macrophages and fibroblasts) and replated, and the subsequent adherent cells were maintained in monolayer culture. For preparation of lysates, cells were seeded in 10-cm dishes 48 h prior to treatment. For preparation of confocal microscopy slides, cells were seeded in 4-well multichamber culture slides (BD Biosciences) 48 h prior to treatment.

Magnetic sorting of CD133-hi and CD133-lo cells. The primary ovarian carcinoma cells from patient ascites were separated by magnetic cell sorting using the EasySep PE selection kit (StemCell Technologies) according to the manufacturer’s protocols. Briefly, cells were digested by 0.25% Trypsin-EDTA (Invitrogen), resuspended in recommended medium (PBS with 2% fetal bovine serum and 1 mmol/L EDTA), containing species-specific FcR blocking antibody. After gently agitating the mixture for 15 min, mouse anti-human CD133/1(Ac-133)-PE antibody was added and the cells were incubated at room temperature for 15 min. The cells were washed once with recommended medium before EasySep PE Selection Cocktail was added and were incubated at room temperature for 15 min. Subsequently, EasySep Magnetic Nanoparticles were added and the cells were separated according to the manufacturer’s recommendations using the EasySep Magnet. Both the CD133-hi and CD133-lo fractions were subsequently cultured in RPMI feed medium, as described above, for 48 h prior to in vitro experiments.

is enriched in the tumor stroma and pericellular matrix around cancer cells in a variety of malignant cancer types, including ovarian carcinomas (9). In previous work, we observed that hyaluronan accumulates rapidly in ascites and at sites of initial attachment of tumor cells to the mesentery and peritoneal wall following inoculation of murine ovarian or mammary tumor cells into the peritoneum of syngeneic mice (10). Other investigators have shown functional involvement of hyaluronan and CD44 in ovarian cancer cell adhesion and migration in culture systems (11, 12). In addition, we and others have shown that hyaluronan-CD44 interactions coregulate several oncogenic signaling pathways in various cancer cells (8, 13–15). Of particular interest is the apparent role of hyaluronan-CD44 interactions in the assembly or stabilization of signaling complexes containing receptor tyrosine kinases or transporters in the plasma membrane and the functional importance of these interactions in malignant and drug-resistant properties of cancer cells (16–21).

CD133 is a stem cell marker that is commonly used for identifying highly malignant, tumor-initiating subpopulations (4), although, as with all such markers, its usefulness varies widely with respect to tumor type or stage and details of technical application (22). Nevertheless, recent studies have shown that CD133-expressing cells are present within ovarian carcinomas and that these cells exhibit properties associated with tumor-initiating cells (23, 24). Consequently, we employed CD133 to separate tumorigenic and nontumorigenic subpopulations from the ascites of patients with ovarian carcinoma, and used these cells to investigate the role of hyaluronan-CD44 interactions. We found that cells with high levels of CD133 (CD133-hi cells) express elevated levels of hyaluronan, emmprin (CD147), receptor tyrosine kinases (RTK), monocarboxylate (lactate) transporters (MCT), and ATP-binding cassette (ABC) family multidrug transporters, as compared with cells with low levels of CD133 (CD133-lo cells). Surprisingly, we found that CD133-hi and CD133-lo cells express similar levels of CD44. However, in the CD133-hi cells, CD44 is concentrated in the plasma membrane and is associated therein with RTKs, MCTs, and ABC transporters, whereas this was not the case in CD133-lo cells. Moreover, treatment with an antagonist of hyaluronan-CD44 interactions, i.e., small hyaluronan oligosaccharides (8), reduced association of CD44 with the RTKs, MCTs, and ABC transporters, induced their internalization, reduced drug transporter activity, and inhibited tumorigenic capacity in the CD133-hi cells.
Significant differences were found between CD133-hi at 10^4 cells and CD133-lo at 10^5 cells (*, P < 0.05), as assessed by percent change in weight due to ascites accumulation [(weight at week n/weight at day 0) - 1] × 100.

Carcinoma cells from patients R1, magnetically sorted for CD133, were injected into the peritoneum of SCID mice as indicated. CD133-hi cells.

Alternatively the cells were counted, mixed with 1:10 PBS-diluted, growth factor-reduced Matrigel, and injected directly into the mice.

**Immunoblot analysis.** Whole cell lysates were prepared for immunoblotting using a radioimmunoprecipitation assay buffer modified to contain 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL aprotinin and leupeptin, 2 mmol/L sodium orthovanadate, and 10 mmol/L sodium fluoride. Protein content was determined by BCA assay (Pierce), and aliquots containing 50 μg of protein were solubilized in SDS sample buffer, resolved on a 4% to 20% reducing polyacrylamide gel, transferred to nitrocellulose (Osmonics) with a Pierce transfer buffer, and subjected to immunoblot analysis as previously described (19).

**Microscopy.** Cells were plated, fixed, permeabilized, blocked for nonspecific binding, incubated with the indicated antibodies, and washed as previously described (21). Cells were then incubated with AlexaFluor secondary antibodies and Draq5 nuclear stain for 2 h. Slides were processed as described (21) and viewed on a Leica Total Confocal Microscopy, System, Spectral Prism 2, Acoustic Optical Beam Splitter (TCS SP2 AOBS) in the Josh Spruill Molecular Morphology and Imaging Center in the Department of Cell Biology and Anatomy. Alternatively, slides were viewed by epifluorescence using an Olympus BX-60 microscope. Images were acquired using a 12.5-megapixel cooled digital camera (BP70; Olympus) and DP Controller software. Image processing and compilation were done using Adobe Photoshop Software (Adobe Systems, Inc.).

**Hyaluronan production.** Hyaluronan levels were assayed in conditioned medium by an ELISA-like assay (25).

**FURA 2-AM assay.** FURA 2-AM efflux was measured as described (26). Ovarian carcinoma cells were seeded in 96-well plates and incubated for 48 h prior to the experiment, to a final density of ~70% confluence. Cells were treated with 100 μg/mL hyaluronan oligomers in feed medium containing 2.5 μmol/L FURA 2-AM. Following a 1-h incubation, plates were read (excitation, 340 nm; emission, 500 nm) in an FLx800 Microplate Fluorescence Reader (Biotek Instruments, Inc.) from the bottom to determine FURA 2-AM levels in the cell layer.

**Tumorigenicity in vivo.** Approximately 5-week-old severe combined immunodeficient (SCID) mice provided by the Hollings Cancer Center, Medical University of South Carolina (MUSC), Xenograft Core Facility were used. Employing a 27-gauge syringe. CD133-hi cells or CD133-lo cells suspended in 400 μL of 1:10 PBS-diluted Matrigel were injected into the i.p. cavity of the SCID mice. The animals were weighed weekly (as a measure of ascites accumulation) and sacrificed after 7 wk. Alternatively, the animals inoculated with CD133-hi cells were treated by i.p. injection of 500 μg hyaluronan oligomers dissolved in 200 μL PBS, or PBS alone, once per week for 6 wk, following 1 wk without treatment. Immediately after euthanasia, ascites fluid was removed by syringe, volume was measured, and the cells were processed for further analysis. All surgical procedures were carried out in an animal surgery unit within the facilities for laboratory animals provided by the Division of Laboratory Animal Resources, and these studies were approved by Medical University of South Carolina’s Institutional Animal Care and Use Committee. It should be noted that extensive experience has shown that the hyaluronan oligomers have no significant adverse effects in mice (20, 27–29).

**Results**

**Identification of a CD133-hi tumorigenic subpopulation of ovarian carcinoma cells in patient ascites.** Carcinoma cells were...
isolated from the ascites of three patients (R1, R2, and R3) with stage III/IV ovarian carcinoma by routine procedures and monitored for the presence of cytokeratin to identify carcinoma cells and CD45 for cells derived from the circulation, such as macrophages. One hundred percent of the cells in these carcinoma preparations stained positively for cytokeratin and negatively for CD45 (Fig. 1A).

Immunostaining of the carcinoma cells revealed a minor subpopulation of cells that stained strongly for CD133 (Fig. 1B). These cells were smaller and less flattened than the CD133-lo carcinoma cells. Although cytokeratin-positive, the CD133-hi cells expressed lower levels of cytokeratin than did the CD133-lo cells, consistent with previous observations that less differentiated epithelial cells exhibit low keratin levels. In addition, the CD133-hi cells did not stain with antibody against CD45, showing that they are not leukocytic in origin. Magnetic sorting of the carcinoma cells from patients R1, R2, and R3 for the CD133 antigen, as described in Materials and Methods, consistently resulted in a CD133-hi fraction that was <10% of the total starting pool.

To confirm the relative tumorigenicity of CD133-hi and CD133-lo subpopulations found in previous studies (23, 24), we seeded them into the i.p. cavity of SCID mice. Injection of these carcinoma cells within the same cultures of cells obtained from the ascites of animals receiving CD133-hi cells failed to yield significant numbers of viable cells, whereas large numbers of cells were obtained from the ascites of animals that had been injected with CD133-hi cells (e.g., see Fig. 6B). We analyzed the cells obtained from ascites of animals receiving CD133-hi cells by immunocytochemistry and found that most cells were positive for CD44 but few cells stained for CD45, indicating that most cells were not macrophages or other cells from the circulation (Fig. 1D). These results confirmed the efficacy of CD133 as a marker for identifying cells with high tumorigenic capacity, as previously reported (23, 24).

Elevated expression of drug transporters, lactate transporters, RTKs, emmprin, and hyaluronan in CD133-hi primary ovarian carcinoma cells. In past studies we and others found that the RTKs ErbB2 and EGFR (16, 19, 30), the ABC drug transporters P-glycoprotein/ABCB1 (Pgp) and breast cancer resistance protein/ABCG2 (BCRP; refs. 20, 31, 32), MCT1 and MCT4 (21), and the matrix metalloproteinase inducer emmprin (21), are present in close association with CD44 in the plasma membrane of various types of malignant tumor cell lines. Consequently, we examined the distribution of Pgp, BCRP, MCT4, EGFR, emmprin, and CD44 by immunostaining of the ovarian carcinoma cells. We found that each type of protein is enriched in the CD133-hi cells as compared with CD133-lo cells within the same cultures of cells obtained from patients R1, R2, and R3 (Figs. 2 and 5A). Closer examination showed that in each case, these proteins were concentrated in the plasma membrane of the CD133-hi cells (see below). Similar results were obtained with ErbB2 and MCT1 (not shown). Previous studies showed that organization of these various proteins in the plasma membrane of tumor cell lines is dependent on hyaluronan–CD44 interaction (16, 19–21, 30), and that in some cases they are stimulated by increased hyaluronan production (18, 19). Thus, we also measured the amounts of hyaluronan secreted by CD133-hi and CD133-lo cells, after separating the cells by magnetic sorting. We found 2.5- to 5-fold more hyaluronan in media from cultures of the CD133-hi subfractions from patients R1, R2,
and R3, when compared with the corresponding CD133-lo subfractions (Fig. 3A).

To confirm the results obtained by immunostaining, we also used Western blotting to measure the relative amounts of the various proteins, after separating patient R1 cells by magnetic sorting. As expected, CD133 was highly enriched in the CD133-hi subfraction compared with the CD133-lo sub-fraction (Fig. 3B-D). In addition, we found that expression of lactate transporters (MCT1 and MCT4), ABC-family drug transporters (Pgp and BCRP), RTKs (ErbB2 and EGFR), and emmprin is elevated in the CD133-hi fraction when compared with the CD133-lo fraction (Fig. 3B-D). Unexpectedly, we did not find significant differences in levels of CD44 between the two fractions (Fig. 3B).

Colocalization of transporters and RTKs with CD44 in CD133-hi primary ovarian carcinoma cells and disruption by treatment with hyaluronan oligomers. Examination of cells from patients R1, R2, and R3 by immunostaining indicated enrichment of multidrug transporters and MCTs in the plasma membrane of CD133-hi cells when compared with their CD133-lo counterparts (Fig. 2A-C). Confocal analysis of these unsorted, immunostained carcinoma cells showed clearly that membrane localization of Pgp (red; Fig. 4A) and BCRP (red; Fig. 4B) is elevated in CD133-hi versus CD133-lo cells. Furthermore, CD44 (green) was found to colocalize with Pgp and BCRP in the membrane of the CD133-hi cells to a much greater extent than in the CD133-lo cells (Fig. 4A and B), although the total amounts of CD44 were similar in both cell types (Fig. 3B). Similar results were obtained when the cells were stained for CD44 and MCT4 (Fig. 5C) or MCT1 (data not shown). In addition to these transporters, we found that the RTKs, EGFR is codistributed with CD44 in CD133-hi cells (Fig. 5A) and is colocalized with CD44 in the plasma membrane of these cells (Fig. 5B). Similar results were obtained with ErbB2 (not shown).

We have previously found that treatment of malignant cell lines with an antagonist of hyaluronan-CD44 interaction, i.e., hyaluronan oligomers, results in dissociation of CD44-drug transporter and CD44-MCT complexes from the plasma membrane, internalization of the respective transporters into the cytoplasm, and loss of their function (20, 21). In analogous fashion, treatment of the ovarian carcinoma cells for 1 hour with hyaluronan oligomers resulted in dissociation of Pgp (Fig. 4A), BCRP (Fig. 4B), and MCT4 (Fig. 5C) from CD44 at the plasma membrane of CD133-hi cells and internalization into the cytoplasm. We obtained similar results with MCT1 (not shown) and the RTKs, EGFR (Fig. 5B) and ErbB2 (not shown). Emmprin, which is associated with drug resistance in cancer cells (15) and is highly expressed in malignant ovarian carcinomas (33), is also enriched in CD133-hi cells and colocalizes with CD44 at the plasma membrane (Figs. 2D and Fig. 5D). However, treatment with hyaluronan oligomers had little or no effect on membrane localization of emmprin (Fig. 5D), in similar fashion to our previous findings in a breast carcinoma cell line (21).

Because the CD133-hi cells express higher levels of Pgp than the CD133-lo cells, we compared the ability of magnetically

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**Fig. 3.** Elevated expression of hyaluronan, signaling proteins, and transporter proteins in CD133-hi primary ovarian carcinoma cells. A, ovarian carcinoma cells from patients R1, R2, and R3, magnetically sorted for CD133, were plated in 6-well dishes for 24 h. Conditioned media were collected and hyaluronan was quantified by an ELISA-like assay. Results were normalized to cell number. Error bars for R1 cells, SD of the mean of three separately sorted batches; significant differences (***, P < 0.001) between CD133-lo and CD133-hi cells were observed. Error bars for R2 cells, range of two measurements. Due to limitations in amounts of primary material, the statistical significance of differences could not be determined in R2 and R3 cells because n < 3; however, the differences obtained were large. B to D, ovarian carcinoma cells from patient R1, magnetically sorted for CD133, were plated in 6-well dishes for 24 h. Western blot analysis of whole cell lysates (50 μg/lane) showed elevated expression of CD133, MCT1, MCT4, Pgp, BCRP, ErbB2, EGFR, and emmprin in CD133-hi cells. Expression of CD44 (B) was approximately equivalent in CD133-hi and CD133-lo cell lysates. B, blot obtained with lysates from one batch of sorted cells. C and D, two separate blots obtained with lysates from a second sort.
sorted CD133-hi and CD133-lo cells to efflux FURA 2-AM, a fluorescent substrate for Pgp. In cells expressing Pgp, intact FURA 2-AM is rapidly transported out of the cell, whereas in the absence of Pgp it cleaves to FURA-2 and accumulates in the cytosol (26, 34). Thus, transporter activity is inversely proportional to accumulation of cytosolic FURA-2 fluorescence. We found that CD133-hi cells efflux higher levels of FURA-2 than their CD133-lo counterparts, indicating that transporter activity is indeed greater in the CD133-hi cells (Fig. 4C). Because treatment with hyaluronan oligomers caused internalization of Pgp, we also measured the effect of the oligomers and found that they caused accumulation of FURA-2 in the cytoplasm (Fig. 4D), indicating that they inhibit transporter activity, as found previously in malignant cell lines (20).

Inhibition of CD133-hi ovarian carcinoma growth by hyaluronan oligomer treatment in vivo. As described above, we found that CD133-hi cells gave rise to tumors after injection of relatively low cell numbers but CD133-lo cells failed to give tumors, even with inoculates of high cell numbers (Fig. 1). We tested the effect of hyaluronan oligomers on the growth of tumors initiated by injection of CD133-hi cells. Animals were injected i.p. with 10⁷ CD133-hi cells. Treatment with PBS or hyaluronan oligomers in PBS, by i.p. injection, was initiated after allowing these cells to grow in vivo for a week. Treatment continued subsequently once per week for 6 weeks. Analyses of ascites accumulation (Fig. 6A) and numbers of cells obtained from the ascites after 7 weeks (Fig. 6B) indicated that the hyaluronan oligomers abrogate tumor growth.

Discussion

The origins and defining characteristics of solid tumor cancer stem/tumor-initiating cells are controversial, and these cells may simply represent a highly malignant, drug-resistant tumor cell subpopulation within a hierarchy of cells with various levels and combinations of oncogenic characteristics (4–6). Nevertheless, increased understanding of the properties of this subpopulation of cells may provide important insights into tumor malignancy and resistance to therapy. Several groups have isolated cells with the apparent properties of tumor-initiating cells from ovarian carcinoma cell lines or primary ovarian tumors (7, 23, 24, 35, 36). CD133 has been shown to be an effective marker for identifying these cells from ovarian carcinomas (23, 24), as has the combination of CD44 and CD117 (c-kit; ref. 7), albeit no single marker or combination of markers is likely to be completely selective (5). Although CD44 and CD133 are commonly used to identify tumor-initiating cells from a variety of tumor types (4), little is known about their functions in these cells. In this study we have examined the potential role of interactions of CD44 with its major ligand, hyaluronan, in the drug-resistant and tumorigenic properties of a subpopulation of ovarian carcinoma cells that were isolated using CD133 as a marker. We have found that cells with high levels of CD133 express elevated levels of hyaluronan, emmprin, RTKs (ErbB2 and EGFR), MCT-1 and MCT-4, and ABC-family multidrug transporters (Pgp and BCRP), compared with cells with low levels of CD133. Although CD44 is present in similar amounts in CD133-hi and CD133-lo cells, CD44 is enriched in the plasma membrane of CD133-hi cells, and is colocalized with the RTKs, MCTs, and ABC transporters in the membrane of CD133-hi cells but not CD133-lo cells.

Past studies using coimmunoprecipitation and confocal microscopy have provided strong evidence for association of CD44 subfractions with RTKs, MCTs, or ABC transporters, and most of these studies have shown dependence of these interactions on binding of hyaluronan to CD44 (16, 19–21, 21, 22). However, increased understanding of the properties of this subpopulation of cells may provide important insights into tumor malignancy and resistance to therapy.
In this study, we probed the role of hyaluronan-CD44 interactions in CD133-hi cells by treating the cells for a short period of time (1 hour) with small hyaluronan oligomers. Past studies have shown that these oligomers, which interact monovalently with CD44, compete for multivalent, polymeric hyaluronan-receptor interactions (37–40) and mimic the effects of other antagonists of hyaluronan-CD44 interaction, such as soluble hyaluronan-binding proteins and antibodies or small interfering RNA (siRNA) against CD44 (19, 27). In addition, the oligomers inhibit hyaluronan synthesis but this effect requires a much longer time period (20). We found that treatment of the CD133-hi cells with hyaluronan oligomers...

Fig. 5. Colocalization of EGFR and MCT4 with CD44 in CD133-hi cells and disruption by hyaluronan oligomer treatment. A, low-power confocal images of unsorted ovarian carcinoma cells from patients R2 and R3 that were plated on chamber slides for 24 h and stained for CD44, CD133, and EGFR. CD133-lo cells, which comprised the majority of cells, showed dispersed staining for CD44 and low levels of EGFR. CD133-hi cells stained strongly for CD44 and EGFR. B to D, higher-power images of unsorted ovarian carcinoma cells from patient R1, which were plated on chamber slides for 24 h, treated with and without 100 μg/mL hyaluronan oligomers for 1 h, and then processed for immunostaining. Cells were stained for CD44 (green); for EGFR (B), MCT4 (C) or emmprin (D; red); and for CD133 to distinguish CD133-hi and CD133-lo cells (not shown). CD133-hi and CD133-lo cells were visualized by confocal microscopy at a z-plane corresponding to the approximate center of the cell. Note colocalization (yellow) of CD44 with EGFR, MCT4, and emmprin at the plasma membrane of untreated CD133-hi cells and internalization of EGFR (B) and MCT4 (C), but not emmprin (D), in hyaluronan oligomer-treated CD133-hi cells. Similar results were obtained for MCT1 and ErbB2 (not shown). Note that internalization of MCT4 is difficult to visualize here due to dispersion throughout the cytoplasm, but is clearly visible on increasing gain (e.g., see ref. 21). Increased gain did not reveal intracellular emmprin. Arrows, areas of the plasma membrane and adjacent cytoplasm shown at higher magnification in the insets.

Fig. 6. Hyaluronan oligomer treatment inhibits growth of CD133-hi ovarian carcinomas in vivo. CD133-hi cells (10⁶), obtained by magnetic sorting for CD133, were injected in Matrigel into the peritoneum of SCID mice at day 0. Beginning 1 wk after injection of cells, PBS (200 μL) or hyaluronan oligomers (500 μg; 25 mg/kg) in PBS were injected i.p. once per week for 6 wk. A, tumor growth, as assessed by percent change in weight due to ascites accumulation, was calculated as in Fig. 1C. B, numbers of cells that accumulated i.p. after 7 wk. Error bars, SD of the mean of results from four or more animals. Significant differences were observed between treated and untreated animals as determined by Student’s t-Test; *, P < 0.05 (A); **, P < 0.01 (B).
inhibits association of CD44 with the RTKs, MCTs, and ABC transporters, induces their internalization, reduces drug transporter activity, and inhibits the capacity for tumor growth in the CD133-hi cells. We showed previously that treatment with hyaluronan oligomers also inhibits tumor growth by a highly malignant, drug-resistant subpopulation of C6 rat glioma cells prepared by the side-population method (29). These results strongly support an important role for hyaluronan-CD44 interactions in the characteristic properties of highly tumorigenic subpopulations such as tumor-initiating cells. However, this study has also highlighted another important point, i.e., that hyaluronan-dependent organization of CD44 into membrane complexes is more likely to be significant for tumor-initiating cell properties than levels of CD44 expression.

The usefulness of CD44 as a marker of malignant properties has been a dilemma for many years. For example, in some studies evidence was obtained for CD44 as an essential participant in metastasis (41, 42), whereas in other studies opposing relationships have been documented (43). Likewise, although CD44 has been useful as a marker for tumor-initiating cells in many human carcinomas, it has not been useful for several other human cancers (4). One obvious reason for this discrepancy is that CD44 is widely expressed in numerous cell types, and that in tumors such as gliomas most of the cancer cells express CD44. Despite this, certain subfractions of CD44-expressing human tumor cells reproducibly possess malignant or tumor-initiating cell characteristics, especially in human carcinomas and carcinoma cell lines (44–47). We find that, although CD133-hi and CD133-lo subfractions of ovarian carcinoma cells express similar levels of CD44, only in CD133-hi cells is CD44 localized to plasma membrane complexes containing RTKs and transporters. Moreover, constitutive multivalent interactions of CD44 with hyaluronan seem to be crucial because the small, monovalent hyaluronan oligomers destabilize these complexes and inhibit tumor progression in vivo. Moreover, even though CD44 has not been a useful marker for identifying mouse carcinoma tumor-initiating cells (4), overexpression of an antagonist of hyaluronan-CD44 interactions, in this case soluble CD44, inhibited mouse mammary carcinoma growth (48) and lung colonization (49) in a highly malignant syngeneic mouse model; a mutant form of soluble CD44 defective in hyaluronan binding did not have these effects, indicating that hyaluronan interactions were involved. These results suggest that the organizational status rather than level of expression of CD44 is critical to malignant and tumor-initiating cell phenotypes and that hyaluronan-CD44 interaction is required to activate this status.

The question remains how hyaluronan-CD44 interaction leads to organization of signaling complexes. In past studies it has been shown that cell-autonomous hyaluronan-CD44 interactions regulate several oncogenic pathways that promote malignant and drug-resistant properties of cancer cells (8, 15). Several studies suggest that hyaluronan-CD44 interactions influence these pathways by stabilizing RTK and transporter-containing signaling complexes in the plasma membrane of malignant cells (16, 17, 19–21, 30). Moreover, emmprin, which is highly expressed in ovarian carcinomas (33) and is enriched in the CD133-hi cells, cooperates in the assembly or stabilization of these complexes (19, 21). At this point, it is not clear whether these widespread effects of hyaluronan-CD44-emmprin interactions on complex formation are due to unique involvement of specific subfractions of CD44 splice variants with each complex, general interactions with membrane subcompartments, or global effects of a hyaluronan-based pericellular matrix. However, considerable evidence points towards the involvement of CD44 variants (16, 21) and CD44-mediated complex assembly within lipid microdomains (17, 19, 50). Despite these caveats, the present study suggests that these interactions drive the properties of tumor-initiating cells by stabilizing oncogenic complexes, and may explain why cell surface CD44 is so commonly associated with these cells.

In summary, our results indicate that hyaluronan-CD44 interactions have functional importance in the properties of highly tumorigenic subpopulations of ovarian carcinoma cells and that utilization of antagonists of these interactions, such as small hyaluronan oligomers, may provide an improved therapeutic approach.

Disclosure of Potential Conflicts of Interest

B.P. Toole, inventor on patent related to article. The other authors declare they have no conflicts.

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References


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