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HYALURONIDASE REDUCES HUMAN BREAST CANCER XENOGRAFTS IN SCID MICE

Svetlana SHUSTER[1], Gregory I. FROST[2], Antonei B. CSOKA[1], Bent FORMBY[3] and Robert STERN[1]*

[1]*Department of Pathology, School of Medicine, University of California, San Francisco, San Francisco, CA, USA*
[2]*Sidney Kimmel Cancer Center, San Diego, CA, USA*
[3]*The Rasmus Institute for Medical Research, Santa Barbara, CA, USA*

A hyaluronan-rich environment often correlate with tumor progression. and may be one mechanism for the invasive behavior of malignancies. Eradication of hyaluronan by hyaluronidase administration could reduce tumor aggressiveness and would provide, therefore, a new anti-cancer strategy. Hyaluronan interaction with its CD44 receptor and the resulting signal transduction events may be among the mechanisms for hyaluronan-associated cancer progression. We have shown previously that hyaluronidase treatment of breast cancer cells *in vitro* not only eradicates hyaluronan but also modifies expression of CD44 variant exons of tumor cells. We now determine if such effects occur *in vivo* and if it is accompanied by tumor regression. SCID mice bearing xenografts of human breast carcinomas were given intravenous hyaluronidase. Tumor volumes decreased 50% in 4 days. Tumor sections showed decreased hyaluronan. Intensity of staining for CD44s was not affected, whereas staining for specific CD44 variant exon isoforms was greatly reduced in residual tumors. Necrosis was not evident. Hyaluronidase, used previously as an adjunct in cancer treatment, presumably to enhance penetration of chemotherapeutic drugs, may itself have intrinsic anti-cancer activity. Removing peritumor hyaluronan appears to cause an irreversible change in tumor metabolism. Continuous hyaluronan binding to CD44 variant exon isoforms may also be required to stabilize inherently unstable isoforms that participate perhaps in tumor progression. Further investigation is required to confirm a cause and effect relationship between loss of hyaluronan, changes in CD44 variant exon expression and tumor reduction. If confirmed, hyaluronidase may provide a new class of anti-cancer therapeutics and one without toxic side effects.

Key words: *Hyaluronan; hyaluronidase; extracellular matrix; CD44; breast cancer; cancer xenografts; SCID mice*

Hyaluronan, a non-sulfated glycosaminoglycan polymer, is prominent in the extracellular matrix surrounding cancer cells, as well as normal cells undergoing rapid turnover and movement. During embryonic development, hyaluronan is elevated early, during the phase of migration and expansive growth of pluripotential cells. Degradation of the hyaluronan-rich matrix by hyaluronidase corresponds to the cessation of cell movement and the onset of differentiation.[1] Hyaluronan promotes growth. and spread of tumor cells. Levels on the surface of tumor cells often correlate with invasive and metastatic behavior.[2,3] Elimination of pericellular hyaluronan by hyaluronidase may be a mechanism for reducing tumors and slowing malignant progression, as has already been documented in some experimental tumor systems.[4,5] Hyaluronidase may function therefore as a new class of anti-cancer agent. This potential treatment modality would have few toxic side effects. An LD_{50} could not be achieved in cats and dogs despite administration of increasing doses by either oral or intravenous routes, as indicated by the specifications of the drug insert for a clinical preparation of hyaluronidase (Wydase®).

The large volume of water of hydration associated with hyaluronan creates spaces through which cells move, while simultaneously conferring motility upon cells. Mechanisms for the cell motility involve binding of hyaluronan to its CD44 receptor. The intracellular component of CD44 interacts with the cytoskeleton,[6] stimulating motility and various signal transduction pathways.[7,8]

CD44, a transmembrane glycoprotein, is the prominent receptor for hyaluronan.[9] It exists in a number of isoforms, products of a single gene[10] generated by alternative splicing of variant exons inserted into a single extracellular membrane-proximal site.[11] Several splice variants of CD44 have been associated with malignant progression, presumably through their ability to enhance the invasive motility of tumor cells. Correlation of particular CD44 variant exons with clinical prognoses are claimed, though no consistent patterns have been observed.[12] We postulate that hyaluronan acts in concert with a specific array of variant exons of CD44 in the ability to promote cancer growth and spread and that the putative anti-cancer effect of hyaluronidase might be coordinated with a loss of such CD44 exons together with the eradication of hyaluronan.

We and others have shown *in vitro* that degradation of cancer cell-associated hyaluronan by hyaluronidase can modulate CD44-splice variant expression.[13,14] Such studies are extended here to *in vivo* experiments. Hyaluronidase was administered to SCID mice bearing human breast cancer xenografts, to determine the effects on such tumors *and* to examine the effect on expression of CD44 and its exon splice variants.

MATERIAL AND METHODS

Preparation of hyaluronidase

Bovine testicular hyaluronidase (PH-20) was preparation Type VI-S, a product of the Sigma (St. Louis, MO). The testicular hyaluronidase was purified to near homogeneity using 2 sequential chromatographic steps: Concanavalin-A and Mono-S cation exchange. The purified enzyme had 2 bands on SPAGE and corresponded to 2 bands of activity on hyaluronan-substrate gel electrophoresis,[15] as shown in Figure 1 (*lane 1*). The 2 bands may represent the 2 forms of the enzyme, related to the soluble and membrane-bound forms of PH-20.[16,17] Separation of the 2 forms of the enzyme, the 75 and 58 kDa bands, was achieved by tandem gel filtration on Superose 12. Single bands were obtained on SPAGE, indicating that there was not equilibrium between the 2 forms of the enzyme (Fig. 1, *lanes 2,3*). The preparation used for administration to animals contained both enzyme isoforms. The purified material had a range of specific activities of from 80,000–105,000 relative turbidity reducing units (rTRU)/mg protein, a unit of enzyme activity that has been described previously.[18] In the

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*Correspondence to: Robert Stern, MD, Department of Pathology, School of Medicine, UC San Francisco, San Francisco, CA 94143-0511. Fax: +415-476-5669. E-mail: rstern@itsa.ucsf.edu

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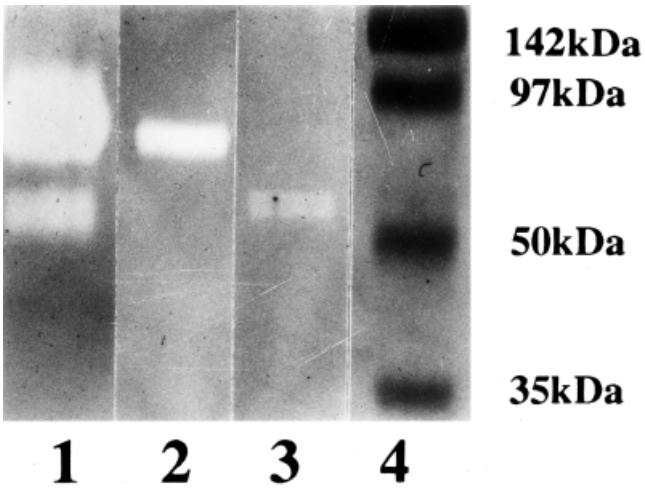

FIGURE 1 – Hyaluronan-SDS substrate gel electrophoresis of purified testicular hyaluronidase. The partially purified enzyme preparation was subjected to substrate gel electrophoresis. Approximately 0.05–0.50 rTRU of hyaluronidase enzyme activity were electrophoresed in the presence of SDS under non-reducing conditions in a 10% polyacrylamide gel containing hyaluronan. After electrophoresis, gels were incubated overnight in Na formate buffer, pH 4.5, in 0.1% Triton X-100 detergent, to substitute the SDS. Gels were then stained for carbohydrate with Alcian blue followed by Coomassie blue. The cleared bands in *lanes 1–3* represent the positions of the hyaluronidase that had hydrolyzed the hyaluronan within the gel. The hyaluronidase preparation, purified by sequential Concanavalin-A and Mono S cation exchange chromatographies (*lane 1*), was further separated into two peaks of activity by Superose 12 gel filtration, *lanes 2* and *3*, representing proteins of 75 and 58 kDa molecular size, respectively. *Lane 4* contains prestained molecular weight markers. The enzyme preparation used in these experiments was material shown in *Lane 1*, containing the 2 peaks of enzyme activity. The two enzymes contained in the PH-20 preparation may represent PH-20 plus a second-neutral-active enzyme. HYALP1, a pseudogene, is one of the six hyaluronidase-like genes in the human, one of the three on 7q31.3. The mRNA transcription contains an aberrant stop codon, and is not translated into protein. However, active enzyme is present in the mouse, and possibly in other mammals, such as bovine. This observation may also explain why the mouse with a null mutation in the PH-20 gene is fertile. (Baba et al., Mouse sperm lacking cell surface hyaluronidase PH-20 can pass through the layer of cumulus cells and fertilize the egg. J. Biol. Chem, in press).

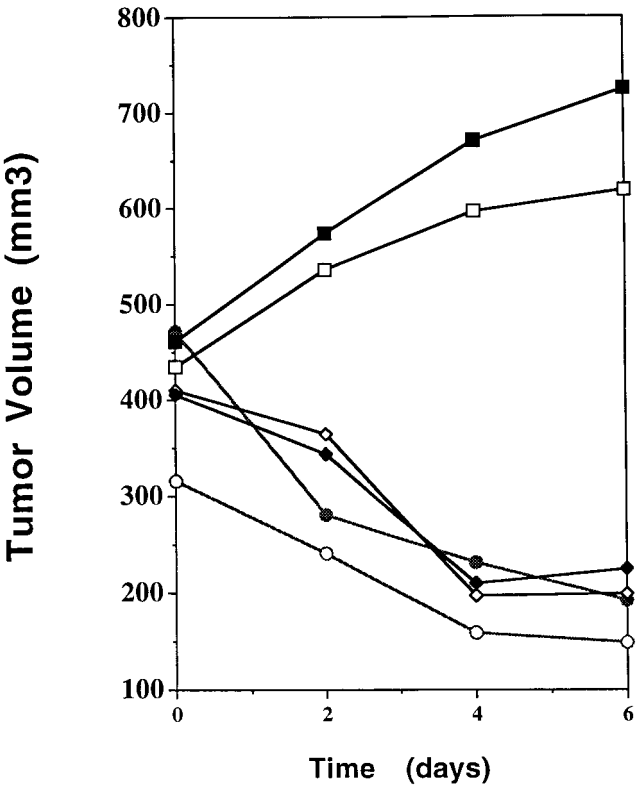

FIGURE 2 – Effect of hyaluronidase treatment on tumors in SCID mice derived from human breast carcinoma cell line MDA435. Reduction in size of established tumors was observed after hyaluronidase administration *in vivo*. The MDA435 cells, 5×10^6 cells in each inoculum, produced tumor nodules of ~400 mm^3 within 3 weeks. Hyaluronidase or saline was then injected using an i.v. route (retro-orbitally). Open and closed squares represent tumor volumes in the 2 control animals receiving saline treatment; the open and closed circles, the 2 animals injected with 300 rTRU of hyaluronidase on Day 0; the open and closed diamonds, the 2 animals injected with 75 rTRU on Days 0, 2, 4 and 6. Tumor volumes were determined every second day. These experiments were repeated several times, with a total of 17 control and 25 hyaluronidase-treated animals. Consistent reductions in tumor sizes were obtained.

ELISA-like assay for enzyme activity,[19] a standard hyaluronidase preparation from bovine testes, Wydase® (Wyeth-Ayerst Labs., St. Davids, PA) was used as a reference.

Cell culture

The human breast cell lines MDA435 was obtained from the American Type Culture Collection. The cells were grown routinely in RPMI-1640 in the presence of 10% FCS in a humidified chamber with 5% CO_2. The breast cancer cell line had a hormone-containing mixture added. This culture media addition contained a final concentration of hydrocortisone, 0.5 μg/ml, β-estradiol and progesterone, each at 1 ng/ml, transferrin, 1 ug/ml, triiodo L-thyronine, 10 μM, insulin, 0.5 μg/ml and 2 mM glutamine. Flasks of cells were scraped with a rubber policeman and cells gently centrifuged, rinsed with media and recentrifuged before inoculation. An additional flask was used for cell counts, in which case, cells were trypsinized.

For experiments in which cells were pretreated with hyaluronidase, cells were incubated in this same medium in the presence and absence of purified testicular hyaluronidase, 100 rTRU/ml diluted in the culture medium.

In vivo *experiments*

For each inoculum, 5×10^6 tumor cells, concentrated by centrifugation, were suspended in media (1:1) with Matrigel (Collaborative Biomed, Bedford, MA). This suspension was inoculated orthotopically into the mammary fat pad of 6-week-old homozygous, female ICR SCID mice (Taconic Farms, Inc., York, ME). Tumors were measured in 3D by digital caliper. All experiments were carried out with IACUC approval.

Histochemistry of hyaluronan

For the histolocalization of hyaluronan, a biotinylated hyaluronan-binding peptide was utilized, derived from a trypsin digestion of bovine nasal cartilage. The peptide was isolated by affinity chromatography using a column of hyaluronan-Sepharose.[20] Staining was similar to that described previously.[21] Slides were incubated in 3% BSA for 30 min, rinsed with PBS-CMF and then 0.3 ml of the diluted biotinylated hyaluronan binding peptide solution (1:200 of a 0.5 mg/ml preparation) and incubated overnight at 4°C. Slides were then washed for 10 min with calcium- and magnesium-free PBS. Endogenous peroxidase activity was blocked using 0.6% hydrogen peroxide in methanol and incubating for 30 min at room temperature. Slides were rinsed for 20 min in PBS-CMF and then incubated for 45 min with the avidin-labeled horseradish peroxidase solution, prepared as specified by the manufacturer (Vecstatin ABC Peroxidase Kit PK-4000, Vector, Burlington, CA). Slides, washed for 15 min in PBS-CMF, were incubated for five min in peroxidase substrate solution (Peroxidase Substrate Kit,

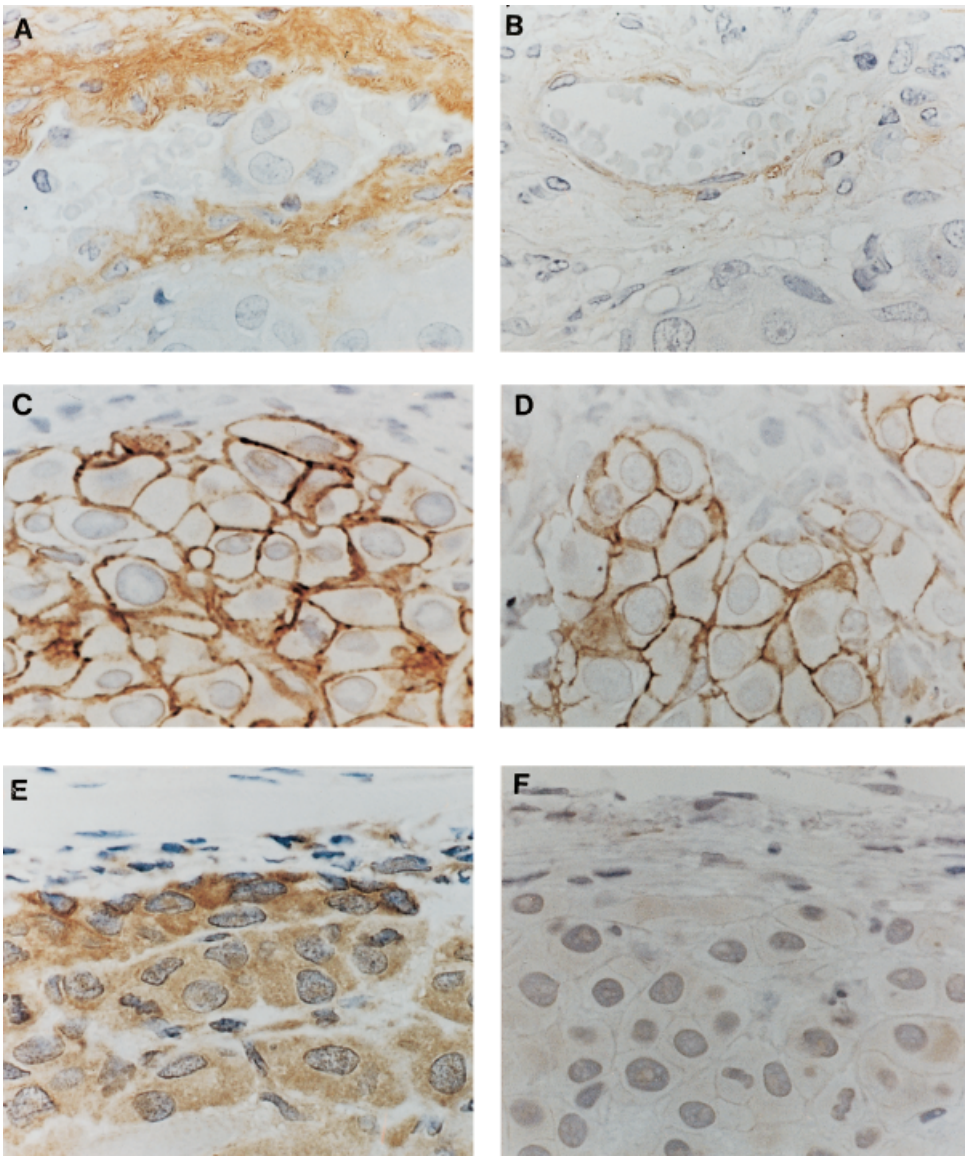

Figure 3 – Staining of xenograft breast tumor sections. Tumors were obtained from SCID mice bearing human tumors derived from the breast carcinoma cell line MDA435. Sections were stained for HA, CD44s and CD44 variant v7-8. Tumors were harvested on Day 12 after the start of hyaluronidase treatment. Treatment consisted of 4 i.v. injections of 75 rTRU of hyaluronidase on Days 0, 2, 4 and 6. Histolocalization of HA using the biotinylated hyaluronan-binding peptide in (*a*) untreated mice with mock inoculation and (*b*) after hyaluronidase treatments. Immunolocalization of CD44s using monoclonal antibodies in (*c*) untreated mice and (*d*) after hyaluronidase treatment. Immunolocalization of CD44 variant v7-8 in (*e*) untreated mice and (*f*) after hyaluronidase treatment. Magnification = ×200.

DAB SK-4100, Vector), washed for 5 min in tap water, counterstained with hematoxylin, cleared and mounted with a coverslip. For controls, slides were either preincubated with bacterial hyaluronidase in a solution containing 0.1 M sodium acetate, pH 5.0, 50 mM NaCl, 0.1 mg/ml bovine serum albumin and 100 rTRU/ml *Streptomyces* hyaluronidase (Calbiochem, San Diego, CA), or by the preincubation of the HABP with excess HA before application to the slides. Each of these controls gave comparable results.

Immunolocalization of CD44

For immunohistochemistry, slides were incubated in 3% goat serum (Vector) in PBS for 30 min at 37°C to block non-specific binding sites. Slides were then incubated with the primary monoclonal antibodies. Two antibodies were used, BM-CD44-UN [BMS 113] for anti-CD44s and BM-CD44-V7V8 [BMS 118] for anti-CD44 v7-8, both from BioSource International (Camarillo, CA), at a dilution of 1:30 in 3% goat serum in PBS-CMF for 1 hr at 37°C and then overnight at 4°C. Slides, washed for 10 min in PBS-CMF, were then incubated with biotinylated goat anti-mouse IgG (Vector) diluted 1:100 with 3% normal goat serum in PBS-CMF for 45 min at room temperature. Slides (were again washed, as above. Endogenous peroxidase activity was blocked as above using 0.6% hydrogen peroxidase in methanol, incubating for 30 min at room temperature. Slides were then processed as described above following the endogenous peroxidase blocking reaction. For negative controls for CD44 v7-8, mouse IgG2A from BioSource International was utilized at a dilution of 1:150 in 3% goat serum in PBS-CMF. Dilutions were used that provided comparable final protein concentrations.

Photographs were taken on an Olympus Vanox AHBT3 Microscope fitted with an integrated Olympus C-35 AD-4 camera, using Kodak Gold Plus 100 film.

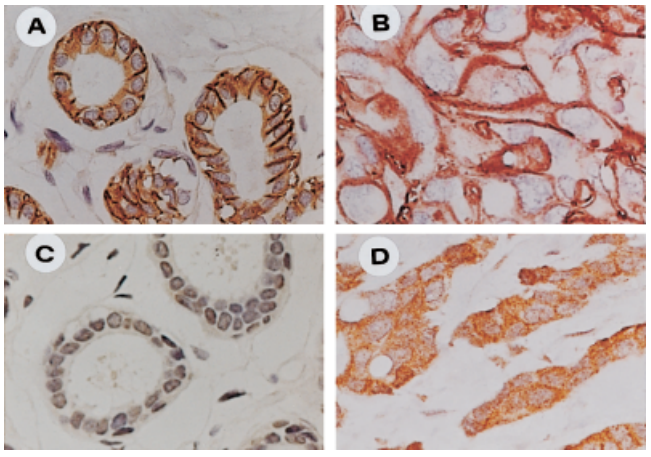

FIGURE 4 – Staining of normal and malignant human breast tissue. The infiltrating ductal carcinoma was obtained from the formalin-fixed, paraffin embedded archival material of the Department of Pathology (UC San Francisco). Normal breast tissue was obtained at autopsy from an age-matched (48 y) subject, as control. Sections cut at 4 um were stained using monoclonal antibodies specific for CD44s (*a*,*c*) and for CD44v7-8 (*b*,*d*) from normal and cancerous tissue, respectively.

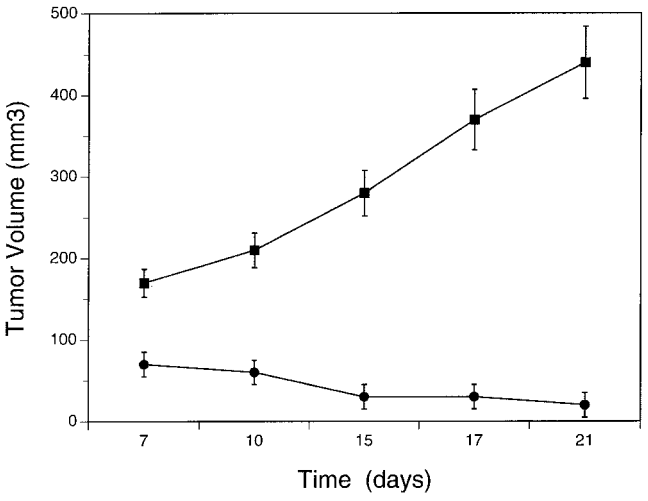

FIGURE 5 – Effect of *in vitro* hyaluronidase treatment on *in vivo* growth potential in SCID mice. The MDA435 cells were incubated *in vitro* for 20 hr in the presence or absence of 100 rTRU of purified testicular hyaluronidase. The cells were then inoculated into the mammary fat pads of 12 female mice, 5×10^6 cells in each inoculum. Six mice received the hyaluronidase pretreated cells and 6 mice received the mock preincubated cells. The sizes of the tumors were measured in 3D every other day using a micrometer caliper. Three animals from each group of 6 were sacrificed on Day 10 for examination of their tumors. The remaining animals continued to be observed until Day 21. The values given for tumor volumes represent the mean and SD for six animals in each group at Days 7 and 10 and for the remaining three animals in each group on Days 15, 17 and 21, closed squares for mice receiving mock-treated cells, closed circles for mice receiving the hyaluronidase pretreated cells.

RESULTS

The cultured human breast cancer cell line, MDA435, a well-characterized cell line readily forms xenograft tumors.[22] Cultured MDA435 cells were allowed to form tumors in the mammary fat pad of female SCID mice. In the presence of Matrigel, each cancer cell inoculum produced a tumor. These grew at a consistent and rapid rate, in marked contrast to earlier experiments carried out without Matrigel. Tumors did grow in the absence of Matrigel but at a much slower rate (data not shown). Tumor metastases were observed occasionally in such, reflecting more accurately the course of the human disease. There was great variability, however, in the rate of growth and in the size of tumors grown without Matrigel. Such a protocol would have required a far greater number of animals to obtain statistical significance. The decision was made, therefore, to proceed with tumors grown in the presence of Matrigel.

Tumors were measured in 3D by a micrometer caliper every other day and permitted to grow until they reached a size of approximately 400 mm^3 This took about 12–30 days. Purified testicular hyaluronidase was then injected intravenously by the retro-orbital route. A marked reduction in tumor size was observed. Pairs of animals were either injected 4 times with 75 rTRU every other day, or were given a single injection of 300 rTRU. In both cases, after 4 days, tumor volume had decreased by 50%. In the control animals, injected with comparable volumes of saline, tumors continued to grow in size (Fig. 2). The purified enzyme had no apparent toxic effects on these animals. No change in behavior was apparent, in grooming, eating, drinking, or respiration. The experiments were repeated three times and gave comparable results on each occasion. After 1 month, without further hyaluronidase administration, major differences continued to be observed between tumors in the treated and non-treated animals.

Sections from all tumors were taken for histologic examination. Tumors were harvested on Day 12 after the beginning of hyaluronidase treatment, as shown in Figure 2. Sections were stained for hyaluronan using the biotinylated hyaluronan-binding probe, for CD44s and for CD44 isoforms, using specific antibodies. The hyaluronan in tumors from untreated animals was predominantly in the stroma surrounding clumps of tumor cells (Fig. 3*a*). Such staining was much reduced in tumors from animals treated *in vivo* with hyaluronidase. The residual staining appeared to be associated with the endothelial cells of the tumor vasculature (Fig. 3*b*). Staining for CD44s was associated predominantly with tumors (Fig. 3*c*). This remained unchanged in the residual tumors taken from animals treated with hyaluronidase (Fig. 3*d*). A modest decrease in staining intensity was observed, but no change in the staining pattern. Staining for the CD44v7-8 isoform had a more diffuse pattern in tumor cells from sections taken from untreated animals, with very little staining observed in the surrounding tumor stroma (Fig. 3*e*). Such staining was largely eliminated from tumor nodules obtained from hyaluronidase-treated animals (Fig. 3*f*). Necrosis was not apparent in association with the small tumors or tumor nodules taken from the hyaluronidase-treated animals.

Archival material of human breast tissue from the Department of Pathology, UC San Francisco was utilized to compare breast cancer with the breast cancer xenografts growing in the SCID mice. A case of infiltrating ductal carcinoma was obtained. The block containing the primary lesion was sectioned at 4 um for immunohistochemistry. A block containing normal breast, obtained at autopsy from an age-matched control (48y) was also selected. Staining was observed for CD44s in both normal ductal epithelium (Fig. 4*a*) and clumps of infiltrating ductal carcinoma cells (Fig. 4*b*). Using antibodies specific for CD44v7-8, very little staining was observed in normal breast ducts (Fig. 4*c*), whereas intense staining was seen of breast ductal carcinoma cells (Fig. 4*d*). There is a similarity in patterns of staining of CD44s and CD44v7-8 between the untreated human xenograft (Fig. 3*c*,*e*) and actual human breast cancers (Fig. 4*b*,*d*), respectively.

The ability of the human breast cancer cells MDA435 to grow in SCID mice after *in vitro* hyaluronidase treatment was examined. Cultured cells were treated with purified testicular hyaluronidase by incubation with 100 U/ml of enzyme for 20 hr. Control cells were incubated in medium for the same length of time. breast cancer cells (5×10^6) were then inoculated orthotopically into the mammary fat pads. Cells that had been pre-incubated with hyaluronidase failed to form tumors subsequently in SCID mice,

whereas the untreated cells produced tumor nodules, reaching 400–500 mm^3 in 3 weeks (Fig. 5). Necropsy inspection of all animals failed to show metastatic spread of tumors.

DISCUSSION

Hyaluronidase administration to SCID mice bearing human breast tumor xenografts caused eradication of hyaluronan and rapid reduction in tumor size. The hyaluronan and CD44 staining patterns of the xenografts growing in nude mice and actual breast carcinomas were similar, suggesting that the SCID mouse tumor model may have relevance for human disease.

Hyaluronan is particularly prominent in the matrix of tissues and cells undergoing rapid proliferation,[23] including the matrix of malignancies.[24] Previously, it was shown that hyaluronidase treatment of cultured tumor cells caused eradication of hyaluronan, as well as several CD44 variant isoforms. The current experiments confirmed that CD44s is relatively resistant, whereas some variant exon-containing isoforms of CD44 are sensitive to enzyme treatment, intrinsically unstable isoforms requiring continuous hyaluronan for their expression.

The hyaluronidase treatment of tumor cells appears to have induced an irreversible change in cell cycle kinetics. There was sufficient time for these cells to have resynthesized a hyaluronan-containing matrix. In embryology, removal of hyaluronan initiates programs of differentiation,[1] a commitment that is irreversible. The same may be true of cancer cells. Dedifferentiation or a return to the hyaluronan rich pluripotential state does not appear to be an option. This may explain why changes in hyaluronidase treated cells continued to be observed for several weeks.

A number of observations support the model that hyaluronan promotes malignant progression.[2,25] Interaction of hyaluronan with CD44 enhances tumor survival and invasiveness.[26,27] Hyaluronan promotes maintenance of the undifferentiated oncogenic-like state,[28] and its removal initiates programs of differentiation.[1] Hyaluronan is an effective space-filler and tissue expander. This opens up intercellular spaces facilitating cancer cell movement. Eradication of hyaluronan disrupts matrix structures,[29,30] destabilizing the scaffolding on which tumor cells grow.

The binding of hyaluronan to the CD44 receptor promotes interactions with the cytoskeleton and initiates signal transduction pathways that support cancer growth.[6–8,31,32] Hyaluronan binds and concentrates growth factors such as TGF-β and protects them from proteolytic degradation.[33] The hyaluronan and CD44 interaction provides a cell surface docking receptor for metalloproteinase-9 that facilitates invasion and also activates latent TGF-β.[27,34] Hyaluronan provides an emergency matrix for tumor cells that have lost attachment to a basal lamina, cells that would otherwise undergo apoptosis.[35–39] Hyaluronan also protects cells from immune surveillance and from immune-mediated cytotoxic agents.[40,41]

Hyaluronan is taken up by cells,[42,43] and cancer cells take up more hyaluronan than do normal cells.[42] Hyaluronan is transported to the nucleus and nucleolus,[43–45] where it stimulates cell cycle progression. Some nuclear proteins involved in cell cycle progression[46] and RNA splicing[47] are hyaluronan-binding proteins.

Hyaluronidase has been utilized as an adjunct in anticancer chemotherapeutic regimens.[48–50] It is assumed to facilitate penetration and to decrease interstitial fluid pressure, permitting anticancer agents to reach malignant cells.[51] As demonstrated here, however, reduction in tumor size can occur in the absence of chemotherapeutic drugs, suggesting that hyaluronidase has intrinsic anticancer properties.

Experimental tumor models support this hypothesis. Hyaluronidase-treated mice exposed to carcinogens develop fewer and smaller tumors.[4] Growth rates of transplantable tumors in mice are inversely proportional to serum hyaluronidase.[5] In a mouse model for T cell lymphoma, hyaluronidase prevents tumor cell invasion of lymph nodes.[52] Hyaluronidase also blocks TNF-mediated cancer cell death, reverses multi-drug resistance,[53] and alters cell-cycle kinetics of chemo-resistant carcinomas.[54]

The hyaluronidase enzymes have recently been explored and much new information is now available.[55–57] There are 6 hyaluronidase-like sequences in the human genome, 3 each at 2 chromosomal locations, at 3p.21.3 and 7q31.3. Both regions map to tumor suppressor genes including those for lung[58] and breast cancer[59] at 3p21.3 and for lymphomas[60] and ovarian carcinomas[61] at 7q31.3.

All of these observations support the concept that accumulation of HA is an important step in the development of some malignancies and that hyaluronidase can function as an anti-cancer agent. Inconsistencies in this pattern, however, are also documented, suggesting that individual cancers utilize widely differing pathways during the process of malignant transformation and progression.[62–65]

There was no toxicity observed with the hyaluronidase treatment. The specification insert for the testicular hyaluronidase preparation available for clinical use (Wydase®) indicates that an LD_{50} in cats and dogs could not be achieved. Hyaluronidase thus is unlikely to have the toxic side effects encountered with most other anti-cancer agents.

The results of the current experiments are merely suggestive of a cause and effect relationship between hyaluronan levels, certain CD44 isoforms, malignant growth and progression and their reversal by hyaluronidase. Clearly, far greater numbers of animals are needed, to establish dose-response curves, variations in inoculation schedules, sites of inoculation, as well as to examine longer periods of treatment. Studies on such a magnitude are beyond the means of an academic laboratory. These preliminary experiments, however, suggest that hyaluronidase has the potential of providing a new class of anti-cancer therapeutics, and is worthy of further exploration.

ACKNOWLEDGEMENTS

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