Hyaluronan on the Surface of Tumor Cells Is Correlated with Metastatic Behavior

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ABSTRACT

In the present study, we examined the metastatic potential of tumor cells expressing different levels of cell surface hyaluronan. We used flow cytometry to isolate subsets of the B16-F1 mouse melanoma cell line that expressed either high (HA-H) or low (HA-L) amounts of hyaluronan on their surfaces. These two subsets of cells showed a 32-fold difference in the amount of cell surface hyaluronan, due to its rate of synthesis. However, these cell lines did not differ from each other with regard to their in vitro growth rates, susceptibility to natural killer-mediated cytotoxicity, or the expression of the cell surface proteins CD44, ICAM-1, and GMP-140. When these cells were injected s.c., they both formed s.c. tumors of approximately the same size. However, when injected into the tail vein of mice, the HA-H cells formed a greater number of nodules in the lungs and caused a faster rate of mortality than the HA-L cells. The presence of hyaluronan did enhance the interaction of the HA-H cells with cultured endothelial cells that expressed CD44. Thus, it is possible that enhanced interactions between hyaluronan and CD44 promoted the formation of tumor emboli which, in turn, increased the chances that the tumor cells would be trapped in the lungs. Taken together, these results suggest that hyaluronan may play a critical role in the process of tumor metastasis.

INTRODUCTION

HA represents one of the main structural components of the extracellular matrix. It is particularly abundant in connective tissues such as the dermis of the skin, lamina propria of mucous membranes, and in the adventitia surrounding blood vessels (1, 2). In these locations, HA is often associated with a variety of proteins to form a large, complex network, which is responsible for maintaining the space between cells.

HA is also located directly on the surfaces of cells. In the case of some cultured cells, this pericellular coat of HA can be visualized by its ability to exclude particles such as RBC (3, 4). This coat may also act as a physical barrier to protect cultured cells from viral infection (5, 6) and lysis by cytotoxic leukocytes (7, 8), although it is unclear whether the HA coat offers a similar type of protection in vivo. This HA appears to interact with binding protein on the cell surface, termed the hyaladherins (9, 10). In addition, the reducing terminal of HA may play a role in determining the metastatic behavior of tumor cells. We used repeating cell sorting by flow cytometry to isolate cell lines with high and low levels of HA on their surface (termed HA-H and HA-L cells, respectively). Subsequent characterization of these cells revealed that the HA-H cells were more highly metastatic than the HA-L cells, as judged by both number and size of metastases formed in the lungs after tail vein injection. Mice injected with the HA-H cells also had a shorter survival time. These results indicate that cell surface HA may play an important role in the metastatic process.

MATERIALS AND METHODS

Preparation of CD44-immunoglobulin. The CD44-immunoglobulin expression plasmid (23) was generously provided by Dr. Alejandro Aruffo (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA). Bacteria transfected with the plasmid were cultured in LB media containing 100 μg/ml of ampicillin, and the CD44-immunoglobulin plasmid was purified by using the Magic Maxi-preps kit (Promega, Madison, WI). The plasmids were then used to transfec T7 COS 7 cells using the DEAE dextran procedure (24). Following transfection, the COS 7 cells were cultured in serum-free DMEM. The media was collected from these cultures and passed through a 10 ml column of protein A-Sepharose 4B (Pharmacia, Piscataway, NJ), which was then washed extensively with 0.1 M NaHPO4 (pH 7.35) and immediately neutralized with 2 ml of 1 M Tris-HCl buffer (pH 9.0). This material was dialyzed against PBS, concentrated with a Centricon 10, and kept at 4°C until use.

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Cell Lines and Culture Conditions. A variety of cell lines derived from different forms of human cancers were obtained either from the ATCC (Rockville, MD) or were part of a collection maintained at the Bristol-Myers Squibb Pharmaceutical Research Institute. The cells were cultured in IMDM supplemented with 10% FCS.

Isolation of the HA-H and HA-L Cell Lines. B16-F1 cells (ATCC) were grown to 90% confluence in 100-mm plates, released from the plate with EDTA, and then centrifuged into a pellet containing about 5 × 10^6 cells. The cells were resuspended in a solution of CD44-immunoglobulin solution (100 μl of 100 μg/ml) and incubated at 4°C for 1 h. After washing, the cells were further incubated at 4°C for 45 min with FITC-labeled goat anti-human IgG (100 μl of 100 μg/ml; PharMingen, San Diego, CA). Following washing, the stained cells were kept on ice and processed by flow cytometry within 1 h.

The HA-H and HA-L cell lines were isolated using an electronic cell sorter (EPICS C; Coulter, Hialeah, FL). Typically, viable cells were separated by setting bitmap 1 to forward light scatter (reflection of cell size) and 90° light scatter (reflection of cellular granules). Among the living cells, 0.1—0.2% with highest or lowest fluorescence were chosen by bitmaps 2 or 3 and sorted at a flow rate of 15,000—25,000 cells/s. About 5000 cells with high or low levels of HA on their surfaces were collected in 1 ml of 90% IMDM and 10% FCS and cultured at 37°C in 5% CO_2. After growth expansion (generally 1 week), the cells were subjected to repeated rounds of sorting in which either the highest or lowest HA-expressing cell populations were collected and expanded. After eight repetitions, the resulting cells stably expressed either high or low levels of HA on their surfaces and were defined as the HA-H and HA-L, respectively.

Assay of Cell Surface HA. To determine the rate at which HA reappeared on the cell surface, 1 × 10^6 HA-H or HA-L cells were treated with 200 μl of 0.5 mg/ml bovine testicular hyaluronidase (Sigma Chemical Co., St. Louis, MO) at 37°C for 1 h to remove endogenous HA and washed extensively with serum-free IMDM. The enzyme-digested cells were suspended in serum-free IMDM and divided equally into several culture dishes. At various times thereafter, the cells were harvested, and the cell surface HA was stained with CD44-immunoglobulin as described above and fixed in 1% paraformaldehyde. The flow cytometric analysis of cell surface HA for each sample was done under identical conditions. To calculate the relative levels of fluorescence intensity, the 0-h sample was used as background or control level. The difference in the intensity was determined as follows: (a) the mean channel of the control was subtracted from that of each sample; and (b) this value was converted to linear fluorescence equivalence.

Measurement of Cell Growth Rate. Cell growth rate was measured by [3H]thymidine incorporation. The HA-H and HA-L cell lines were subcultured into 96-well plates at a concentration of 10^5 cells/well in IMDM with or without 10% FCS. Four h later, [3H]thymidine (0.5 μCi; New England Nuclear, Boston, MA) was added to each well, and at various times thereafter, the cells were collected with a LKB cell harvester. The amount of [3H]thymidine incorporation was measured with a scintillation counter. All experiments were done in triplicate.

Assay of Cell Surface Molecules. HA-H and HA-L cells (1 × 10^6) were harvested and stained at 4°C for 45 min with 10 μg of anti-mouse ICAM-1 (PharMingen, San Diego, CA), anti-mouse CD44 (PharMingen), GMP-140-immunoglobulin (generously provided by Dr. A. Aruffo), or an irrelevant IgG or CD7-immunoglobulin (as a control). After washing, the cells were mixed with 10 μg of appropriate FITC-labeled secondary antibody, incubated for 45 min, and washed. The fluorescence on the cell surface was analyzed by flow cytometry standardized with Coulter beads (Coulter Electronics, Hialeah, FL). Both the mean and peak channels of fluorescence intensity for each sample were determined.

To determine whether HA masks cell surface CD44, 2 × 10^6 HA-H or HA-L cells were incubated at 37°C for 1 h in the presence or absence of 10 μg/ml of bovine testicular hyaluronidase in serum-free IMDM. After washing three times with 4 ml PBS, the cells were incubated for 1 h at 4°C in 100 μl of 100 μg/ml rat anti-mouse CD44 antibody. The cells were washed and resuspended in 100 μl of 100 μg/ml of FITC-labeled goat anti-rat IgG (Calbiochem, La Jolla, CA) and incubated at 4°C for 45 min. After washing, the amount of CD44 staining on the surface of the enzyme-treated and untreated cells was determined by flow cytometry.

NK Cytotoxicity Assay. The NK cell assay was performed as described previously (24). Briefly, cells were prepared from spleens of C57BL/6 mice (Taconic) 48 h after i.p. injection of 1 mg/mice of poly I:C. The target cells were labeled with 200 μCi of 51Cr (New England Nuclear) overnight for the HA-H or HA-L cells or 1 h for YAC-1 cells (as a NK-sensitive control cell line; ATCC). The target cells (5 × 10^6 cells/well) were cocultured at 37°C with the spleen cells at ratios ranging from 1:200 to 1:3. After 4 h, the supernatants were collected, and the released 51Cr was measured with a gamma counter. The percentage of NK mediated lysis was calculated as:

\[
\% \text{ lysis} = \frac{\text{Experimental cpm} - \text{spontaneous cpm}}{\text{Maximal cpm} - \text{spontaneous cpm}} \times 100
\]

In the absence of NKs, the amount of spontaneous release was less than 25% of the maximum obtained by the addition of 1% Triton X-100. All experiments were done in triplicate.

Assay of Experimental Metastasis. HA-H and HA-L cells were injected into the tail veins of syngeneic C57BL/6 mice (2.5 × 10^6 or 10^7 cells/mouse, 4—5 mice/group). For the lung colony assay, mice were sacrificed 14 days later, and the lungs were fixed with Bouin’s solution. The visible tumor nodules were assessed under a dissecting microscope. For the survival study, the mice were monitored on a daily basis throughout the course of the experiment.

Adhesion Assay. For the cell adhesion assay, we used SV40-transformed mouse lymphoid endothelial cells, SVEC4–10 (25), from Dr. Michael Edelid (The Johns Hopkins University, Baltimore, MD). These cells express significant amounts of CD44. The cells were grown to confluence monolayers in 96-well flat-bottomed plates. The HA-H and HA-L cells were isotopically labeled by growing overnight in the presence of [3H]thymidine (100 μCi in 8 ml of complete medium). After washing, the cells were harvested with 0.02% EDTA, resuspended in IMDM, and added to the wells containing confluent monolayers of SVEC4–10 cells (5 × 10^4 cells/well). The cultures were incubated at 4°C with gentle shaking and after 45 min were washed four times with 200 μl/well PBS to remove the unbound cells. The bound cells were detached from the plate using 0.025% of trypsin (200 μl/well), and the radioactivity was measured with a scintillation counter.

In other assays, the suspended cells and/or the monolayers were pretreated with bovine testicular hyaluronidase (10 μg/ml; Sigma) or KM-114, a rat anti-mouse CD44 monoclonal antibody (20 μg/ml; ATCC) in 200 μl of IMDM medium. After 1 h at 37°C, the cells were extensively washed with serum-free IMDM and then used in the binding assay as described above. The percentage of binding was calculated as:

\[
\% \text{ binding} = \frac{\text{Experimental binding cpm/total added cpm}}{\text{Control binding cpm/total added cpm}} \times 100
\]

All experiments were repeated at least 6 times.

RESULTS

Isolation and Characterization of HA-H and HA-L Cell Lines. To examine the potential role of cell surface HA in the metastatic process, we selected high and low HA-expressing variants from a parent B16-F1 cell line, which is heterogeneous in nature (26—28). The amount of cell surface HA was detected with CD44-immunoglobulin, a fusion protein consisting of the HA-binding region of CD44 coupled to the constant region of human immunoglobulin (23). The cells stained with this probe were repeatedly selected by flow cytometry to separate both high- and low-expressing subpopulations. The resulting HA-H cells had approximately 32 times more cell surface HA than the HA-L cells (Fig. 1, A and B). This phenotype was stable in vitro for 2 months and in vivo for at least 1.5 months, according to follow-up observations by flow cytometry. Furthermore, treatment of the HA-H cells with bovine testicular hyaluronidase reduced the staining intensity to a level comparable to that of the
HA-L cells (Fig. 1, C and D). These results verify the specificity of the CD44-immunoglobulin probe.

The rate at which HA appeared on the surfaces of these cells was also examined. The two cell lines were treated with hyaluronidase to remove cell surface HA and then cultured in HA-free media (to avoid the binding of exogenous HA to the treated cells). At various time intervals, the amount of cell surface HA was evaluated using fluorescent staining with CD44-immunoglobulin. Fig. 2 shows that the rate at which HA reappeared on the surfaces of HA-H cells was approximately 15 times faster than that of the HA-L cells, and the HA-H cells regained their original HA level (before the digestion) on the cell surface within 20 h.

Growth Rates and Cell Surface Markers. While the HA-H and HA-L cell lines were selected solely on the amount of cell surface HA, it was possible that other properties might be sorted out as well. For example, the growth rate of cultured cells has been shown to influence the rate of HA synthesis (29, 30). To test this possibility, we examined the growth rate of the two cell lines. The two cell lines had similar growth rates, both in the presence and in the absence of serum (data not shown). When injected s.c. into syngeneic mice, the two cell line gave rise to tumors which grew at approximately the same rates (Table 1). Thus, the two cell lines grew at the same rate, both in vitro and in vivo.

We also examined whether the HA-H and HA-L cells expressed different levels of cell surface proteins by flow cytometry. There were no obvious differences in the amounts of ICAM-1, GMP-140 ligand, and CD44 between the two cell lines (Table 2).

HA-H Cells Have a Higher Potential to Induce Experimental Metastasis than HA-L Cells. The metastatic potential of the HA-H and the HA-L cell lines were then compared in a model of experimental metastasis. Equal number of cells were injected i.v. into syngeneic mice to establish lung metastasis. After a period of two weeks or longer, the number of lung colonies and survival of the mice were determined. The injection of HA-H cells resulted in a greater number and larger lung metastases than that of the HA-L cells (Fig. 3; Table 3). Similarly, the HA-H cells also caused a higher mortality rate than the HA-L cells, with a 50% survival rate at 25 days after injection, as compared to 37 days for the HA-L cells (Fig. 4). However, as described above, both cell type were able to form comparable tumor masses when injected s.c. (Table 1). These results suggest that cell surface HA enhances the metastatic potential of cells, as judged by the lung model of experimental metastasis.

HA Does Not Mask the Cell Surface. One possible explanation for the enhanced metastatic behavior of the HA-H cell line was that the HA masked other cell surface components. To test this possibility, we removed the cell surface HA from the HA-H cells with hyaluronidase and then examined the ability of antibodies to bind to CD44, the HA receptor. The results of this experiment indicated that pretreatment with hyaluronidase did not significantly increase the access of

**Table 1** Growth kinetics HA-H and HA-L cells injected s.c. into syngeneic mice

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Days postinjection</th>
<th>HA-L cells</th>
<th>HA-H cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>8.3 ± 0.8</td>
<td>7.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>11.6 ± 2.4</td>
<td>12.0 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>7.0 ± 1.3</td>
<td>7.6 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>13.8 ± 2.0</td>
<td>14.3 ± 3.1</td>
</tr>
</tbody>
</table>

**Table 2** Expression of adhesion molecules on HA-H and HA-L cells

<table>
<thead>
<tr>
<th>Molecule</th>
<th>HA-L cells</th>
<th>HA-H cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>170</td>
<td>165</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>49</td>
<td>50</td>
</tr>
<tr>
<td>GMP-140 ligand</td>
<td>78</td>
<td>87</td>
</tr>
<tr>
<td>HA*</td>
<td>59</td>
<td>156</td>
</tr>
</tbody>
</table>

*P < 0.01; the mean channel of fluorescence intensity of the background was <30.
antibodies to cell surface CD44 over that of untreated cells, as judged by flow cytometry (data not shown). Thus, it is unlikely that HA blocks the access of antibodies to CD44 or other components on the cell surface.

**HA Does Not Change Sensitivity to NK Cells.** Another possibility is that the HA acts as a physical barrier to block access of cytotoxic cells to the surface of the HA-H cells. Indeed, a pericellular coat of HA has been shown to protect cultured cells from cellular immune attack (7, 8). To test this possibility, the susceptibility of the HA-H and HA-L cell lines to NK cell-mediated cytotoxicity was examined. As shown in Fig. 5, both cell lines were resistant to lysis by NK cells, while the YAC-1 cells were quite susceptible. These results demonstrate that there was no significant difference in the sensitivity of the HA-H and HA-L cells to NK-mediated cytoly sis.

**HA-H Cells Bind to CD44-positive Cells.** Another possible explanation for the high metastatic potential of HA-H cells is that they have an enhanced interaction with CD44 on the surfaces of other cells. To test this possibility, we examined the binding of both the HA-H and HA-L cells to SVEC4—10 cells, a SV40 virus-transformed endothelial cell line that expresses CD44. The HA-H and HA-L cells were labeled with [H3]thymidine and added to monolayers of SVEC4—10 cells. As shown in Table 4, the HA-H cells bound to the monolayer of SVEC4—10 cells to a greater extent than the HA-L cells (75% versus 44—48%). Furthermore, pretreatment of the HA-H cells with hyaluronidase or the SVEC4—10 cells with antibodies to CD44 blocked most of this binding (Fig. 6). In contrast, if the treatment of the two cell types was reversed (i.e., HA-H pretreated with antibodies and SVEC4—10 treated with hyaluronidase), then only a modest inhibition was observed. This suggests that the binding was due to the interaction between HA on HA-H cells and the CD44 on SVEC4—10 cells.

**DISCUSSION**

We have presented evidence that the levels of cell surface HA are correlated with the metastatic behavior of B16 tumor cells. Following injection into the tail vein of syngeneic mice, the HA-H cells formed larger and more numerous lung metastases than an equivalent number of HA-L cells. Furthermore, the rate of mortality in mice injected with the HA-H cells was greater than those injected with the HA-L cells.

In contrast to their differential metastatic properties, when the HA-H and HA-L cell lines were injected s.c. into mice, they both gave rise to tumors of approximately the same size. This suggests that, while both cell types have comparable abilities to grow in vivo, the HA-H cells have a distinct advantage in forming lung metastases.

In a second possible mechanism for the HA-induced enhancement of metastatic behavior is by increased trapping of tumor cells in the capillaries of the lungs. The HA can extend some distance from the cell surface, making the cells physically larger so that they are more likely to be trapped in the capillaries. In addition, it is possible that the HA interacts with surfaces of endothelial cells. While histochemical studies suggest that CD44 is absent from endothelial cells of mature mice (31), it is possible that other HA binding proteins are present, which could trap the HA-expressing tumor cells in the lungs (9).

### Table 3

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of cells injected x 10^6</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-L</td>
<td>2.5</td>
<td>3 ± 1.6</td>
<td>0–5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>115 ± 35</td>
<td>80–150</td>
</tr>
<tr>
<td>HA-H</td>
<td>2.5</td>
<td>172 ± 66.5</td>
<td>90–200</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt;200^b</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

*a* *p* < 0.01, *b* *p* < 0.05.
Table 4 Binding of HA-H and HA-L cells to endothelial cells

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Cell type</th>
<th>cpm ± SD</th>
<th>% total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HA-L</td>
<td>92,966 ± 30,886</td>
<td>48 ± 16</td>
</tr>
<tr>
<td>2</td>
<td>HA-H</td>
<td>254,878 ± 20,269</td>
<td>75 ± 6a</td>
</tr>
<tr>
<td></td>
<td>HA-L</td>
<td>94,750 ± 2,250</td>
<td>44 ± 10</td>
</tr>
<tr>
<td></td>
<td>HA-H</td>
<td>249,150 ± 3,850a</td>
<td>75 ± 1a</td>
</tr>
</tbody>
</table>

a P < 0.05.

Alternatively, the HA on the surface of the tumor cells could interact with other cells in the blood to form a cluster or embolus, which would be more likely to be trapped in the lungs. Indeed, some types of leukocytes and platelets express CD44 on their surfaces, and these cells could form clusters surrounding the HA-H cells, which are more likely to be trapped in the capillaries of the lungs (19). As we have also shown in the present study, the HA on the surfaces of the tumor cells can interact with CD44 on the surfaces of cultured cells. It is very likely that a similar type of interaction could occur when the cells are injected into the circulation.

A number of studies have shown that the expression of CD44 enhances the metastatic behavior of tumor cells (20, 21). It is possible that this enhancement is due to the ability of these cells to bind HA to their surfaces in a fashion similar to that described in this study. In addition, the injection of CD44-immunoglobulin has been shown to diminish the metastatic behavior of tumor cells expressing CD44 (22). Perhaps this agent acts by covering the cell surface HA so that it cannot interact with other adhesion molecules. Additional research is required to examine and clarify this point.

It is possible that HA and CD44 contribute to different steps in the metastatic process. The cell surface HA could lead to increased trapping of the tumor cells in the blood vessels of the lungs, while CD44 promotes the invasion of these cells through the extracellular matrix. Indeed, the expression of CD44 has been shown to stimulate the migration of tumor cells (31) on a substratum of HA, which is present surrounding the large blood vessels of the lungs (32). In addition, CD44 also allows tumor cells to endocytose and degrade the HA (33, 34), which may increase their ability to invade through the extracellular matrix. It should be noted that the HA-H cells used in this study expressed high levels of both HA and CD44, suggesting that they can simultaneously synthesize and degrade HA.

Another point demonstrated by this study is that the tumor cells can synthesize their own HA. This was demonstrated by the fact that, following treatment of the HA-H cells with hyaluronidase, the cell surface HA was regenerated over a period of 16 to 24 h. Since the medium does not contain HA, the cells must have been responsible for its synthesis.

In conclusion, HA may be an important factor contributing to the formation of metastases. Clearly, additional research is required to identify the mechanism of action. If cell surface HA is a potentiating factor in tumor metastasis, then it represents an avenue for interven-

Fig. 4. Survival durations of mice receiving injections of HA-H and HA-L cells. Suspensions of HA-L (○) and HA-H (□) cells (2.5 × 10⁶ cells in 0.2 ml of PBS) were injected into the tail veins of two groups of C57BL/6 mice (15 mice for HA-H cells and 13 mice for HA-L cells). The mice were housed under the same conditions, and the death date of each mouse was recorded. All the mice died of lung metastasis. The difference in the survival times between the two sets of mice was statistically significant (P < 0.01).

Fig. 5. Susceptibility of HA-H and HA-L cells to NK cell-mediated cytotoxicity. NK cells were prepared from spleens of mice 48 h after i.p. injection of 1 mg/mouse of poly I:C. The target cells, HA-L (○), HA-H (□), and YAC-1 (■) were labeled with ⁵¹Cr. The results are expressed in terms of percentage of ⁵¹Cr release from triplicate determinations; bars, SD.
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Fig. 6. The binding of HA-H cells to cells expressing CD44. HA-H cells (1 × 10^5) labeled [^3]H]thymidine or adherent monolayer of SVEC4–10 were treated separately with bovine testicular hyaluronidase (10 μg/ml) or purified KM-114 (rat anti-mouse CD44 mAb; 20 μg/ml) in IMDM at 37°C for 1 h and then washed four times. The relative binding was then determined as described in “Materials and Methods.” The results are means of six determinations; bars, SD.

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