Relationship between Hyaluronan Production and Metastatic Potential of Mouse Mammary Carcinoma Cells

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ABSTRACT

To investigate the roles of hyaluronan produced by cancer cells in cancer metastasis, the metastatic potential of the highly metastatic mouse mammary carcinoma FM3A HA1 cell line was compared with those of hyaluronan-deficient mutant cells. Five different mutant clones showed markedly reduced hyaluronan production and lacked the ability to form hyaluronan-rich pericellular coats. These mutant clones displayed significant decreases in metastatic ability compared with the parental cells after i.v. injection into syngeneic mice. These results suggested that the decreased hyaluronan production caused not only the lack of matrix formation but also decreased metastatic potential of the cancer cells. Expression of mouse hyaluronan synthase 1 (HAS1) by transfection into HAS− cells defective in hyaluronan synthase activity rescued hyaluronan matrix formation as well as hyaluronan production. Lung metastasis after i.v. injection of HAS1 transfectants was also recovered significantly. The results provide direct evidence for the involvement of hyaluronan in cancer metastasis.

INTRODUCTION

Significantly increased levels of HA are often associated with certain types of human tumors, and the levels of HA in the serum of some cancer patients were also significantly elevated over those of normal individuals (1–5). Although increased HA synthesis is not a universal characteristic of tumors, there seems to be an overall tendency for transformed cells to exhibit higher levels of HA production. For instance, cells infected with oncoviruses such as SV40 or Rous sarcoma virus synthesize and release elevated amounts of HA (6–8). In addition, a close relationship has been demonstrated between HA production and malignant phenotype. In rabbit V2 carcinoma, elevated levels of tumor-associated HA are correlated with invasiveness (9). We and another group found that highly metastatic cell lines released more HA into culture medium than low metastatic variants (10, 11). Furthermore, Zhang et al. (12) reported that HA on the surface of tumor cells is correlated with metastatic behavior. HA has either directly or indirectly been implicated in a variety of cell behaviors such as adhesion, cell motility, growth, and differentiation (13–15). Furthermore, HA-binding proteins regulate these cellular behaviors through interactions with HA and formation of HA pericellular matrix (16). Increased matrix deposition of HA may favor tumor growth and invasion by increasing tissue hydration and by providing a suitable environment for cell migration analogous to embryonic cell movement. In addition, the HA matrix may favor tumor growth by additional mechanisms. For example, it is possible that the HA pericellular coat reduces the access of host immunocompetent cells to tumor cells (17). In fact, a variety of tumor cells are surrounded by a thick pericellular coat that is sensitive to hyaluronidase. Removal of this coat may allow the lymphocytes to exert their cytolytic effect on the tumor cells. It has also been shown that partially degraded HA fragments promote angiogenesis, an important host contribution to tumor cell viability (18, 19).

Numerous animal cell mutants have been used to study the roles of glycoproteins and glycosaminoglycans in tumorigenesis and metastasis (20–22). Therefore, HA-deficient mutant cells would provide an ideal tool for studying the relationship between HA production and the metastatic ability of cancer cells. In this study, we isolated mutants defective in the formation of HA matrix from mouse mammary carcinoma FM3A HA1 cells by treatment with a chemical mutagen, followed by selection using particle exclusion assay. All of these mutants showed marked reduction in HA production. In the present study, we compared the abilities of these mutants to form metastatic foci in lung to examine whether HA has any role in determining metastatic capacity.

Molecular cloning of the genes encoding HA synthase, which is the key enzyme in HA biosynthesis, is one of essential steps to investigate the biological functions of HA. Recently, we and other groups have isolated three mammalian HA synthase genes, HAS1, HAS2, and HAS3 (23–25). In this study, we also demonstrated that introduction and expression of the HA synthase gene into mutant cells defective in HA biosynthesis enhanced their metastatic ability.

MATERIALS AND METHODS

Cell Culture and Isolation of Mutants. A subline having high capacity of HA production, FM3A HA1, was established from a mouse mammary carcinoma cell line with highly metastatic potential, FM3A P15A, as described previously (23). To obtain mutant cells defective in HA production, FM3A HA1 cells were treated with 0.5 mg/ml of MNNG (Nakalai Tesque, Inc., Kyoto, Japan) for 3 h and selected by particle exclusion assay. These cell lines were maintained on 100-mm Falcon Petri dishes (no. 1005) in Eagle’s MEM with double the normal concentrations of amino acids and vitamins and 10% heat-inactivated calf serum as described previously (23).

Particle Exclusion Assay. Fixed sheep erythrocytes (Inter-Cell Technologies, Inc., Hopewell, NJ) were reconstituted in PBS to a density of 5 × 10^8 cells/ml and used for the particle exclusion assay as described previously (26). HA matrices were visualized by adding 1 × 10^7 erythrocytes to the growth medium and by viewing under an OLYMPUS IMT-2 inverted phase-contrast microscope.

Determination of HA Production by Competitive ELISA-like Assay. The amounts of HA in culture medium were measured by a modification of the procedure described by Tengblad (27). We used the biotinylated HA binding region of bovine nasal cartilage proteoglycan and alkaline phosphatase-conjugated streptavidin (Amersham Pharmacia Biotech, Ltd., Uppsala, Sweden) as primary and secondary probes, respectively. The enzyme activity was measured using p-nitrophenyl phosphate (Nakalai Tesque, Inc.) as the substrate. The amounts of HA in cell layers were lower than the limit of detection, compared with that in culture medium.
HA Synthase Assays. HA synthase activity was monitored using UDP-[14C]GlcA (272.5 mCi/mmol; NEN Life Science Products, Inc., Boston, MA) as described previously (23).

Construction and Transfection of HAS1 Expression Vectors. pcDNA3-HAS1 plasmid was prepared as described previously (23). HAS1 cells were transfected either with pcDNA3-HAS1 or with pcDNA3 control vector by the lipofection procedure as described previously (23) and then selected in the medium containing 500 μg/ml G418 (Life Technologies, Inc., Grand Island, NY). Cloned cell lines were obtained by limiting dilution.

The pRESneo-HAS1 plasmid was made from the pRESneo bicistronic expression vector (Clontech Laboratories, Inc., Palo Alto, CA) and the pFLAG-HAS1 plasmid containing the mouse HAS1 cDNA. To construct a pFLAG-HAS1 plasmid, a mouse HAS1 PCR fragment was amplified using Pfu DNA polymerase (Stratagene, La Jolla, CA) and the following primers: forward, 5'-GATAGATCTGAGACAGGACATGCCAAAGCCCTCA-3' and reverse, 5'-CAGAGCTACTTCCACTGAGTGAC-3' (corresponds to amino acids 204 LVRTRRCV of HAS1). The PCR reaction conditions were as follows: 1 cycle at 94°C for 45 s, 20 cycles at 94°C for 45 s, 60°C for 45 s, 72°C for 4 min, and 1 cycle at 72°C for 10 min. The resulting PCR fragment, which includes a BspHI site, was excised at the BgII and BspHI sites and gel purified. A 3'-fragment, excised from a full-length HAS1 cDNA at BspHI and BgII sites, was also gel purified. These two HAS1 fragments were subcloned into the BgIII site of pFLAG-CMV2 vector (Eastman Kodak Co., Rochester, NY) to create a pFLAG-HAS1 construct. The pFLAG-HAS1 plasmid was digested with SacI and EcoRV, and the cohesive end was repaired by incubation with T4 DNA polymerase (Boehringer Mannheim Biochemicals, Indianapolis, IN). The gel-purified HAS1 cDNA fragment was subcloned into the EcoRV site of pRESneo vector to create the pRESneo-HAS1 plasmid. HAS1 cells were transfected either with pRESneo-HAS1 or with pRESneo control vector by the lipofection procedure and then selected in the medium containing 1 mg/ml G418. The cells surviving during selection were pooled and used for the experiments.

Northern Blot Analysis and Real Time Quantitative RT-PCR Analysis. Total RNAs were prepared using TRIZOL reagent (Life Technologies, Inc.) from exponentially proliferating FM3A HA1, mutants and transfectants. Poly(A)+ RNAs were prepared using oligo-dT cellulose (Amersham Pharmacia Biotech, Ltd.). To monitor gene expression, we used Northern blot analysis and real time quantitative RT-PCR analysis (28). For Northern blot, 2 μg of poly(A)+ RNA were separated by formaldehyde agarose gel electrophoresis and transferred to Hybond N+ nylon membranes (Amersham Pharmacia Biotech, Ltd.) overnight. RNAs were fixed, prehybridized, and hybridized according to the manufacturer’s recommendations. High stringency hybridization was performed using partial cDNA probes representative of mouse HAS1, mouse HAS2, mouse HAS3, and the housekeeping gene GAPDH. These probes were labeled with [-32P]dCTP by random priming. Blots were washed to high stringency and exposed at −80°C to autoradiographic film with intensifying screens.

Real time RT-PCR analysis was performed according to the reported method (28). Briefly, within a gene-specific PCR oligonucleotide primer pair, a fluorogenic oligonucleotide probe is designed. When the probe is intact, reporter dye emission with the quenching dye emission and calculated.

RESULTS

Isolation and Characterization of HA-deficient Mutant Cells. We first isolated mutant clones defective in formation of the HA-rich pericellular coat from the highly metastatic mouse carcinosarcoma cell line FM3A HA1 (Fig. 1). FM3A HA1 cells were mutagenized with MNNG under strict conditions and then screened for deficiency of HA coat formation using particle exclusion assay. Five different mutant clones were isolated by limiting dilution. The clones were again confirmed to be unable to assemble the HA-rich pericellular coat. No pericellular coats were detected around the mutant cells (Fig. 1, B–F).

HA production of the parental and mutant cells was assessed by measuring the amount of HA secreted into the culture medium. HA production of all of the mutants was significantly lower than that of the parental cells, suggesting that the deficiency in HA coat formation of the mutant cells was mainly due to the decrease in HA production (Fig. 2A). These phenotypes of the mutant cells were stable in vitro in each descendant during culture for 2 months.

To identify primary defects of the mutant cells in the HA synthetic pathway, HA synthase activity was measured and compared with that of the parental cells. As shown in Fig. 2B, clones HA-m2, HA-m14 (HAS1), and HA-m20 had <5% of the parental cell activity. These results suggested that the loss of HA synthase activities of these mutant cells caused the observed defects in HA production. In contrast, clones HA-m11 and HA-m12 retained >40% of the enzyme activity of the parental cells, suggesting that some aberration in the process of HA secretion or biosynthesis may be responsible for the defects observed in HA production and matrix deposition. A previous complementation experiment also demonstrated that some altered phenotypes in HA-m11 and HA-m12 cells were caused by some aberration other than that in HA synthase (23).

We examined the growth characteristics of the parental and mutant cells in vitro. No correlation was found between in vitro growth properties and HA production (data not shown).

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Tumor Growth and Experimental Lung Metastasis of Parental and Mutant Cells. To examine how HA production by cancer cells modifies their in vivo behavior, $1 \times 10^5$ cancer cells were injected s.c. into syngeneic mice. The animals were monitored every 5 days for tumorigenicity of the parental and mutant cells. The tumor mass in animals injected with the parental and mutant cells was first visible within 10 days. By 15 days, 100% of animals injected with parental and mutant cells had grossly visible tumors. No correlation was found between tumor growth properties and HA production (data not shown).

To evaluate the relationship between HA production and metastatic ability of cancer cells, we injected the parental and mutant cells into the tail vein of syngeneic mice and examined their lungs for tumor colonies after 25 days. The statistical significance of the apparent differences between the parental and mutant cells was tested by Student’s $t$ test. The extent of experimental metastasis of all mutants was significantly decreased and correlated with HA production (Fig. 3). On the other hand, no significant differences were observed in pulmonary metastasis between the parental and MNNG-treated cells (Fig. 3).

Establishment of Clones Stably Expressing HAS1. Northern blot analysis showed that of the three mammalian HA synthase genes, HAS1, HAS2, and HAS3, parental FM3A HA1 cells highly expressed only HAS1 (Fig. 4B). In contrast, the level of expression of the HAS1 gene in HAS$^-$ cells was 0.24% of that of parental cells, as measured by real time quantitative RT-PCR analysis (Fig. 4C). To confirm the contribution of HA produced by cancer cells to the metastatic process, the HAS$^-$ mutant was stably transfected with the cDNA encoding mouse HAS1, which was constructed into two different types of vectors (Fig. 4A). Neomycin-resistant colonies arising from single cells transfected with pcDNA3-HAS1 plasmid were grown to subconfluency in 35-mm dishes and screened for HA synthetic activity. Two independent clones, HAS1-N1 and HAS1-N6, producing different levels of HA, were selected and characterized. Furthermore, neomycin-resistant cells

Fig. 1. Visualization of HA matrices around FM3A HA1 and HA-deficient mutant cells. The particle exclusion assay was used to outline the HA matrix surrounding the cells. The HA matrix occupies the clear area (arrowheads) between the fixed erythrocytes and the parental FM3A HA1 cells. These photomicrographs were taken under an inverted phase-contrast microscope at ×200. A, FM3A HA1 cells; B, HA-m2 mutant cells; C, HA-m11 mutant cells; D, HA-m12 mutant cells; E, HA-m14 (HAS$^-$) mutant cells; F, HA-m20 mutant cells.

Fig. 2. HA production and HA synthase activity of the parental and HA-deficient mutant cells. HA production (A) and HA synthase activity (B) were determined as described in “Materials and Methods.” Because the amounts of HA in cell layers were lower than the limit of detection, HA production represents that in culture medium. Columns, mean values of three determinations; bars, SD.

Fig. 3. Experimental pulmonary metastasis of the parental and HA-deficient mutant cells. Animals were given i.v. injections of $1 \times 10^6$ tumor cells via tail vein. Mice were killed after 25 days, and the numbers of lung metastatic colonies were counted. Data points, number of lung metastatic colonies. Bars, mean values. *, $P < 0.01$ as compared with the parental FM3A HA1 by Student’s two-tailed $t$ test.
transfected with pIREsneo-HAS1 plasmid were pooled and designated as HAS1-BS. The HAS1-N6 and HAS1-BS cells had elevated levels of HAS1 expression and HA production (Figs. 4C and 5A). On the other hand, HAS1-N1 had a moderate level of HAS1 expression and showed reduced HA production (Figs. 4C and 5A). These HAS1 transfectants also showed significant elevation in their synthase activity (Fig. 5B). In addition, expression of HAS1 cDNA in HAS cells complemented the deficient matrix deposition of HA (Fig. 6). Mock-transfected cells were selected as controls and designated as mock (DNA3) and mock (IRES1), respectively. These mock transfectants showed comparable levels of HAS1 expression, HA production, and HA synthase activity to HAS cells (Figs. 4C and 5).

**Experimental Lung Metastasis and Tumor Growth of HAS1 Transfectants.** When HAS1 transfectant cells were injected i.v. into syngeneic mice, the cells showed a significant increase ($P < 0.01$) in the ability to form lung colonies compared with the HAS cells and with mock transfected cells (Table 1). HAS1-N6 and HAS1-BS transfectants gave rise to metastases with median numbers of 27 and 36, respectively, whereas a median number of 17 metastases was observed in the clone HAS1-N1, which showed moderate levels of HA production. The number of metastatic colonies with mock transfectants was similar to that with the HAS cells. Taken together, these results indicate that the high HA production of mouse mammary carcinoma cells is related to their enhanced metastatic abilities.

**Growth of tumors after s.c. injection of $1 \times 10^6$ cells into syngeneic C3H/He mice was compared among HAS1 transfectants, control transfectants, parental FM3A HA1, and HAS cells. The mean value of tumor size was measured on day 15 after injection. No apparent correlation was found between their tumor growth properties and HA production (Table 1). These findings suggested that the difference in metastatic ability between the HAS1 and control transfectants is not primarily due to different rates of in vivo proliferation.

**DISCUSSION**

HA, a ubiquitous glycosaminoglycan, has long been suggested to be linked to both transformation and malignant tumor progression (13, 16, 29). However, there is no direct evidence that HA is involved in malignant phenotypes. In this study, we demonstrated that HA is an important factor contributing to the formation of pulmonary metastasis.

Several mechanisms have been proposed for the involvement of HA in tumor malignancy. By analogy to embryonic cell movement,
increasing tissue hydration and by providing a suitable environment for cell migration (1, 29). Toole et al. (9) observed that the HA content of invasive V2 carcinomas grown in rabbits was 3–4-fold greater than that of the same tumors grown in the nude mouse, in which it was noninvasive (9). In addition, the HA matrix may favor tumor growth by some other mechanism. For example, the HA-rich pericellular coat constitutes a barrier that impedes both the generation of cytolytic T lymphocytes specific to antigens on the tumor cells and the lysis of tumor cells by cytolytic lymphocytes (17, 30). Furthermore, angiogenesis should be considered as another mechanism of HA involvement, which is vital to tumor growth and is strongly stimulated by low molecular weight HA fragments (18, 19).

Enhanced metastasis is the result of various cellular processes. Previous studies have shown that highly metastatic tumor cells produce higher levels of HA than low metastatic counterparts (10, 11). A recent pathological study also showed a positive correlation between HA content in human colorectal cancer and Dukes’ classification and stage (35). However, metastasis is by increased trapping of tumor cells in the capillaries of the lungs. Yoneda et al. (35) showed that lung colonization of the highly metastatic mouse mammary carcinoma cell line FM3A P15A was markedly inhibited by pretreatment of the cells with specific hyaluronidase to degrade the pericellular HA. In addition, HA oligosaccharides (>10 monosaccharide units), which are known to competitively inhibit the interaction of HA with most HA-binding molecules, significantly decreased the lung colonization of FM3A P15A cells after i.v. injection. Although the effects may be transient and reversible, it is possible that these treatments might have decreased the arrest of the microcirculation in organs and extravasation. Zhang et al. (12) also reported that a highly metastatic melanoma cell line expressing a high level of cell surface HA showed high-affinity binding to the SV40 virus-transformed endothelial cell line SVEC4–10 via HA-CD44 interaction. Therefore, it is likely that the interaction of the pericellular HA of cancer cells with other molecules in the circulation may be involved in high metastatic potential. In addition, we cannot rule out the possibility that HA induces cellular activation and novel gene expression through HA-receptor interaction (36, 37). Further investigation will be required to fully understand the mechanisms by which HA enhances the metastatic process, and genetic manipulation of HA using mammalian HA synthesize genes may offer some advantage for additional studies.

Table 1 In vivo tumorigenicity and experimental pulmonary metastasis of the HAS1 transfectants

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mean tumor diameter (mm)</th>
<th>Incidence</th>
<th>Metastasis to lungs</th>
<th>Foci, no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM3A HA1</td>
<td>8.8 ± 0.9</td>
<td>7/7</td>
<td>16, 23, 33, 41, 45, 50, 59</td>
<td></td>
</tr>
<tr>
<td>HAS^</td>
<td>8.5 ± 0.9</td>
<td>4/7</td>
<td>0, 0, 0, 2, 4, 5, 12</td>
<td></td>
</tr>
<tr>
<td>Mock (DNA3)</td>
<td>8.1 ± 0.8</td>
<td>4/7</td>
<td>0, 0, 0, 4, 5, 8</td>
<td></td>
</tr>
<tr>
<td>HAS1-N1</td>
<td>8.4 ± 0.3</td>
<td>7/7</td>
<td>5, 8, 16, 19, 22, 26</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HAS1-N6</td>
<td>8.0 ± 1.2</td>
<td>7/7</td>
<td>14, 20, 22, 34, 40, 41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mock (IRE1)</td>
<td>8.4 ± 1.0</td>
<td>6/7</td>
<td>0, 3, 4, 5, 5, 15, 18</td>
<td></td>
</tr>
<tr>
<td>HAS1-BS</td>
<td>7.3 ± 1.0</td>
<td>6/6</td>
<td>13, 21, 27, 44, 56, 58</td>
<td>0.003</td>
</tr>
</tbody>
</table>

^ The tumor sizes were measured at 15 days after s.c. injection. The average of the short and long diameters was used in the determination: Mean ± SD (n = 4).

^ Statistical analysis was performed by Student’s two-tailed t test.
ACKNOWLEDGMENTS

We thank Michiyo Matsumoto for technical assistance and the members of the Institute for Molecular Science of Medicine, Aichi Medical University, for helpful discussion.

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