Overproduction of Hyaluronan by Expression of the Hyaluronan Synthase Has2 Enhances Anchorage-independent Growth and Tumorigenicity

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ABSTRACT

Hyaluronan (HA) has long been implicated in malignant transformation and tumor progression. However, due to the lack of molecular tools to directly manipulate production of HA, which does not require a core protein for its synthesis, our understanding of the role of HA in tumor cells has been largely circumstantial. In this study, we genetically manipulated the production of HA by transfection of a mammalian HA synthase Has2 into human HT1080 cells and examined the malignant phenotype of transfected cells. We found that increased production of HA promotes anchorage-independent growth and tumorigenicity of the cells. Has2-transfected cells formed greater numbers of colonies in semisolid medium. Tumors in nude mice derived from Has2-transfected cells grew more rapidly and were 2–4 times larger than those derived from control cells at termination of experiments. Histological and biochemical analyses of tumors revealed no significant differences in cell density and tissue structures between them, indicating that the larger size of the tumors was due to enhanced cell proliferation, not to increased accumulation of tumor stroma or increased angiogenesis. These results demonstrate that HA production by tumor cells per se promotes proliferation of these cells in tissues and provides direct evidence for the role of HA in tumorigenesis.

INTRODUCTION

The ECM is one of the crucial environmental elements that affects tumor cell behavior. ECM serves as a scaffold to which tumor cells adhere and migrate. ECM is also thought to act as a reservoir of growth factors and cytokines that are potentially beneficial to malignant cells (1). HA is a large glycosaminoglycan containing repeating disaccharide units of N-acetylgalactosamine and glucuronic acid residues. Several studies have shown that HA plays significant roles in matrix assembly, cell proliferation, and migration (2–5). Spatiotemporal distribution of HA during embryogenesis and wound healing indicates that HA plays important roles in tissue remodeling processes (6, 7).

There is increasing evidence that HA plays significant roles in tumor progression (8–11). HA is present in a greater amounts in a variety of tumors than in normal counterparts, including colon cancer (12), breast cancer (13–15), glioma (16), lung carcinoma (17), and Wilm’s tumor (18). It has also been shown that high HA production correlates with invasive and metastatic activities of tumors (19–23). HA is also implicated in tumor angiogenesis (9). These observations suggest that increased levels of HA may provide an environment facilitating various aspects of tumor progression.

Despite this wealth of data, whether increased cellular HA production has direct effects on tumor cell proliferation has not been addressed because HA, which does not contain any protein components, cannot be directly manipulated by molecular biological techniques. Cloning of mammalian HA synthases by our and other laboratories now allows genetic manipulation of the cellular HA production. Here, we show that increased production of HA directed by human HA synthase Has2 (24) promotes anchorage-independent growth and tumorigenicity of human HT1080 cells. These changes toward a more malignant phenotype did not, however, accompany increased growth rate of cells in monolayers, suggesting that the HA exerts its growth-promoting effect mainly in a three dimensional environment. These results provide the first evidence for a direct relationship between cell-autonomous HA production and malignant transformation.

MATERIALS AND METHODS

Materials. The CellPhect transfection kit and the HA Test HA assay kit were purchased from Pharmacia (Piscataway, NJ). Polyclonal antihuman factor VIII antibody and MU were obtained from Sigma Chemical Co. (St. Louis, MO), BCA Protein Assay Reagent kit and Vectastain ABC kit were purchased from Pierce (Rockford, IL) and Vector Laboratories (Burlingame, CA), respectively. Monoclonal antibody 12CA1 against the influenza virus hemagglutinin epitope was purchased from Boehringer Mannheim (Indianapolis, IN). Type I collagen was purchased from Collagen Corp. (Palo Alto, CA), and bovine plasma fibronectin was a kind gift from Dr. E. Ruoslahti (The Burnham Institute, La Jolla, CA).

Stable Transfectants. A human fibrosarcoma cell line HT1080 was obtained from American Type Culture Collection (Manassas, VA) and maintained in αMEM supplemented with 10% FCS, 2 mm glutamine, 100 unit/ml penicillin, and 100 µg/ml streptomycin (growth medium). For isolation of stable transfectants, subconfluent cultures of HT1080 cells in 10-cm culture dishes were transfected with 10 ng of pHAStagC or an insertless pCDNA3 using the CellPhect transfection kit and selected in the presence of 500 µg/ml G418. pHAStagC contains a full-length human Has2 cDNA tagged with the influenza hemagglutinin epitope sequence at its COOH terminus (24). From each transfection experiment, 10 G418-resistant colonies were picked with cloning rings. The expression of Has2 was examined by immunoblotting with 12CA1 antibody against the influenza hemagglutinin epitope, as described previously (24). Three Has2 transfected clones and two control clones were randomly selected for these studies.

Assay of HA Production. The HA production of transfected clones into culture supernatants was measured by the PharmaCR Test HA assay kit as described previously (24). Briefly, 1 × 105 cells were plated in a 35-mm wells in growth medium. Eighteen h after plating, cells were fed with fresh medium and cultured for another 24 h, and their culture supernatants were collected for HA assay. For inhibition of HA production, cells were pretreated with 0.05 mm or 0.1 mm MU for 3 days before the assay. MU was used to inhibit production of HA.

Adhesion Assays. Wells of 96-well plates (non-tissue culture-treated; Flow Laboratories, McLean, VA) were coated with 10 µg/ml type I collagen or fibronectin at room temperature for 4 h. After washing, wells were blocked with 3 mg/ml BSA in PBS for 1 h. Cells (2.5 × 104/well) suspended in 0.1% DMSO and added to culture medium. The final concentration of DMSO was 0.1%. Pretreated cells were then used in the standard HA production assay in the presence of MU. Control cultures were treated with 0.1% DMSO only.

Growth in Monolayers. Has2- and control-transfected HT1080 cells were plated in 35-mm wells of six-well culture plates at 5 × 104 cells/well in the growth medium. Because plating efficiencies of all these transfected cells were shown to be the same, no adjustment was made in terms of numbers of cells plated. At 1, 2, 3, and 4 days after plating, cells in triplicate wells were trypsinized and counted on a hemocytometer. For inhibition of HA synthesis, cells were pretreated with 0.1 mm MU-0.1% DMSO or 0.1% DMSO alone as described above, and the growth assay was performed in medium containing MU-DMSO or DMSO alone.

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4 The abbreviations used are: ECM, extracellular matrix; HA, hyaluronan; MU, 4-methylumbelliferone.
Anchorage Independence. Has2- and control-transfected HT1080 cells were suspended in 2 ml of growth medium containing 0.4% (w/v) low melting temperature agarose and plated onto a layer of 0.53% agar prepared in growth medium in a 60-mm dish. Dishes in triplicate were incubated at 37°C under 5% CO₂, and the number of colonies consisting of more than 30 cells was determined for each dish at 10 days after plating. For inhibition of HA synthesis, cells were pretreated with 0.1 mM MU as described above and plated on agarose plates containing 0.1 mM MU. As a control, cells were pretreated with 0.1% DMSO only and tested on agarose plates containing 0.1% DMSO.

In Vivo Tumor Growth. Six-week-old female BALB/c nude mice were given bilateral s.c. injections of transfected and control cells in both flanks. Each injection consists of 2 × 10⁶ cells in 200 μl of PBS. Growth of the tumors was evaluated by calculating tumor volume from the width and length of palpable tumor according to the following formula: volume = (width)² × length/2 (25). When the first sign of distress was observed in any mouse in the experiment (which usually occurred at 2–3 weeks after inoculation for Has2-transfected cells), all mice in the experiment were euthanized, and their tumors were excised for examination. Wet weight of tumor tissues free of attached nontumor tissues was measured immediately after excision. Some of the tumors were fixed in 4% paraformaldehyde and embedded in paraffin. Sections from these tissues were examined by H&E staining and by immunohistochemistry with anti-factor VIII antibody. Immunohistochemistry was performed with the ABC method as described previously (26). For measurement of DNA content, tumor tissues were homogenized using a Polytron homogenizer, followed by sonication method as described in “Materials and Methods.” Columns, means of triplicate determinations; bars, SD.

RESULTS

Transfection of Has2 cDNA into HT1080 Cells. Human fibrosarcoma HT1080 cells were transfected with pHASTagC (24), which is a pcDNA3-based expression vector containing human Has2 cDNA tagged with the influenza hemagglutinin sequence recognized by the 12CA5 monoclonal antibody (28). After G418 selection, several resistant colonies were randomly isolated and established as stable cell lines. For generation of control cell lines, HT1080 cells were transfected with pcDNA3 without an insert. Here, two Has2-transfected cell lines (designated as Has2-1 and Has2-2) and two control cell lines (designated as Cont-1 and Cont-2) were used. Expression of Has2 in transfected was examined by immunoblotting with the 12CA5 monoclonal antibody, which recognizes the hemagglutinin tag attached to the COOH terminus of Has2. As shown in Fig. 1A, a Mr ∼75,000 band reactive to 12CA5 was detected in all Has2-transfected clones. We previously showed that, in transfected human 293 cells, the Has2 protein is present in both the membrane and soluble fractions (24). This observation was also the case in HT1080 transfecants. Small quantities of Has2 were detected in the soluble fraction (Fig. 1A, Lanes 3 and 4), whereas the majority of the protein was in the membrane fraction (Lanes 1 and 2).

HA Production in Transfected Cells. HA production in transfected HT1080 cells was quantitated by a radiometric HA binding assay as described previously (24). Like many malignant tumor cells, HT1080 cells produce larger amounts of HA than do cell lines derived from normal tissues, such as 293 and CHO cells. Parental and control transfected HT1080 cells produce 350–450 ng of HA per day (Fig. 1B). Transfection of Has2 cDNA increased HA production by HT1080 cells. HA production by the Has2-transfected clones was in the range of 1.2–1.5 μg per day (Fig. 1B), which is >3 times the production of parental and control cell lines. This level of fold increase is not as remarkable as that seen in Has2-transfected 293 and CHO cells; 293 and CHO cells showed 23- and 6-fold increases by Has2 transfection (average of three clones), respectively (24). However, this is partially due to the higher baseline HA production of HT1080 cells compared to 293 and CHO cells. Untransfected 293 and CHO cells produced only 80 and 25 ng of HA per day, respectively, under the same assay conditions (24).

Adhesion and Growth in Monolayers. We first examined whether overproduction of HA affects adhesive properties of HT1080 cells. As shown in Fig. 2, no significant differences were found with regard to the adhesion to fibronectin and type I collagen between Has2-transfected and control cells. We have also examined the plating efficiency on tissue culture plastic using regular culture medium containing 10% FCS. We found no differences in plating efficiency between Has2-transfected and control transfected cells (data not shown). These results demonstrate that general adhesive properties of HT1080 cells are not affected by overproduction of HA.

The growth of Has2-transfected cells was examined by a standard growth assay in monolayer cultures. Because, as noted above, the plating efficiencies of the cell lines are not different, no adjustment was made with regard to the number of cells plated. As shown in Fig. 3, there were no significant differences in growth rate and saturation density between Has2-transfected and control transfected clones.

Overproduction of HA Makes Cells More Anchorage Independent. Anchorage-independent growth correlates well with tumorigenicity in vivo (29). We examined this parameter using the soft agar assay. Although the growth rate of Has2-transfected cells in monolayers is not different from that of control cells (see Fig. 3A), the soft agar assay demonstrated that Has2-transfected cells consistently form greater numbers of colonies than do control cells (Fig. 4A; see also Fig. 5C). Moreover, the size and shape of these colonies were significantly different (Fig. 4B). Has2-transfected cells were larger than control cells and tended to exhibit expanding morphology. These results show that Has2-transfected cells are more anchorage independent than control HT1080 cells.
There is a possibility that overexpression of Has2 protein itself rather than overproduction of HA caused the more transformed phenotype observed in Has2 transfectants, i.e., that an activity of Has2 unrelated to its enzymatic activity promotes transformation. To rule out this possibility, we examined whether the suppression of HA synthesis by chemical inhibitors would eliminate the difference in anchorage independence between Has2-transfected and control transfected cells. Because such chemical agents would suppress HA synthesis but not expression of Has2 protein, any differences observed in anchorage dependence of control and transfected cells following such treatment might be attributable to overexpression of Has2 protein. Although its biochemical mechanism of inhibition is not well understood, MU has been shown to inhibit the synthesis of HA in human fibroblasts without affecting the synthesis of other proteoglycans (30). As shown in Fig. 5A, 0.1 mM MU indeed suppressed HA production in both Has2 transfectants and control cells to a similar level. We also ascertained that this concentration of MU does not adversely affect the growth of HT1080 transfectants (Fig. 5B). Soft agar assays showed that there were no differences in anchorage independence between Has2-transfectants and control cells following treatment with 0.1 mM MU (Fig. 5C). These results indicate that the increased anchorage independence of Has2-transfected cells is due to increased production of HA.

**HA Overproduction Increases Tumorigenicity in Vivo.** The *in vitro* assays described above suggest that tumorigenicity *in vivo* may also be promoted by HA overproduction. To examine this possibility, we performed tumorigenesis assays in nude mice. We found that

![Figure 2](image2.png) **Fig. 2.** Adhesion of Has2-transfectants to substrates of fibronectin (A) and type I collagen (B). Cell adhesion was quantitated by staining attached cells with toluidine blue as described in “Materials and Methods.” Columns, means (n = 5) of A590 values; bars, SD.

![Figure 3](image3.png) **Fig. 3.** Growth of Has2-transfected cells in monolayer cultures. Cells were plated in 35-mm wells in six-well tissue culture plates at 5 × 10^4 cells/well and cultured in growth medium. At time points indicated in the figure, duplicate wells were trypsinized, and cell numbers were counted using a hemacytometer.

![Figure 4](image4.png) **Fig. 4.** Anchorage-independent growth of Has2-transfected cells in soft agar. A, Has2- and control-transfected cells were examined by the soft agar assay as described in “Materials and Methods.” Dishes in triplicate were incubated at 37°C under 5% CO₂, and the number of colonies consisting of more than 30 cells was determined for each dish at 10 days after plating. Columns, mean numbers of colonies; bars, SD. Experiments were repeated three times with similar results. B, phase contrast micrographs of colonies of Has2- and control-transfected cells in soft agar. Has2, Has2-transfected HT 1080 cells (clone Has2-1); Cont, control-transfected HT1080 cells (clone Cont-1).

Has2-transfected cells are more tumorigenic than control HT1080 clones (Fig. 6). Has2-transfected cells grew more rapidly, and differences in tumor size between Has2 transfectants and control cells increased as tumor size increased (Fig. 6A). By the termination of experiments, Has2 transfected cells had formed tumors 3–4-fold larger than those derived from control cells (Fig. 6, B and C).

These results and those derived from soft agar assays suggest that the proliferation in three-dimensional environments, such as colonies in soft agar and tumor mass, is promoted in HA-overproducing cells. However, there is a possibility that the increased tumor size may be due to increased amounts of tumor stroma rather than to the increased growth of tumor cells. To examine this possibility, we analyzed tumor tissues by histological and biochemical methods. Macroscopically, there were no substantial differences between tumors derived from Has2-transfectants and control cells. Both were well encapsulated, and no sign of increased infiltration into surrounding tissues was observed. H&E staining of tumor sections showed no significant histological differences between Has2-transfectants and control cells (data not shown). Furthermore, there is no indication that angiogenesis was enhanced in Has2-transfectant-derived tumors. The numbers of capillaries identified by immunostaining for factor VIII, a marker for capillaries, were not different between Has2-transfectants and control cells (data not shown). Finally, we quantitatively analyzed cell...
density in tumor tissues (Table 1). We determined nuclear density in H&E-stained tumor sections and DNA concentration in extracts of tumor tissues. Neither nuclear density nor DNA content was significantly different between tumors derived from Has2-transfectants and control transfectants. These results indicate that the larger sizes of Has2-transfectant-derived tumors are primarily due to increased proliferation of tumor cells in vivo. Although some contribution of tumor stroma to the increased tumor size cannot be ruled out, it does not account for the observed increases in tumor size.

DISCUSSION

A number of studies have suggested that HA is involved in proliferation, motility, and metastasis of tumor cells (3, 4, 10). HA is increased in tumor tissues compared to normal tissues (10). It has also been demonstrated that some transformed cells in culture produce larger amounts of HA than do untransformed cells (19–22). However, these reports have not demonstrated a direct causal relationship between cellular HA synthesis and malignant transformation.

This study provides the first direct evidence that genetic manipulation of HA synthesis enhances the malignant phenotype of tumor cells. We found that increased HA synthesis promoted colony formation in soft agar and tumor formation in nude mice. The increased tumor size in nude mice from Has2-transfectants was due not to accumulation of tumor stroma or increased angiogenesis. Therefore, these results indicate that the cell-autonomous production of HA enhances proliferation of tumor cells.

The cellular origin of HA in tumors can be parenchymal cancer cells and/or stromal cells (11). Although there is strong evidence that some cancer cells stimulate adjacent noncancerous stromal cells to produce HA (10, 31), a number of tumor cells have been shown to produce greater amounts of HA than their benign counterparts (11, 21, 22, 32). At present, it is not well understood how these different mechanisms are involved in tumor progression. Our results demonstrate that increased HA production by cancer cells has enhancing effects on the growth of tumors in vivo. Whether increased HA production by stromal cells has similar effects on the growth of tumors is not known. With HA synthase genes and their cDNAs available, this problem can now be tested by generating transgenic mouse models in which HA synthases are overexpressed in stromal cells. It is possible that cancer cell- and stromal cell-derived HA are involved in different aspects of malignant transformation.

Fig. 5. Effects of chemical inhibition of HA synthesis on anchorage-independent growth of Has2-transfected cells. A, treatment with 0.1 mM MU reduces HA production. HA production was assayed as described above in the presence of 0.1 mM MU dissolved in 0.1% DMSO (□) or 0.1% DMSO alone (●). B, treatment with 0.1 mM MU does not adversely affect the growth of cells in monolayers. Growth assay was performed in wells of 24-well tissue culture plates with Cont-1 cells in the presence of 0.05 mM and 0.1 mM MU in 0.1% DMSO or 0.1% DMSO alone. C, enhanced anchorage independence of Has2-transfected cells was abolished by suppression of HA synthesis. Soft agar assays were performed in the presence of 0.1 mM MU in 0.1% DMSO (□) or 0.1% DMSO alone (●). This result demonstrates that increased anchorage independence observed in Has2 transfectants is due to enzymatic activity of Has2 protein rather than to any unrelated activity of Has2 protein.

Columns and data points, mean numbers of colonies in soft agar; bars, SD.

Fig. 6. Enhanced tumorigenicity of Has2-transfected cells. In vivo tumor growth assays in nude mice were performed as described in “Materials and Methods.” A, time course of tumor growth. Growth of the tumors was evaluated by measuring tumor volume from the width and length of a palpable tumor as described in “Materials and Methods.” Cells examined in this experiment were: ● parental HT1080; ■ Has2-1; ■ Has2-2. Data points, mean tumor volumes from four (parental HT1080) or five (Has2-1 and Has2-2) animals. B and C, tumor weights at termination of experiments. Results of two separate experiments (B and C) are shown. All animals involved in each experiment were sacrificed when the sign of distress was observed in any one animal in the experiment, which occurred at day 16 in B and day 13 in C. Columns, mean tumor weights per animal (i.e., the sum of tumor weights in both flanks) for each cell line; bars, SD. Numbers of animals examined were four for parental HT1080 and five for Has2-1 and Has2-2 in B and four for each cell line in C.
There are several ways that high levels of HA might promote anchorage-independent growth and tumorigenesis of cells. For example, increased amounts of HA may facilitate cell division. It has been shown that HA synthesis occurs during cell division (33, 34), and it is thought that HA synthesis is important for cell detachment that occurs during cell division. It is also possible that an HA-rich environment may provide hydrated spaces that facilitate the migration of cells following mitosis. It has been suggested that high levels of HA within the ECM cause tissue spaces to become highly hydrated and to expand because of increased osmotic pressure (35). This expanded and water-enriched environment is thought to deform the compact, restrictive architecture of extracellular matrices and facilitate cell movement (8, 10). Thus HA-overproducing tumor cells might migrate outwardly more efficiently, resulting in faster expansion of the tumor mass. Alternatively, HA-mediated cell signaling might be responsible for increased tumorigenicity. It has been reported that binding of HA to its cell surface receptors leads to activation of intracellular signaling pathways (36–40). Notably, an HA receptor, RHAMM, has been shown to be involved in a signaling pathway leading to H-ras-mediated malignant transformation of fibroblastic cells (38).

This work establishes that cell-autonomous overproduction of HA enhances transformation of tumor cells, although further study will be required to elucidate its mechanism. Because tumorigenicity in nude mice is the experimental parameter closely correlated with malignancy at the clinical level, it will be interesting to determine whether levels of cellular HA might also correlate with the level of clinical malignancy. A recent report by Ropponen et al. (12) on the correlation between tumor HA levels and the prognosis of patients with colorectal cancer appears to be consistent with this notion.

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REFERENCES

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