Elevated Hyaluronan Production Induces Mesenchymal and Transformed Properties in Epithelial Cells*

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During carcinoma progression, tumor cells often undergo changes similar (but not identical) to epithelial-mesenchymal transitions in embryonic development. In this study, we demonstrate that experimental stimulation of hyaluronan synthesis in normal epithelial cells is sufficient to induce mesenchymal and transformed characteristics. Using recombinant adenoviral expression of hyaluronan synthase-2, we show that increased hyaluronan production promotes anchorage-independent growth and invasiveness, induces gelatinase production, and stimulates phosphoinositide 3-kinase/Akt pathway activity in phenotypically normal Madin-Darby canine kidney and MCF-10A human mammary epithelial cells. Cells infected with hyaluronan synthase-2 adenovirus also acquired mesenchymal characteristics, including up-regulation of vimentin, dispersion of cytokeratin, and loss of organized adhesion proteins at intercellular boundaries. Furthermore, we show that the transforming effects of two well described agents, hepatocyte growth factor (HGF) and β-catenin, are dependent on hyaluronan-cell interactions. Perturbation of endogenous hyaluronan polymer interactions by treatment with hyaluronan oligomers is shown here to reverse the transforming effects of HGF and β-catenin in Madin-Darby canine kidney and MCF-10A human mammary epithelial cells. Also, HGF and β-catenin induced assembly of hyaluronan-dependent pericellular matrices similar to those surrounding mesenchymal cells. Thus, increased expression of hyaluronan is sufficient to induce epithelial-mesenchymal transition and acquisition of transformed properties in phenotypically normal epithelial cells.

The transition from epithelial to mesenchymal cell phenotype involves morphological and biochemical changes in cell-cell adhesion and cytoskeletal organization and the acquisition of migratory ability (1–4). Epithelial-mesenchymal transition (EMT)1 during development is a precisely regulated event that is integral to proper embryonic morphogenesis. Many changes that occur during EMT are also seen in epithelial cells that have undergone malignant transformation. However, these changes in cancer cells do not follow an orderly program and are not identical to developmental EMT. In addition to changes in cell-cell adhesion and cytoskeletal organization, common hallmarks of malignant cells are loss of adhesion requirements for survival and proliferation (anchorage independence) and the acquisition of invasive ability.

Normal and malignant cell behavior is dependent on the composition and state of the pericellular matrix microenvironment. A prominent pericellular matrix component is hyaluronan, a high molecular mass glycosaminoglycan that is ubiquitous to the matrices of adult and embryonic tissues. In particular, however, hyaluronan is concentrated in the pericellular matrix of migrating and proliferating cells, e.g. during development (5). A definitive role for hyaluronan in development has recently been demonstrated in the hyaluronan synthase-2 (Has2) gene knockout mouse (6). Pericellular matrices in Has2-null mice are less hydrated than normal, with altered organization of matrix proteins. These mice show abnormal heart development due to defective cell migration and failure of cushion cell endothelium to undergo the EMT necessary for cardiac tissue development. The latter defect is due to loss of hyaluronan-induced ErbB2/3 and Ras signaling (6, 7). Thus, these experiments demonstrate an important and direct role for hyaluronan in EMT in this system.

Many reports have provided evidence for a strong relationship between hyaluronan and tumor progression (8, 9). Most malignant tumors contain elevated levels of hyaluronan (10), and poor prognosis is correlated with elevated hyaluronan levels in malignant tumors (11–13). Experimentally increased production of hyaluronan in tumor cells via overexpression of hyaluronan synthases enhances tumor growth and metastasis in animal models (14–17), whereas antisense cDNAs to these synthases markedly inhibit subcutaneous growth of tumor cells (18). In addition, perturbation of hyaluronan-cell interactions has been shown to inhibit growth and metastasis of several tumor types (19–23). Although the underlying mechanism whereby hyaluronan influences cell behavior is not completely understood, several studies indicate that hyaluronan influences a number of signaling pathways that affect cell growth and survival (24). In this study, we demonstrate that experimental stimulation of hyaluronan synthesis in normal epithelial cells is sufficient to induce mesenchymal and transformed characteristics. Furthermore, we show that the transforming effects of two well described agents, HGF and β-catenin, are dependent on hyaluronan-cell interactions and that hyaluronan appears to act via stimulation of the PI 3-kinase/Akt cell survival pathway.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—MDCK and MCF-10A human mammary cells were obtained from American Type Culture Collection (Ma-

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1 The abbreviations used are: EMT, epithelial-mesenchymal transition; HGF, hepatocyte growth factor; PI, phosphoinositide; MDCK, Madin-Darby canine kidney; HAS2, hyaluronan synthase-2; MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase.

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1 The abbreviations used are: EMT, epithelial-mesenchymal transition; HGF, hepatocyte growth factor; PI, phosphoinositide; MDCK, Madin-Darby canine kidney; HAS2, hyaluronan synthase-2; MMP, matrix metalloproteinase; ERK, extracellular signal-regulated kinase.

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Hyaluronan and Epithelial Transformation

Hyaluronan—Hyaluronan content was determined using a previously described competitive enzyme-linked immunosorbent assay (30, 31) and standards. Cells, standards, and blanks were incubated with biotinylated hyaluronan-binding protein (Seikagaku America, Inc.) and applied to hyaluronan-coated plates. Excess biotinylated hyaluronan-binding protein binds to the hyaluronan coating the plate and is detected with peroxidase-conjugated mouse anti-biotin monoclonal antibody (03-3720, Zymed Laboratories Inc.), followed by color development with 3-amino-9-ethylcarbazole (Anika Therapeutics) (23) or 12.5 units of Streptomyces hyaluronidase (Sigma) for 1 h. An erythrocyte suspension (750 µl, -10 ×10^6 fixed human erythrocytes/ml) was added to each well and allowed to settle. Cells were viewed under an Olympus microscope, and pictures were taken with a Fuji Finepix 4700 digital camera. We used the Scion Image for Windows program to measure the coat size as defined by the area surrounded by red blood cells minus the cell area defined by the cell membrane.

Quantitation of Hyaluronan—Hyaluronan content was determined using a previously described competitive enzyme-linked immunosorbent assay (30, 31) and standards. Cells, standards, and blanks were incubated with biotinylated hyaluronan-binding protein (Seikagaku America, Inc.) and applied to hyaluronan-coated plates. Excess biotinylated hyaluronan-binding protein binds to the hyaluronan coating the plate and is detected with peroxidase-conjugated mouse anti-biotin monoclonal antibody (03-3720, Zymed Laboratories Inc.), followed by color development with 3-amino-9-ethylcarbazole (Anika Therapeutics) (23) or 12.5 units of Streptomyces hyaluronidase (Sigma) for 1 h. An erythrocyte suspension (750 µl, -10 ×10^6 fixed human erythrocytes/ml) was added to each well and allowed to settle. Cells were viewed under an Olympus microscope, and pictures were taken with a Fuji Finepix 4700 digital camera. We used the Scion Image for Windows program to measure the coat size as defined by the area surrounded by red blood cells minus the cell area defined by the cell membrane.

Results—Cell lysates were preincubated with antibody G Plus/Protein A-agarose beads (Oncogene Science Inc.) for 10 min at 4 °C on an orbital shaker. Beads were removed by centrifugation, and lysates were normalized to 1 mg/ml total protein. The β-catenin immunoprecipitating antibody (1:200; BD Transduction Laboratories) was added to cell lysate and incubated at 4 °C overnight on an orbital shaker. To capture the immunocomplex, agarose beads were added to the lysate and incubated at 4 °C overnight. The agarose beads were collected, washed three times with ice-cold phosphate-buffered saline, and resuspended in 2× sample buffer. Beads were boiled for 5 min and centrifuged, and the supernatant was subjected to SDS-PAGE. Membranes were incubated with antibody against E-cadherin (1:250; Sigma), followed by horseradish peroxidase-conjugated anti-rat secondary antibody (1:2000; Cell Signaling Technologies). ECL reagent (PerkinElmer Life Sciences) was used for detection. Lysates of MCF-10A cells infected with recombinant adenoviruses were processed for Western blotting of MMP-9 (anti-MMP-9 antibody, 1:200; Oncogene Science Inc.) in a similar manner. Band densitometry was performed with an Eagle Eye camera. Representative results are shown and are representative of three separate experiments. Gelatinase zymography was carried out as described previously (33).

Immunoprecipitation—Cell lysates were preincubated with protein G Plus/protein A-agarose beads (Oncogene Science Inc.) for 10 min at 4 °C on an orbital shaker. Beads were removed by centrifugation, and lysates were normalized to 1 mg/ml total protein. The β-catenin immunoprecipitating antibody (1:200; BD Transduction Laboratories) was added to cell lysate and incubated at 4 °C overnight on an orbital shaker. To capture the immunocomplex, agarose beads were added to the lysate and incubated at 4 °C overnight. The agarose beads were collected, washed three times with ice-cold phosphate-buffered saline, and resuspended in 2× sample buffer. Beads were boiled for 5 min and centrifuged, and the supernatant was subjected to SDS-PAGE and transblotted. Membranes were incubated with antibody against E-cadherin (1:250; Sigma), followed by horseradish peroxidase-conjugated anti-rat secondary antibody (1:1000; Amersham Biosciences).

Immunostaining—Cells were seeded the day after adenoviral infection in 2-chamber slides (Falcon) in the presence or absence of 100 µg/ml hyaluronan oligomers. After 24 h, the cells were washed three times with 1× phosphate-buffered saline and fixed with cold (−20 °C) 100% methanol for 5 min. Cells were washed with 1× phosphate-buffered saline (3× 5 min), followed by blocking in 3% bovine serum albumin/phosphate-buffered saline overnight. Cells were washed as described above and incubated at 4 °C overnight with antibody against β-catenin (1:500; BD Transduction Laboratories), E-cadherin (1:250; BD Biosciences), CD44 (1:250; Calbiochem), cytokertatin (1:250; Sigma), or vimentin (3–5 µg/ml; Oncogene) prepared in blocking solution for 1 h at room temperature. Cells were washed as described above, coverslipped, and examined using the SPOT advanced program (Diagnostic Instruments). Duplicate chambers were plated in three separate experiments.

RESULTS—Increased Expression of Hyaluronan Promotes Anchorage-independent Growth in Normal Epithelial Cells—Perturbation of cell-surface hyaluronan-tumor interactions by addition of hyaluronan oligomers or by experimentally induced expression of soluble hyaluronan-binding proteins leads to inhibition of cell survival pathway activity and malignant cell behavior (20–23, 27). Thus, we postulated that increased levels of hyaluronan synthesis in non-transformed epithelial cells may lead to enhanced epithelial-cell interactions and cell survival signaling pathway activity and thus to acquisition of...
transformed properties. To address this question, we experimentally elevated hyaluronan levels in phenotypically normal epithelial cells via increased expression of HAS2. This was achieved by infecting MDCK epithelial cells or MCF-10A human mammary epithelial cells with a recombinant HAS2 adenovirus. As expected, cells infected with the HAS2 adenovirus produced 2–4-fold higher levels of hyaluronan than cells infected with a control β-galactosidase adenovirus or uninfected cells, and these increases were observed in both the cell layer and the medium.

We found that increased hyaluronan production in either MDCK or MCF-10A cells caused a clear-cut induction of anchorage-independent growth, as assessed by colony formation in soft agar (Fig. 1, A and B). To illustrate further that endogenous hyaluronan-cell interactions are important in this phenomenon, we tested whether hyaluronan oligomers reverse the effect of elevated hyaluronan production. The hyaluronan oligomers compete for binding of endogenous hyaluronan polymer to the cell surface, thus replacing high affinity, multivalent endogenous interactions with low affinity, low valence interactions (34, 35). Induction of anchorage-independent growth was reversed by addition of hyaluronan oligomers, but not by chitin oligomers (Fig. 1, C). The chitin oligomers were used as a negative control because they are very similar in structure to the hyaluronan oligomers, to the extent that hyaluronan synthases are capable of synthesizing chitin oligomers (36). Anchorage-independent growth was also reversed by addition of antibody against CD44, implicating enhanced hyaluronan-CD44 interaction in the onset of anchorage-independent growth.

We have found, in previous studies performed with tumor cells, that inhibition of anchorage-independent growth by hyaluronan oligomers is due to suppression of the PI 3-kinase/Akt pathway (23). Thus, we asked whether promotion of anchorage-independent growth by increased hyaluronan production is associated with increased activity of this pathway. We first tested the effect of the PI 3-kinase inhibitors LY294002 and wortmannin on HAS2 stimulation of anchorage-independent growth. Both these inhibitors reversed the effect of increased hyaluronan production (Fig. 1 C). We then measured the effect of increased hyaluronan production on phosphorylation of Akt (protein kinase B), a major downstream effector in the PI 3-kinase pathway, and of glycogen synthase kinase-3β, one of its targets (37, 38). We found that the MDCK cells infected with the HAS2 adenovirus had greatly increased levels of phosphorylated Akt and glycogen synthase kinase-3β compared with the control cells (Fig. 1 D). We did not detect any significant change in the total levels of Akt in these experiments (data not shown).

In our previous experiments with tumor cells, we have shown that hyaluronan oligomers have a negligible effect on cell growth under anchorage-dependent conditions, but induce apoptosis under anchorage-independent conditions (23). As
with the tumor cells, we observed no effect of the hyaluronan oligomers on growth of H9252-galactosidase or HAS2 adenovirus-infected cells in routine monolayer culture, i.e. anchorage-dependent conditions, using the same concentrations of hyaluronan oligomer as in the experiments above (data not shown). This result also indicates that these oligomers do not have a toxic effect on the cells. We then tested the effect of the oligomers on growth in suspension, i.e. under anchorage-independent conditions, and found that they induced high levels of apoptosis (~80%) in the HAS2 adenovirus-infected cells (see below).

**Increased Expression of Hyaluronan Promotes Cell Invasion in Normal Epithelial Cells**—Increased hyaluronan production also caused increased invasiveness in MDCK and MCF-10A cells, and hyaluronan oligomers reversed this effect (Fig. 2, A and B). The degree of stimulation by increased hyaluronan production depended on time of incubation and cell number. For example, in the experiment with MDCK cells in Fig. 2A, incubation was for 24 h, and apparent stimulation by HAS2 was only ~2-fold. However, at shorter times of incubation, as shown for MCF-10A cells in Fig. 2B, a greater level of stimulation was detected. Reversal by the hyaluronan oligomers was ~85% in both cases. Antibody against CD44 also had an inhibitory effect, but the degree of this effect was variable and not as great as that of the hyaluronan oligomers (Fig. 2, A and B). As with anchorage-independent growth (Fig. 1C), inhibitors of PI 3-kinase, e.g. LY294002, reversed the effect of increased hyaluronan production (Fig. 2, A and B).

Because increased invasiveness is usually associated with increased production of MMPs (39), we also determined whether increased hyaluronan production leads to increased MMP production. We found that HAS2-overexpressing MDCK and MCF-10A cells produced highly elevated levels of MMP-9 (Fig. 2, C and D). MMP-2 was also elevated in the HAS2-treated MDCK cells, but to a lesser extent than MMP-9 (Fig. 2C). Interestingly, however, these increases in MMP production were not reversed by treatment with hyaluronan oligomers (Fig. 2C).

**Increased Hyaluronan Expression Induces Mesenchymal Characteristics in Epithelial Cells**—The MDCK cells grew in a typical epithelial “cobblestone” pattern, forming tight colonies with smooth borders that did not extend cell projections (Fig. 3A, panel A). In contrast, the HAS2 adenovirus-infected MDCK cells showed a spindle-shaped, more mesenchymal phenotype, with dispersed cells that did not form tight cell-cell contacts (Fig. 3A, panel D). Because vimentin expression is a reliable mesenchymal marker, we examined HAS2-overexpressing cells by immunocytochemistry for the presence of vimentin. Whereas the control MDCK cells showed little vimentin staining (Fig. 3A, panel C), the cells overexpressing HAS2 exhibited strong vimentin staining (panel F), indicative of a shift toward a mesenchymal phenotype. To further explore this apparent shift, we stained the cells for cytokeratin, an epithelial marker. In this case, the control MDCK cells showed strongly stained cytoskeletal fibrils (Fig. 3A, panel B), whereas the HAS2-overexpressing cells showed a weaker diffuse staining pattern (panel E).

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**Fig. 2. Increased hyaluronan levels promote invasiveness and gelatinase expression in epithelial cells.** A and B, treatment with recombinant HAS2 adenovirus increased invasion through Matrigel by MDCK and MCF-10A cells, respectively. However, this effect varied with the time course of invasion. Invasion was inhibited by treatment with 100 μg/ml hyaluronan oligomers (HA-o) or the PI 3-kinase inhibitor LY294002, but not by treatment with 100 μg/ml chitin oligomers (Ch-o). Antibody (Ab) against CD44 partially inhibited invasion. C and D, treatment with recombinant HAS2 adenovirus induced expression of gelatinases A and B in MDCK cells and gelatinase B in MCF-10A cells, respectively. Gelatinases were detected by zymography for both cell types. In the case of MCF-10A cells, Western blotting (D, right panel) was used to confirm the presence of MMP-9.
were predominantly located along intercellular borders in the control MDCK cells (Fig. 3B, panels A–C). In contrast, cells overexpressing HAS2 showed a marked decrease in intensity of staining at the intercellular boundaries and a diffuse, disorganized, cytoplasmic distribution of these adhesion proteins (panels D–F). Treatment with hyaluronan oligomers did not appear to affect β-catenin or E-cadherin distribution. However, CD44 staining increased at cell borders following hyaluronan oligomer treatment of HAS2-overexpressing cells (data not shown).

We used Western blot analysis to examine whether changes in β-catenin or E-cadherin expression levels occur in HAS2-overexpressing cells. We found little change in expression levels between the control and HAS2-infected cells (data not shown), suggesting that overexpression of HAS2 affects the distribution of cell adhesion proteins and induces loss of intercellular contacts, but does not greatly affect the expression levels of these proteins. To determine whether β-catenin-E-cadherin complex formation was affected, we examined their interaction by immunoprecipitation with antibody against E-cadherin, followed by Western blotting with antibody against β-catenin. As expected, less complex formation between β-catenin and E-cadherin was observed in the HAS2-overexpressing cells compared with the control cells, even though the total levels of E-cadherin remained approximately the same (Fig. 3B, panel G).

Hyaluronan Oligomers Inhibit Induction of Anchorage-independent Growth and Cell Invasion by HGF or β-Catenin—Because we found that elevated levels of hyaluronan led to the induction of transformed characteristics in normal epithelial cells, we also examined whether hyaluronan is an important effector in well described systems of cell transformation. Treatment of MDCK epithelial cells with HGF induces anchorage-independent growth and enhanced cell invasion (40, 41), two phenomena associated with malignant cell behavior. We found here, as expected, that treatment of either MDCK cells or MCF-10A human mammary epithelial cells with HGF induced the ability of these cells to form colonies in soft agar (Fig. 4) and to invade reconstituted basement membrane (Fig. 5). Overexpression of β-catenin via stable transfection also induces anchorage-independent growth in MDCK cells (25), as reproduced here in Fig. 4. In addition, increased cytoplasmic β-catenin has been linked to invasiveness (42, 43); and accordingly, we found here that overexpression of β-catenin in MDCK cells increased their ability to invade reconstituted basement membrane (Fig. 5). To determine whether endogenous hyaluronan-cell interactions are important in these phenomena, we tested whether treatment with hyaluronan oligomers reverses the effects of HGF or β-catenin.

We first tested the effect of hyaluronan oligomers on HGF-induced anchorage-independent growth and found that the oligomers reversed this induction in both MDCK (Fig. 4A) and MCF-10A (Fig. 4B) cells. Partial reversal was obtained upon addition of 1–10 μg/ml hyaluronan oligomers, and almost complete reversal was obtained with 100 μg/ml. However, no effect was obtained with 1–100 μg/ml chitin oligomers. Reversal of HGF induction was also obtained upon treatment with antibody against CD44 (Fig. 4, A and B), implying that the hyaluronan oligomers act by competing for endogenous hyaluronan polymer-CD44 interactions. Reversal was also obtained with inhibitors of PI 3-kinase activity, e.g. LY294002 and wortmannin, confirming previous findings that HGF acts via the PI 3-kinase cell survival pathway (44, 45). We then tested the effects of the above reagents on induction of anchorage-independent growth by overexpression of β-catenin. Addition of 1–100 μg/ml hyaluronan oligomers reversed the effects of
transfection of MDCK cells with either wild-type β-catenin (Fig. 4C) or a degradation-resistant mutant (Fig. 4D). Again, chitin oligomers had no effect, whereas antibodies against CD44 and LY294002 mimicked the effect of the hyaluronan oligomers. We also tested the effect of hyaluronan oligomers on the levels of apoptosis in HGF- and β-catenin-treated cells under anchorage-independent conditions and found that they stimulated apoptosis to an extent similar to that observed upon treatment of cells infected with the HAS2 adenovirus, i.e. ~80% (Fig. 4E).

We next examined the effects of hyaluronan oligomers on induction of invasiveness by HGF and β-catenin. We found that the oligomers had a strong inhibitory effect; but as described above for HAS2, the level of this effect depended on the conditions of the assay, especially the time course of treatment and the number of cells used. Fig. 5A shows a 12-h time course of the effect of 100 μg/ml hyaluronan oligomers on MDCK cells transfected with the wild-type β-catenin construct or treated with HGF. For β-catenin, the oligomers inhibited invasion by almost 100% over a 6-h period; but in this experiment, inhibition was reduced to ~20% by 12 h. In a separate 6-h experiment, we showed that the hyaluronan oligomers inhibited invasion of S37A β-catenin-transfected cells by ~80%, whereas chitin oligomers did not have a significant effect (Fig. 5B). Antibodies against CD44 and LY294002 had inhibitory effects similar to those of the hyaluronan oligomers (Fig. 5B), as was also found for anchorage-independent growth in soft agar (Fig. 4). A similar pattern was seen upon hyaluronan oligomer treatment of MDCK cells transfected with the degradation-resistant mutant of β-catenin (data not shown). In the experiment shown in Fig. 5A, HGF was slower to stimulate invasion to the same extent as β-catenin, and inhibition by the oligomers was 40–50% over
Fig. 5. Hyaluronan oligomers reverse invasiveness induced by HGF or β-catenin in epithelial cells. A, HGF-treated and β-catenin-transfected MDCK cells invaded Matrigel-coated chambers in greater numbers compared with untreated cells over a 12-h period. Hyaluronan oligomers reduced invasion to varying extents over this period. B, MDCK cells transfected with wild-type (WT) β-catenin invaded Matrigel in greater numbers compared with the control cells over a 6-h period. This invasion was inhibited by treatment with 100 μg/ml hyaluronan oligomers (HA-o), antibody (Ab) against CD44, or LY294002, but not by treatment with chitin oligomers (Ch-o). C, MDCK cells treated with HGF invaded Matrigel in greater numbers compared with the control cells over a 24-h period. This invasion was partially inhibited by 100 μg/ml hyaluronan oligomers, antibody against CD44, or LY294002, but not by treatment with chitin oligomers.

this period. Fig. 5C gives the result of another experiment in which MDCK cells were treated with HGF over a 24-h period. In this particular experiment, 100 μg/ml hyaluronan oligomers caused ~60% inhibition of invasion, and 1–10 μg/ml had intermediate effects. Chitin oligomers had no effect, whereas inhibitors of PI 3-kinase had an effect similar to that of the hyaluronan oligomers. Antibody against CD44 had a significant but lesser effect than hyaluronan oligomers. Similar results were obtained with HGF-stimulated MCF-10A cells (data not shown). In each case, addition of hyaluronan oligomers inhibited invasion to a significant extent, but the degree of inhibition varied with the conditions of the experiment.

HGF and β-Catenin Stimulate Assembly of Hyaluronan-dependent Pericellular Matrices around Epithelial Cells—The data presented above imply that endogenously produced hyaluronan is required for the effects of HGF and β-catenin on anchorage-independent growth and, in a somewhat more complex fashion, on invasiveness of epithelial cells. Thus, we determined whether these agents stimulate production of hyaluronan. We measured hyaluronan levels in the media of MDCK and MCF-10A cells treated with HGF and of MDCK cells transfected with wild-type or mutant β-catenin. We found that each of these treatments led to ~30–60% increases in hyaluronan accumulation in the media over 24 h. A similar level of stimulation of secreted hyaluronan was reported previously for HGF (46).

We then measured the assembly of hyaluronan-dependent pericellular matrices in these cultures. To visualize these matrices, we used a particle exclusion assay in which the particles (in this case, fixed red blood cells) are added to subconfluent monolayer cultures (28, 29). Control epithelial cells do not exhibit these matrices; in which case, the particles closely abutted the surface of each cell, either outlining or covering the cells (Fig. 6A, control). For cells that exhibited significant pericellular matrix assembly, the particles could not penetrate the matrices, and the cells appear to be surrounded by a clear halo.
HAS2 adenovirus stimulated assembly of pericellular matrices, and assembly was inhibited by treatment with hyaluronidase or hyaluronan

or coat (Fig. 6A, Has2 panel). The HGF-stimulated and β-catenin-transfected cells exhibited dramatically larger pericellular matrices than the control cells (Fig. 6, A and C). On average, HGF stimulated assembly of these matrices ~2.5-fold and β-catenin ~6-fold (Fig. 6C), compared with ~4-fold after experimental up-regulation of hyaluronan production using HAS2 (Fig. 6B). In each case, treatment with Streptomyces hyaluronidase, an enzyme that is specific for hyaluronan, removed the matrices, confirming that they are hyaluronan-dependent. Especially significant is the observation that treatment of the HGF-, β-catenin-, or HAS2-stimulated cells with hyaluronan oligomers inhibited matrix assembly.

**DISCUSSION**

Elevated levels of hyaluronan are present at sites of EMT in the developing embryo, e.g. during formation of cardiac cushion mesenchyme (47), palatal shelf fusion (48), and neural crest emigration (49). Conversion of cardiac endothelium to cushion mesenchyme is defective in the Has2-null mouse, and this defect has been directly linked to loss of hyaluronan-induced ErbB2/3 and Ras signaling, indicating that hyaluronan is crucial to EMT at this site (6, 7). In addition, numerous studies have indicated that hyaluronan is important in tumor progression (8), several characteristics of which resemble changes that occur during EMT (3). In particular, it has been shown that treatment with exogenous hyaluronan (50, 51) or overexpression of hyaluronan via transfection with Has (52) stimulates fibroblast growth and motility in a PI 3-kinase- and Src-dependent manner. Also, HGF stimulation of hyaluronan production is dependent on PI 3-kinase activity (46), implying that both the synthesis and function of hyaluronan may require PI 3-kinase under some circumstances. The induction of anchorage-independent growth by increased hyaluronan production or by HGF or β-catenin is reversed by treatment with hyaluronan oligomers, indicating the continued requirement for hyaluronan in this phenomenon. Reversal of anchorage-independent growth in this way was also shown previously for tumor cells (23, 27). In each case, we have shown that hyaluronan promotes anchorage-independent growth by inhibiting apoptotic pathways via stimulation of the PI 3-kinase/Akt signaling pathway. Recent data obtained in our laboratory indicate that endogenous hyaluronan also stimulates ERK-mediated phosphorylation of BAD, again promoting cell survival (53). Thus, we conclude that hyaluronan plays a central role in anchorage-independent growth and cell survival, properties that distinguish carcinoma cells from normal epithelia (54, 55).

Our evidence also suggests that the effects of hyaluronan on anchorage-independent growth are mediated by interaction with the hyaluronan receptor CD44. CD44 interacts with and is required for the activity of receptor tyrosine kinases that regulate the PI 3-kinase and other signaling pathways, e.g. c-Met (56) and ErbB2/3 (57, 58). Furthermore, CD44 interacts with ezrin (59, 60), another regulator of PI 3-kinase activity (61). Possibly then, interaction of hyaluronan with CD44 induces activity of these agents, stimulating further hyaluronan production, thus setting up a stimulatory loop that augments transformed cell behavior. Such a loop has been suggested previously in the case of the tpr-met oncogene (46). However, the involvement of CD44 in cell invasion is not as clear as for
anchorage-independent growth in that treatment with anti
body against CD44 was more variable and less efficient than
treatment with hyaluronan oligomers in reversing HAS2-me-
diated increases in invasion. Numerous studies have indicated
that binding of hyaluronan to RHAMM induces cell transforma-
tion (24, 50, 62, 63). Thus, it will be of interest to determine
whether hyaluronan oligomers also inhibit endogenous hya-
loronan-RHAMM interactions involved in cancer cell invasion.

Another effect of increased production of hyaluronan in ep-
ithelial cells is induction of gelatinase production, especially
gelatinase B (MMP-9). Recent reports have also shown that
addition of exogenous hyaluronan stimulates gelatinase pro-
duction in tumor cells (64, 65). MMPs have been implicated in
numerous aspects of transformation and malignancy, including
promotion of epithelial-mesenchymal transition and malignant
transformation (66, 67). MMP-9 in particular is important in
the relationship of these observations to stimulation of MMP-9
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