Hyaluronan (HA) is enriched in the pericellular matrices of many malignant human tumors, and manipulations of HA interactions have strong effects on tumor progression in animal models. Increased HA production stimulates ERBB2 activation, leading to increased cell survival activities and several malignant cell properties. On the other hand, inhibition of constitutive HA-tumor cell interactions in malignant cells inhibits these properties. We have now investigated the role of HA in activation of several additional receptor tyrosine kinases (RTKs), i.e. IGF1R-β, PDGFR-β, EGFR and c-MET, in colon, prostate, and breast carcinoma cells. In each case we show that antagonists of endogenous HA interactions inhibit their tyrosine phosphorylation, i.e. activation. On the other hand, we show that these RTKs are activated in phenotypically normal or relatively benign tumor cells by experimentally increasing HA production. We also investigated the role of HA in constitutive versus ligand-induced activation of RTKs. In HCA7 colon and C4-2 prostate carcinoma cells, ERBB2 is constitutively activated in a ligand-independent manner, whereas IGF1R-β and PDGFR-β require ligand interaction for activation. We show that both constitutive activation of ERBB2 and ligand-mediated activation of IGF1R-β and PDGFR-β are reversed by co-treatment of the cells with a HA antagonist. We conclude that HA serves a general function in RTK activation.

Aberrant activities of several types of RTKs have been implicated in the genesis of a significant proportion of human cancers, including breast, colon, and prostate carcinomas. Increased RTK activity can arise from gene amplification, activating mutations, or altered regulation, e.g. by cross-talk between RTKs, integrins, and other receptors or by autocrine and paracrine stimulation by various regulatory factors. These changes lead in turn to enhanced tumor cell growth, motility, survival, and resistance to therapies (1–4).

HA is a very large, anionic polysaccharide that is localized at the cell surface and in the extracellular matrix of numerous tissues, wherein it plays an important structural role under homeostatic conditions. However, when cells proliferate or migrate, e.g. in embryonic processes, tissue remodeling, inflammation, and diseases such as cancer and atherosclerosis, HA-induced signaling is activated (5–10). High levels of HA in tumors are a prognostic factor in several malignancies (5), and manipulations of HA production or interaction with cell surface receptors strongly influence tumor growth and metastasis (7, 10). CD44 is a major cell surface receptor for HA and also plays an important role in tumor progression (11, 12). We and others have shown that interaction of HA with CD44 induces formation of complexes containing CD44 and ERBB2 or EGFR in a variety of tumor cells (13–16). We have shown that HA constitutively regulates formation of a signaling complex containing phosphorylated ERBB2, CD44, ezrin, PI3K, and the chaperone molecules HSP90 and CDC37 (16, 17). In this work we have used various antagonists of interactions of HA with CD44. These antagonists include HA oligomers that compete for endogenous HA interactions, soluble HA-binding proteins that also act as competitive inhibitors, and RNA interference silencing of CD44. Each of these antagonists inhibits ERBB2 activation and formation of the ERBB2-containing complex in malignant colon and breast cancer cells, whereas experimentally increased HA production induces activation and assembly of this complex (16). These manipulations of HA lead to alterations in cell survival pathways, anchorage-independent growth, invasiveness, epithelial-mesenchymal transition, drug sensitivity, and tumor progression in animal models (7).

In this study, we show that HA has a general effect on RTK activation. In malignant colon, prostate, and breast carcinoma cells, HA antagonists inhibit activation of multiple RTKs, including ERBB2, EGFR, IGF1R-β, PDGFR-β, and c-MET, as well as assembly of signaling complexes containing these activated RTKs. Increased HA production, however, induces RTK activation and signaling complex assembly in phenotypically normal epithelial cells. We find that these manipulations of HA affect both ligand-dependent and constitutive RTK activation. Therefore, we conclude that HA plays an intrinsic role in RTK-induced oncogenic pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—HA oligomers were prepared as described previously (18). Mouse monoclonal antibody against human CD44...
(HCAM; 1:2000), rabbit polyclonal antibodies against PDGFR-β (P-20), and PI3K/p110α (H-201;1:2000) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rat monoclonal anti-CD44 (1:2000) was from EMD Biosciences (La Jolla, CA). Rabbit polyclonal antibodies against ERBB2 (1:3000), phospho-ERBB2 (Y1248; 1:3000), phospho-MET (Y1234, Y1235; 1:3000) and phospho-PDGFR-β (Tyr716) (1:1000), and mouse monoclonal antibody against phospho-IGF-1R (Tyr-1131; clone JY202) were from Upstate Biotechnology (Lake Placid, NY). Horseradish peroxidase-linked sheep anti-rabbit and goat antimouse antibodies were purchased from Amersham Biosciences. Galardin (GM6001) and its negative control, adenine, biotin, and triiodothyronine were from EMD Biosciences. DMEM (high glucose), RPMI 1640, Ham’s F-12, penicillin/streptomycin, insulin, insulin-transferrin-selenium-G supplement, and human recombinant EGF were from Invitrogen. Recombinant human PDGF-BB was purchased from BD Biosciences, (Bedford, MA). IGF1 was purchased from Sigma. All other reagents were of analytical grade and purchased from Sigma.

Cell Culture—LNCaP and C4-2 human prostate carcinoma cells were from Dr. C. Vokel-Johnson, Medical University of South Carolina, Charleston, SC. LNCaP cells were grown in RPMI 1640 medium, 200 mM pyruvate, 0.15 g/ml sodium bicarbonate, 0.45 g/ml glucose, 10 mM HEPES buffer, pH 7.4, 5 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum. C4-2 cells were grown in Ham’s F12/DMEM (20:80 v/v), 1× insulin-transferrin-selenium-G supplement, 13.6 ng/ml triiodothyronine, 25 ng/ml biotin, 2.5 ng/ml adenine, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM glutamine, and 10% fetal bovine serum. PC-3 and DU145 cells (ATCC, Manassas, VA) were grown in RPMI 1640 medium, Glutamax 1 plus 10% fetal bovine serum. MCF-7/Adr drug-resistant human mammary carcinoma cells (K. Cowan, University of Nebraska) and MCF-7 cells (C. Sonnenschein, Tufts University, Boston, MA) were grown in the same medium as PC-3 and DU145 cells. HCA7 (Colon 29) human colon carcinoma cells (European Collection of Cell Cultures) were maintained in DMEM, 10% fetal bovine serum, 2 mM glutamine, 11 mg/ml pyruvate. HT29 cells (ATCC) were grown in McCoy’s 5a medium, 1.5 mM L-glutamine, 2.2 g/liter sodium bicarbonate, 10% fetal bovine serum. IEC6 rat intestinal epithelial cells (ATCC) were grown in DMEM with 4 mM L-Glutamine, 4.5 g/liter glucose, 1.5 g/l. Na bicarbonate, 100 units/liter bovine insulin, 10% fetal bovine serum. HIEC-6 human intestinal cells (J. F. Beaulieu, University of Sherbrook, Quebec, Canada) were maintained in DMEM (high glucose), 4% fetal bovine serum, 20 mM HEPES buffer, pH 7.4, 50 units/ml penicillin, 50 μg/ml streptomycin, 10 μg/ml insulin, 5 ng/ml human recombinant EGF (19) and used between 15th and 17th passage in this study. Cell lines were grown at 37 °C in 5% CO2 and passed every 3–4 days.

RNA Silencing and HAS2 Adenovirus—siRNA for human CD44 and the recombinant HAS2 adenovirus were produced and employed as described previously (16).

Preparation of Cell Lysates—After treatment, cells were washed twice in cold phosphate-buffered saline and then harvested in Versene with gentle pipetting at 37 °C for 15 min, followed by washing twice in cold phosphate-buffered saline. The cell pellet was treated with lysis buffer containing 1% Nonidet P-40, 0.5 mM EGTA, 5 mM sodium orthovanadate, 10% (v/v) glycerol, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, and 50 mM HEPES, pH 7.5. The suspension was vortexed for three cycles of 15 s at maximum speed followed by cooling on ice. The lysate was centrifuged at 12,000 × g for 15 min at
HA Regulates Activation of Multiple RTKs

**RESULTS AND DISCUSSION**

Constitutive and Ligand-dependent Activation of RTKs Are Dependent on Endogenous HA in Malignant Carcinoma Cells—

In previous studies we have shown that antagonists of HA-CD44 interaction inhibit ERBB2 activation (i.e. tyrosine phosphorylation) in colon and breast carcinoma cells (16, 17). We have now investigated the effects of HA antagonists on activation of several RTKs, namely ERBB2, EGFR, IGF1R, c-MET, and PDGFR, in several types of malignant colon, breast, and prostate carcinoma cells. The cells used were selected since they exhibit relatively high levels of tyrosine phosphorylation of the RTKs shown in Fig. 1, when incubated in serum-containing culture medium. However, it can be seen that these RTKs are activated to different degrees in the various cells used (Fig. 1). In each case it was shown that HA-CD44 antagonists, i.e. HA oligomers or CD44 siRNA (16), inhibit activation of all five RTKs to various extents, ranging from 60 to 70% by addition of o-HA. In Fig. 2, A and B, are representative of two independent experiments (quantitation of results appears in the supplemental figure, panel A).

**HA regulates ligand-dependent and constitutive RTK activation**

A. HCA7 colon, C4-2 prostate, and MCF-7 breast carcinoma cells were incubated in the presence or absence of serum, then lysed and immunoblotted with antibodies against phospho-(p)- or total (T)-ERBB2, IGF1R, or PDGFR. Serum was required for activation of IGF1R and PDGFR but not ERBB2, indicating that the latter is constitutively activated in these cells. The cells were incubated in the presence or absence of PDGF-BB (100 ng/ml) or IGF1 (25 ng/ml), with and without addition of o-HA (100 μg/ml). PDGF and IGF1 activated their respective RTK, and this activation was reversed to the extent of 50–70% by addition of o-HA. Results in Fig. 2, A and B, are representative of two independent experiments (quantitation of results appears in the supplemental figure, panel A).

**FIGURE 3. HA antagonists inhibit formation of RTK complexes.** HCA7 colon carcinoma cells (A), HT29 colon carcinoma cells (B), and C4-2 prostate carcinoma cells (C) were incubated in the presence and absence of o-HA (100 μg/ml) or transfected with CD44 siRNA or control RNA. For immunoprecipitation (IP), the cell layer was lysed and incubated with antibody against total c-MET, PDGFR, ERBB2, EGFR, or IGF1R. Aliquots of re-dissolved immunoprecipitates were subject to SDS-PAGE and Western blotting (WB) using antibodies against the respective phospho-(p)- or total (T)-RTK, i.e. c-MET, PDGFR, ERBB2, EGFR, or IGF1R. Results in Fig. 3, A–C, are representative of three independent experiments (quantitation of results appears in the supplemental figure).

4 °C in an Eppendorf 5415R centrifuge. An aliquot of the extract was diluted with 0.5% SDS and protein concentration measured. The remaining supernatant was frozen and stored at −80 °C.

**Immunoprecipitations**—All procedures were done at 4 °C unless otherwise mentioned. Cell lysates (500 μl; 1 μg of protein/μl) were mixed with 5 μg of antibody against ERBB2, EGFR, c-MET, IGF1R, or PDGFR and incubated for 2 h while rotating on a wheel. The immune complexes were captured by incubating with 80 μl of: 1:1 (v/v) protein A-Sepharose 4B suspension and incubated for another 1 h. After collecting by brief centrifugation, the Sepharose 4B beads were washed three times in ice-cold lysis buffer, three times with lithium chloride buffer (5 mM LiCl, 0.1 mM sodium orthovanadate, 0.1 M Tris-HCl, pH 7.4), and three times with buffer containing 150 mM NaCl, 5 mM EDTA, 0.1 mM sodium orthovanadate, 10 mM Tris-HCl, pH 7.4. Finally, the immune complexes were recovered from the beads in 500 μl of SDS-containing denaturing buffer and heated to 65 °C for 5 min.

**SDS-PAGE**—Prestained molecular weight standards or 10–30 μg of denatured protein per lane were loaded onto NuPAGE Novex BisTris gels, electrophoresed in a Novex mini-cell apparatus (Invitrogen) at 200 V for 50 min, transferred to nitrocellulose membranes at 110 V for 40 min. The membranes were blocked with 5% nonfat dry milk in Tris-buffered-saline, 0.01% Tween 20 (TBST) buffer for 1 h, washed in TBST buffer, probed with antibody diluted in 5% bovine serum albumin/TBST (polyclonal antibodies) or 5% nonfat dry milk/TBST (monoclonals), then treated with peroxidase-linked anti-mouse or anti-rabbit secondary antibodies at 1:5000 dilution in 5% milk/TBST for 1 h at room temperature. Bands were revealed by Chemoluminescence Reagent Plus (PerkinElmer Life Sciences Inc.) and protein sizes estimated with size markers. Intensity of the bands was quantified by densitometry.

**Hyaluronan Assay**—HA was assayed in cell culture media collected after 24 h incubation by an enzyme-linked immunosorbent assay-like assay (20).
fate and polymeric HA do not mimic the effects of HA oligomers on various signaling parameters (16, 18).

We then determined whether ERBB2, IGF1R-β, and PDGFR-β are constitutively activated versus ligand-dependent in three of these cell types, i.e. HCA7 colon, C4-2 prostate and MCF-7 breast carcinoma cells. We found that ERBB2 phosphorylation is about the same after incubation in the presence and absence of serum in these cell types, but virtually no phosphorylation of IGF1R-β and PDGFR-β was detected after incubation without serum (Fig. 2A). When cells were treated in the absence of serum but with the appropriate ligand, i.e. IGF1 or PDGF-BB, the corresponding RTK was activated. This activation was reversed to the extent of 68–71% for phospho-IGF1R-β and 50–64% for phospho-PDGFR-β by co-treatment of cells with HA oligomers (Fig. 2B; also see supplemental figure, panel A). Thus, both constitutive activation of ERBB2 and ligand-dependent activation of IGF1R-β and PDGFR-β are dependent on endogenous HA interactions in these cells.

The variable degrees of inhibition of RTK activation in the experiments described here may be due to several factors. For example, inhibition of CD44 expression by the siRNA varied from 65 to 95% (e.g. see Fig. 4E), which was probably due to variability in transient transfection efficiency. Also, efficacy of treatment with HA oligomers will depend on the amount of endogenous HA bound to CD44, as observed before (17). Alternatively, some subpopulations of RTKs may be regulated by HA and others may not.

Assembly of Activated RTK-containing Signaling Complexes Is Dependent on Endogenous HA—We previously showed that formation of a signaling complex containing activated ERBB2, CD44, PI3K, ezrin, and the co-chaperones, HSP90 and CDC37, is dependent on endogenous interaction of HA with CD44 and that this complex is disassembled by treatment of the cells with HA-CD44 antagonists (16). We have now shown that similar complexes form with other RTKs, i.e. EGFR, IGF1R-β, PDGFR-β, and c-MET. We immunoprecipitated selected RTKs from lysates of HCA7 colon, HT29 colon, or C4-2 prostate carcinoma cells and found that CD44, the p110 subunit of PI3K, and CDC37 co-immunoprecipitate with each of these RTKs. Incubation of the cells with either HA oligomers or CD44 siRNA caused a decrease in amounts of activated RTK, CD44, CDC37, and PI3K in these immunoprecipitated complexes (Fig. 3, A–C). Quantitation of three independent experiments showed decreases in RTK phosphorylation of 40–90% (also see supplemental figure, panel B). In the case of the immunoprecipitated IGF1R-β complex, there were decreases of 74–75% for activated IGF1R-β, 41–72% for CD44, 75–83% for CDC37, and 58–82% for PI3K (supplemental figure, panel C). Similar results were obtained for the other RTK complexes. However, the total amounts of RTKs in each of the immunoprecipitates were not affected by the HA antagonists.
These results indicate that the antagonists cause disassembly of CD44, CDC37, and PI3K from the complexes as well as inactivation of the RTKs. Again, the variability observed may be due to differences in effectiveness of the antagonists or differing susceptibilities of sub-populations of RTKs to HA regulation, as discussed above.

Recent studies have shown that HA oligomers can cause partial cleavage of CD44 (22, 23), most likely by matrix metalloproteinases (24, 25). This raises the possibility that the decreased amounts of CD44 observed in RTK complexes may be due to CD44 cleavage. Therefore we examined the relative amounts of CD44 in cell lysates after treatment of cells with HA oligomers or GM6001, a broad specificity matrix metalloproteinase inhibitor. We observed no significant effect with HA oligomers (e.g. see Fig. 4E, right panel) or matrix metalloproteinase inhibitor (data not shown) on the amount or size range of CD44. Thus CD44 cleavage is not a major cause for its decreased interaction with ERBB2 after treatment with HA oligomers.

We conclude that endogenous HA-CD44 interaction promotes assembly of several RTK-containing complexes in malignant cells and that HA-CD44 antagonists cause partial to complete disassembly of these complexes. Our previous results indicate that the ERBB2-containing complex forms within a lipid raft-like structure (16). We do not yet know the degree to which the various RTK complexes form separately or coordinately within lipid microdomains.

Increased HA Production Induces Activation of Multiple RTKs in Intestinal Epithelial Cells—In addition to showing inhibition of ERBB2 activation by HA-CD44 antagonists, we have also shown that increased HA production induces ERBB2 activation in MCF-7 breast carcinoma cells, which exhibit low levels of constitutive ERBB2 activation when untreated (16). Here we show that increased HA production stimulates activation of multiple RTKs in phenotypically normal human HIEC6 and rat IEC6 intestinal epithelial cells, as well as in MCF-7 cells. We treated these cells with a recombinant HAS2 adenovirus, which causes ~2.5-fold increase in HA production in these cells under the conditions used (Fig. 4E). A corresponding increase in activation of ERBB2, EGFR, IGF1R-β, c-MET, or PDGFR-β was observed (Fig. 4, A–D). In most cases RTK activation was reversed to the same or lower levels than controls by co-treatment with HA oligomers or CD44 siRNA (Fig. 4, A–D; also see supplemental figure, panel D for quantitation).

RNA interference silencing of CD44 expression obviously interferes with HA-CD44 interactions, while HA oligomers replace multivalent with monovalent HA polymer-heterodimers (21). Thus we have used these reagents to interfere with HA-CD44 interactions and consequent signaling events. However, it is possible that they might also antagonize HA signaling by reversing the increase in HA production induced by recombinant HAS2 transfection. Therefore we measured their effects on HA production in the human HIEC6 cells. As expected, we found that the CD44 siRNA had no effect, but surprisingly, HA oligomers reversed the HAS2-induced increase in HA production as well as decreasing the basal level of HA; they have no discernible effect on CD44 expression (Fig. 4E). Similar results were obtained with rat IEC6 cells (data not shown). Thus, these oligomers not only compete for endogenous interaction of HA with CD44 but also affect HA production. The mechanism underlying this effect of HA oligomers is currently under investigation.

Conclusions—The data presented here and earlier (16, 17) indicate that HA-CD44 interactions constitutively promote activation of several RTKs, specifically ERBB2, EGFR, PDGFR-β, IGF1R-β, and c-MET, as well as formation of large RTK-containing signaling complexes in malignant carcinoma cells. These interactions lead to numerous downstream events necessary for malignant cell characteristics (7). It should be emphasized that these changes were brought about by alterations in constitutive, cell-autonomous HA levels and interactions. In previous studies, we found that addition of exogenous HA polymer did not duplicate the effects of manipulating endogenous HA on ERBB2 activation or downstream signaling (16, 18), whereas others have found that exogenous HA polymer induces ERBB2 activation (13). Interestingly, a recent study showed that exogenously added HA polymer caused formation of a PDGFR-CD44 complex in fibroblasts but gave rise to inhibition rather than promotion of activation (26).

These results emphasize the different manner by which exogenously added and endogenously produced HA interact with cells (7). The different effects may also be due to the different cells used in these experiments, i.e. normal fibroblasts versus cancer cells. Recent data, however, support the possibility that stromal HA, as opposed to tumor cell-produced HA, may inhibit rather than promote tumor progression (27), despite the fact that high levels of stromal HA are sometimes prognostic for tumor progression (5). Other data indicate that turnover of HA may be an important factor in tumor malignancy, and recent results suggest that the balance between HA synthesis and degradation may be crucial (28–32). Clearly, further work is required to determine the mechanisms whereby HA-induced signaling is activated and regulated and the precise functions of HA signaling in malignant cell behavior.

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