Glycosaminoglycan Synthesis by Subpopulations of Epithelial Cells from a Mammary Adenocarcinoma

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ABSTRACT

Glycosaminoglycan synthesis by two subpopulations of a mouse mammary tumor cell line was compared. The two sublines express distinctly different growth characteristics in vitro and in vivo which indicate differences in growth regulation. Newly made glycosaminoglycans were recovered from the culture media, the cell surfaces, and residual cellular material. The cell population which grows more aggressively in vivo (+SA subline, a subline that grows in soft agarose) incorporated about 8 times more [14C]glucosamine per cell into total glycosaminoglycans than did the slower-growing population (−SA subline, which does not grow in soft agarose). Appropriate control experiments indicated that the apparent difference in rates of synthesis was not due to discrepancies in glucosamine uptake. The main residual cellular molecule labeled was heparan sulfate, but the predominant molecule at the cell surface and in the culture fluid was hyaluronic acid. Overall, +SA cells synthesized more hyaluronic acid and −SA cells synthesized more heparan sulfate; in both cell populations, these two molecules accounted for about 90% of total glycosaminoglycans produced.

INTRODUCTION

Spontaneous mammary tumors are not necessarily homogeneous and may consist of several interacting cell populations with distinctive characteristics (20). This heterogeneity within mammary tumors is probably a consequence of dynamic mutation and selection processes (24). Mechanisms have been proposed by which the degree of cellular diversity can be modulated by cell-cell interactions (16, 20). Regardless of the source of tumor cell heterogeneity, it is obvious that cell populations which proliferate must have the ability to grow within the growth of cell populations within a tumor.

Macromolecules of the extracellular matrix have been implicated in local growth modulation. We have focused our attention on one such class of molecules, the GAGs. A broad range of experimental evidence suggests that the accumulation of particular GAGs stimulates or represses growth of normal and neoplastic cells of several origins (7, 19, 22, 25, 26, 29, 32, 33). Of particular interest is the correlation between growth stimulation and high local concentrations of HA (19, 22, 32). In this regard, it is relevant that levels of HA can be very high within mammary tumors (27, 28, 30). Although fibroblasts were originally thought to be the sole source of these GAGs, it is now clear that normal (4, 6, 9, 14, 34) and neoplastic (1, 6, 15, 17, 23) epithelial cells can also produce and secrete GAGs.

The possibility thus arises that transformed mammary epithelial cells can facilitate or hinder their own growth by producing and secreting GAGs.

To further analyze the role of GAGs in mammary tumor growth, we have chosen to compare GAG production and secretion by various populations of mammary epithelial cells. We describe here GAG production by 2 closely related mammary tumor cell lines isolated from the same tumor which have distinctly different growth patterns in vivo.

MATERIALS AND METHODS

Cells. +SA and −SA sublines were isolated from the WAZ-2T cell line (8). WAZ-2T was established from a spontaneous type B mammary adenocarcinoma in a BALB/c mouse. +SA cells grow in suspension in soft agarose, whereas −SA cells do not, although both are tumorigenic in vivo. +SA cells grow more rapidly s.c. and has a shorter tumor latency period. The 2 sublines are morphologically similar (epithelioid) but differ in a variety of other features. As monolayers on plastic, the growth rates and saturation densities are similar (8). In these studies, cells of passage level 20 to 25 were used.

Culture. Cells were grown exclusively on 60-mm plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.) in DME (Grand Island Biological Co., Grand Island, N. Y.) supplemented with penicillin and streptomycin, 5 μg insulin per ml (Calbiochem-Behring Corp., La Jolla, Calif.) and 10% (v/v) fetal bovine serum (complete growth medium). Cell number was monitored by counting the cells in a hemocytometer after enzymatic removal of all attached cells.

Labeling. Monolayers of cells were labeled by adding 4 μCi of o[1-14C]glucosamine hydrochloride (Amersham/Searle Corp., Arlington Heights, III.) in 0.5 ml of medium to cultures which had been fed 2 hr earlier with 2.5 ml of complete growth medium. All cultures were labeled when confluent.

Collecting [14C]GAGs. After incubation, radioactive fractions were collected from monolayer cultures essentially as described before (2). The culture medium was removed and the cell layer was rinsed with calcium- and magnesium-free saline (0.14 M NaCl, 5 mM KCl, 5 mM glucose, and 4 mM NaHCO3). This volume was pooled with the medium and centrifuged for 15 min at 800 rpm to remove cells and large debris. To obtain the cell surface fraction, the washed cell layer was digested with 2.5 ml of 0.5% trypsin and 0.025% EDTA in calcium- and magnesium-free saline (pH 7.4) for 15 min at 37°. This procedure results in the detachment of all cells from the culture dish surface. This volume was pooled with 1 volume of complete medium and centrifuged to remove the trypsinized cells. The resulting pellet is referred to as the cell surface fraction.
residual cellular fraction. The medium, cell surface, and cell fractions were then assayed for \(^{14}C\)GAGs.

**[^14C]GAG Analyses.** The scheme of analysis is summarized in Chart 1. Fractions were incubated with 0.25 mg Pronase B per ml (Calbiochem) in 0.2 M Tris-CI, pH 7.8, for 72 hr at 37°C, with fresh Pronase added daily. Toluene (0.1%) was added as a bacteriostatic agent. Following proteolysis, a portion of each sample was removed for analysis by CPC precipitation (3). The remaining sample was precipitated with TCA, and the supernatant fluid which was recovered was dialyzed exhaustively against water and used to assay for specific GAGs by digestion techniques.

Total incorporation of radioactivity into GAGs was determined by precipitating the GAGs from 1 volume of sample with 1 volume of 1% CPC in the presence of 1 volume of carrier (1 mg chondroitin sulfate g per ml). The precipitate was collected on a Whatman GF/C glass fiber filter, rinsed (twice with 3 ml of 1% CPC, and once with 10 ml of chilled water), oven dried, and counted in a liquid scintillation system (Packard Tri-Carb scintillation counter) in 10 ml of 3α70B scintillation fluid (Research Products, Inc., Elk Grove Village, III.). Recovery of precipitable radioactivity was not decreased by prior treatment with DNase,7 which suggests that the label was not incorporated into nucleic acids.

The amount of \(^{14}C\)HA was determined by digestion of a known sample volume with 10 units of Streptomyces hyaluronidase per ml (Calbiochem) for 16 hr at 37°C, pH 5.0. Preliminary experiments confirmed that these conditions were sufficient to digest all HA present. The enzyme was then heat inactivated, and the sample was precipitated with CPC as described.

The amount of \(^{14}C\)HS was assayed by digestion of a known sample volume with nitrous acid for 2 hr at room temperature (2). Again, the treated samples were subsequently precipitated with CPC. By comparison with proper controls, the percentages of \(^{14}C\)HA and \(^{14}C\)HS could be calculated (Chart 1). Total radioactivity recovered after these treatments was within 5% of that in the sample before digestion. HA and HS usually accounted for approximately 90% of the total GAGs synthesized. The remaining \(^{14}C\)GAGs were not studied in detail; however, digestion with testicular hyaluronidase and cellulose acetate electrophoresis (2) showed that about one-half of this residual material was chondroitin sulfate (A and C).

**Precursor Uptake.** The uptake of radioactive precursor was monitored according to published procedures (3). In brief, confluent cultures of +SA or -SA cells were incubated with 0.8 μCi of \(^{14}C\)glucosamine per ml for 30 min. The radioactive medium was then removed, and the cell layer was quickly rinsed 5 times with 2 ml of medium. The final wash was counted to establish a maximal background level of "loosely bound" radioactivity. Two ml of chilled 10% TCA were then added, and the cells were extracted in the cold for 15 hr. TCA was removed by ether extraction, and the radioactivity in the resulting sample was quantitated by liquid scintillation counting.

**Colorimetric Assay for Total Secreted GAGs.** Cultures of +SA or -SA were grown to confluency in complete medium. The cells were then rinsed twice with 2.5 ml of DME (serum-free) to lower the serum concentration. This was necessary because serum components interfered with the colorimetric assay for GAGs. The washed cells were then incubated for 9 hr in serum-free DME which had been prequillibrated to the proper temperature and pH. Determinations of cell number and cell viability (by trypan blue exclusion) confirmed that the cells remained viable during this incubation. The medium was then collected and processed for GAG analysis. The media from 4 plates were pooled in 2 batches and the GAGs were precipitated at 4°C by the addition of 3 volumes of 95% chilled ethanol containing 1% potassium acetate. The GAGs in the precipitate were quantitated colorimetrically by the carbazole method (S), using glucuronolactone as a standard.

**RESULTS**

When +SA or -SA cultures were incubated with \(^{14}C\)glucosamine, label was incorporated into GAGs as assayed by CPC precipitation. \(^{14}C\)GAGs were recovered from 3 operationally defined compartments of the tissue culture system: the culture medium; the cell surface; and the residual cellular material (see "Materials and Methods"). The appearance of \(^{14}C\)GAGs in each of these compartments is shown in Chart 2 as a function of labeling time. The amount of cell-associated \(^{14}C\)GAGs per cell reached a maximum value after 12 to 20 hr of labeling (Chart 2, B and C). The rate at which \(^{14}C\)GAGs appeared in the culture medium increased dramatically after a lag period of about 6 hr.

After 6 hr of labeling, +SA cells incorporated about 8 times more radioactivity into total GAGs than did -SA cells (Table 1). The simplest interpretation of these results is that +SA cells synthesize more GAGs than do -SA cells. This result, however, could also be due to different rates of uptake of labeled precursor by +SA and -SA cells. To test this possibility, cultures were incubated with \(^{14}C\)glucosamine and the TCA-soluble radioactivity was measured in thoroughly washed cell layers (see "Materials and Methods"). The amount of radioactive material thereby identified as intracellular radioactive precursor was at least 20 times that remaining loosely bound after washing. More importantly, the intracellular radioactive precursor pools were shown to be essentially the same in both cell types. +SA cells contained 45.3 ± 2.2 (S.E.) x 10⁴ cpm/cell of TCA-soluble radioactivity, and -SA cells took up 40.2 ± 2.4 x 10⁴ cpm/cell. These results, together with the incorporation data, suggest that the actual rate of GAG synthesis is substantially different in +SA and -SA cells.

A second approach to the question of differential GAG synthesis by +SA and -SA cells focused on the actual amount of GAG which was secreted to the culture medium. Using a colorimetric assay (5), we determined that, during a 9-hr incubation, approximately 3.4 times more GAGs appeared in the medium of +SA cultures (1.59 ± 0.10 pg/cell) than appeared in the medium of -SA cultures (0.47 ± 0.06 pg/cell). Considering the limitations of the assay (e.g., the use of serum-free medium), these results are consistent with the interpretation

7 Unpublished observation.
GAG Synthesis by Mammary Tumor Cells

Chart 2. Kinetics of appearance of radiolabeled GAGs in various culture compartments. Confluent cultures of +SA or −SA cells were incubated with [14C]-glucosamine and assayed for [14C]GAGs, as described in “Materials and Methods.” Ordinate, normalized incorporation rate (10^6 × cpm/μCi/ml/cell). The data are the average of triplicate cultures, and the standard errors are less than 10%. , +SA cultures; , −SA cultures.

Table 1
Incorporation of [14C]glucosamine into GAGs of confluent +SA and −SA cells

<table>
<thead>
<tr>
<th>Culture</th>
<th>Total incorporationa</th>
<th>Distribution ( % of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>+SA cells</td>
<td>267.5 ± 14.6b</td>
<td>12.0</td>
</tr>
<tr>
<td>−SA cells</td>
<td>34.7 ± 2.4</td>
<td>14.8</td>
</tr>
</tbody>
</table>

*a 10^6 × cpm/μCi/ml/cell after 6 hr of labeling.

*b Mean ± S.D.

Table 2
Distribution of specific newly synthesized [14C]GAGs

<table>
<thead>
<tr>
<th>Culture component</th>
<th>HA</th>
<th>HS</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+SA</td>
<td>−SA</td>
<td>+SA</td>
</tr>
<tr>
<td>Medium</td>
<td>9.6</td>
<td>8.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Cell surface</td>
<td>37.8</td>
<td>24.2</td>
<td>13.5</td>
</tr>
<tr>
<td>Residual cells</td>
<td>10.3</td>
<td>7.4</td>
<td>14.7</td>
</tr>
<tr>
<td>Total</td>
<td>57.7</td>
<td>40.1</td>
<td>29.9</td>
</tr>
</tbody>
</table>

*a Six hr of labeling with [14C]glucosamine.

that the radioactive incorporation data reflect the actual amount of GAG turnover by +SA and −SA cells. Together with the radioactive precursor uptake data discussed previously, these results support the conclusion that confluent +SA cells synthesize and secrete more GAGs than do confluent −SA cells.

The chemical nature of the radioactive GAG synthesized by +SA and −SA cells was investigated by specific digestion techniques. The results from cultures labeled for 6 hr were selected for comparison because all culture compartments were adequately labeled, but the proportion of [14C]GAG that was secreted to the culture medium was still relatively small. Under these conditions, the best comparison of precursor utilization and GAG distribution by the 2 cell types can be made (summarized in Table 2). The main cell surface GAG of both +SA and −SA cells was HA (65.0 and 47.6%, respectively). In contrast, the predominant GAG remaining with the residual cellular material (i.e., after the trypsin-digestible surface is removed) was HS (49.1 and 67.9%, respectively). This is consistent with reports that much HS associated with the plasma membrane is not easily removed by proteolysis.

For both +SA and −SA cells, approximately 80% of the HA associated with the cell layer (cell surface plus residual cellular material) was removed by trypsin and hence is described in this report as “cell surface” (see Table 2). In contrast, less than 50% of the HS was removed from either cell layer. The data of Table 2 also show that the major GAG synthesized by −SA cells was HS (46.3% of the total CPC-precipitable label). In contrast, the predominant GAG synthesized by +SA cells was HA (57.7%). The compositions of each culture component were similar to these (within 10%) after 12 hr of labeling.

The major GAG in the culture medium of both +SA and −SA cells was HA (80.1 and 57.4%, respectively). This is expected because the other major GAG, HS, was probably internalized and degraded to a great extent (18). In support of this contention, the results in Table 2 show that, while approximately 20% of the newly synthesized HA was found in the culture medium (after 6 hr of labeling), only about 7% of the newly synthesized HS was secreted. Thus, a proportionally smaller amount of newly synthesized HS was released to the culture medium, at least in a form that could be assayed by our procedure.

DISCUSSION

In monolayer culture, +SA cells incorporate much more radioactive precursor into GAGs than do −SA cells. Studies of radioactive pool size and a spectrophotometric assay of total GAGs released to the culture medium support the interpretation that +SA cells synthesize and secrete GAGs, especially HA, at a faster rate than do −SA cells.

These observations relate to 2 aspects of carcinogenesis about which little is known, early tumor development and tumor heterogeneity. Early tumor development requires specific physiological conditions which GAGs can regulate. For example, nutrient transport must be efficient (13, 35), especially prior to...
angiogenesis (11, 12), and cell-cell interactions must permit morphogenesis without restricting cellular proliferation (10, 21, 36). Historically, fibroblastic cells have been considered the primary source of these important interstitial molecules. The possibility that epithelial cells may “condition” their own pericellular environment in this way offers a new perspective from which to consider the tumorigenetic process. Consistent with such conditioning are the observations that tumors which develop in vivo from -SA cells grow more slowly and after a longer latency period than do tumors from +SA cells (8). Tumors of +SA origin appear to contain more extensive areas of intercellular stroma than do tumors of -SA origin (8). Staining patterns with Alcian blue suggests that this stroma contains considerable amounts of hyaluronic acid.7 However, it remains possible that the differences in GAG synthesis by +SA and -SA cells are the result of, rather than the cause of, the differences in their growth properties (22).

Another aspect of carcinogenesis to which these results may relate is the maintenance of intratumor cell heterogeneity. As recently reported in the work of Heppner et al. (16) and Miller et al. (20), 2 mechanisms have been proposed by which mammary tumor heterogeneity might be maintained by cell interaction, the primary effect of which would be to inhibit the growth of the faster-proliferating cells. In a somewhat different system, Todaro and DeLarco (31) have reported that sarcoma virus-transformed cells secrete growth factors which may induce tumorigenic properties in nontransformed cells. By this mechanism, heterogeneity within the tumor population might be abetted by growth stimulation of nontumorigenic, or presumably even slower-growing tumorigenic, cells. All of these investigations describe cell-cell interactions which could increase the phenotypic cellular heterogeneity within a tumor. The results that we have described in this paper may be another such example. The persistence within a tumor of cells with such diverse growth properties as those characteristic of +SA and −SA cells might be explained by the presence of a general “growth stimulation” matrix produced primarily by one cell subpopulation. Mammary adenocarcinomas have previously been reported to contain large amounts of HA (27, 28, 30), a matrix which is conducive to efficient growth (7, 19, 22, 25, 26, 28, 29, 32, 33). We have confirmed this observation in our own histological investigation, using Alcian blue staining of carcinomas obtained by s.c. injection of +SA cells into syngeneic BALB/c mice.7 We propose that the +SA-type cells can establish an HA-rich matrix within which other cells, like −SA cells, also proliferate efficiently.

REFERENCES

6. Chandrasekaran(258,657),(503,959)
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