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Stimulation of Glycosaminoglycan Production in Murine Tumors

Warren Knudson, Chitra Biswas, and Bryan P. Toole

Department of Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111

Three types of murine tumors, B-16 melanoma, A-10 carcinoma, and S-180 sarcoma, were shown to contain elevated glycosaminoglycan (GAG) concentrations in vivo as compared to normal muscle or subcutaneous tissue. Hyaluronate was especially concentrated in the A-10 carcinoma, which contained approximately six times more hyaluronate than subcutaneous tissue and 18 times more than muscle. In all three tumors, chondroitin sulfates, especially chondroitin-4-sulfate, were present in higher concentrations than in the normal tissues. In culture, however, all three tumor cell lines produced less than 5% as much GAG as mouse fibroblasts, when measured by incorporation of [^{3}H] acetate or by chemical analysis. Varying the culture passage number or the medium composition, ie, glucose, serum, and insulin concentrations, had little effect on GAG synthesis by the tumor cells. The low GAG levels in the tumor cell cultures were not due to hyaluronidase activity in their media. In an attempt to mimic possible host-tumor cell interactions that could account for the elevated GAG levels in vivo, tumor cells were cocultured with fibroblasts, but no stimulation above the amount made by the tumor cells alone plus that by the fibroblasts alone was observed. Conditioned media from the tumor cells, either dialyzed or not against fresh complete medium, had no effect on fibroblast GAG synthesis. Tumor extracts, however, were found to stimulate synthesis of hyaluronate by fibroblasts. Stimulation by extracts of A-10 carcinoma was greater than and additive to that of serum. The above results strongly suggest that GAG production in these tumors is in part regulated by host-tumor interactions.

Key words: glycosaminoglycans, murine tumors, host-tumor cell interactions

Extracellular matrix components have received increasing attention in recent years for their contribution to the regulation of various cellular functions [1–3]. One of these extracellular macromolecules, hyaluronate, has been found in high concentrations in tissues where extensive cell proliferation and migration are occurring, for example, in early stages of developing [4–8], regenerating [9], and wound healing [10,11] systems. Chondroitin sulfate is also present in these tissues, usually at a lower concentration than hyaluronate [4,5,8–10], but in some cases at a higher concentration [6,11]. Hyaluronate and chondroitin sulfate are also found in high concentrations in

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many tumors—for example, in human lung cancer [12], Wilms tumor [13], hepatic cancer [14], colon carcinoma [15], and breast tumors [16].

In the rabbit V2-carcinoma, we have demonstrated an increase in hyaluronate in association with invasiveness of this tumor. Specifically, we found that the V2-carcinoma contains large amounts of hyaluronate when implanted in the rabbit, where it behaves as an invasive and metastatic tumor, but contains lower levels when implanted in the nude mouse, where it grows as a noninvasive tumor [17]. In addition, the capsular tissue surrounding the tumor in the rabbit, a tissue that is most likely produced in large part by host rabbit cells in response to the presence of a growing tumor [18], was found to contain three times as much hyaluronate as the tumor itself [17]. This led us to speculate that some form of host-tumor cell interaction may occur that results in stimulation of hyaluronate production. Previous studies by one of us [19,20] have also provided considerable evidence that the high levels of collagenase present in many tumors may be due to stimulation of host fibroblast production by tumor cells rather than synthesis of the enzyme by the tumor cells themselves.

For the above reasons, we have begun to investigate the role of host-tumor tissue interactions in the regulation of production of glycosaminoglycans (GAG). For these studies we have used murine tumor cell lines that can be maintained both as monolayer cell cultures and as transplantable tumors in syngenic hosts. Thus, we have been able to compare the amounts of GAG that accumulate in the tumors in vivo with the amounts produced by the isolated cells in culture. Although these tumors were found to be enriched in hyaluronate and chondroitin sulfate in vivo, the isolated tumor cells synthesized very low amounts of these GAG, suggesting that interaction of tumor and host tissue is necessary for production of the high levels of GAG in the tumors in vivo. Supporting this postulate, and pointing to the possibility that host fibroblasts are the source of the GAG, is our finding that tumor extracts stimulate production of GAG, especially hyaluronate, by fibroblasts in culture.

METHODS

Cell Cultures

Normal fibroblasts were isolated from murine lung tissue by a procedure described by Cahn et al [21]. Briefly, fresh lung tissue was diced and dissociated by repeated digestion in Hanks balanced salt solution (HBSS) containing 0.1% trypsin, 0.1% collagenase, and 1.0% chicken serum. Cells were plated in Roswell Park Memorial Institute medium (RPMI) containing 5% fetal calf serum and antibiotics. Cells isolated from one animal were plated per 60-mm dish (Falcon). After 24 hr the medium was decanted and fresh medium was added. Subsequently, the cells were maintained in Dulbecco modified eagle's medium (DMEM) containing 5% fetal calf serum and antibiotics. The mouse A-10 adenocarcinoma cell line was obtained from Dr. B. Aaranson, the B-16 melanoma and sarcoma S-180 from Dr. J. Folkman. The tumor cell lines were maintained in the same medium as the fibroblasts. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Mouse fibroblasts at fourth passage and tumor cells between fourth to eighth passage were used except where otherwise specified.

Preparation of Conditioned Media

Confluent cultures of A-10 or S-180 cells were incubated for 48 hr in serum-free DMEM. The medium was then collected and centrifuged. A portion of each supernatant was then dialyzed against DMEM in dialysis tubing of porosity excluding proteins of 3,500 molecular weight (mol wt). Dialyzed and nondialyzed samples were then mixed in a ratio of 2:3 with fresh medium, with or without the addition of fetal calf serum, to give a final concentration of 5%.

Analysis of Glycosaminoglycans From Cultured Cells

Incorporation experiments. Unless specified otherwise, cells were plated into 16mm diameter wells (Falcon) at a density of 10^5 cells per well, in 1.0 ml DMEM containing 5% fetal calf serum and antibiotics. After allowing cells to attach for 24 hr, medium was changed to fresh medium containing 20 μCi/ml [^{3}H] acetate (2.3 Ci/mmole, New England Nuclear). At appropriate incubation times, duplicate cultures were processed by a modification of previous methods [22]. Briefly, medium plus cell wash were digested with 0.5 mg/ml protease (Type XIV, Sigma), in 0.1 M Tris, pH 8.0, for 24 hr at 37°C, and heated at 100°C for 10 min. Then 100 μl aliquots were incubated with or without 5 turbidity-reducing units (TRU)/ml streptomyces hyaluronidase (Calbiochem) in 0.05 M NaCl/0. 1 M Na acetate, pH 6.0, or 0.1 mg/ml testicular hyaluronidase (Type I-S, Sigma) in 0.05 M NaCl/0. 1 M Na acetate, pH 5.0, for 12 hr at 37°C. After heating at 100°C for 10 min, carrier hyaluronate and chondroitin sulfate (12.5 μg each) were added and the GAG precipitated with 50 μl 10% cetyl pyridinium chloride (CPC). Incorporation into hyaluronate plus chondroitin sulfate was measured as the difference in radioactivity of the precipitates from the testicular hyaluronidase-treated and control samples and hyaluronate as the difference between streptomyces hyaluronidase-treated and control samples.

Cell-associated material, released from the dish by incubation with 0.25% trypsin (Gibco) for 30 min at 37°C, was processed in a similar manner to that used for medium samples described above.

Chemical anaylsis. Cultures of mouse fibroblasts or tumor cells in log phase of growth were given fresh medium and allowed to incubate for 48 hr. Medium and cell wash were combined, protease digested as above, dialyzed, and analyzed by high-pressure liquid chromatography (HPLC) as described below. Cell-associated material was released from the dish with trypsin and processed as for the medium fraction.

Preparation and Analysis of Tumor and Normal Tissue

Suspensions of tumor cells (5×10^6 cells in 0.5 ml of HBSS) were injected subcutaneously into 4–6-week-old male syngenic mice (Jackson Laboratories: AHe/J for A-10; C57/Bl6 for B-16; CFW for S-180). After approximately 3 weeks the animals were killed and tumors removed. Adhering fat and surrounding connective tissue were dissected away, then the central tumor tissue was cut into small pieces and lyophilized to determine the dry weight. The dried material was then digested with 2.5 mg protease (Type XIV, Sigma) in 2.0 ml 0.1 M Tris, pH 8.0, for 48 hr at 37°C with additional protease added after 24 hr. The digestion was stopped by boiling

for 10 min and clarified by centrifugation. The resulting supernatants, after extensive dialysis against water, were then either (a) analyzed directly for hyaluronate by streptococcal hyaluronidase digestion [17] and measurement of terminal N-acetylhexosamine [23], or (b) precipitated with ethanol and analyzed by HPLC following digestion with chondroitinase (see below). Muscle and subcutaneous tissue, removed from similar sites in nontreated animals, were digested and processed as for the tumor tissue.

Analysis of Glycosaminoglycans by High-Pressure Liquid Chromatography

Two milliliter aliquots of protease-digested samples were precipitated with 3 volumes of 1.0% potassium acetate in ethanol overnight at −20°C. Precipitates, washed twice in phosphate-buffered saline and redissolved in 0.5 ml of H_2O, were then divided into two equal portions, one containing 0.25 units each of chondroitinase ABC and chondroitinase AC (Sigma) in enriched Tris buffer, pH 7.4 [24], the other in buffer only. After 2 hr at 37°C, both portions were heated at 100°C for 10 min. Unsaturated disaccharide products were then separated and quantitated by a modified HPLC method of Delaney et al [25]. A Beckman Model 110A liquid chromatograph equipped with a model 210 injector (20-μl loop) and variable wavelength detector was used. Samples were separated on a 4.6-mm i.d. × 25-cm Ultrasil-Ax strong anion exchange column (Beckman) under isocratic conditions and a constant flow rate of 1.0 ml/min. Effluent fractions were monitored at 232 nm. Disaccharides were identified by their elution times relative to standards and by subtraction of extraneous peaks present in undigested samples. Hyaluronic acid disaccharide, ΔDi-HA, eluted at 11 min in 10 mM KH_2PO_4 + 5% methanol. Chondroitin-6-sulfate, ΔDi-6S, and chondroitin-4-sulfate, ΔDi-4S, disaccharides eluted at 12 and 16 min, respectively, in 75 mM KH_2PO_4 + 5% methanol. Comparison of sample peak areas with reference standards was used for quantitation. This method has a detection limit of 500 ng of disaccharide.

Preparation of Tumor Extracts

A-10 tumors were excised, rinsed twice with HBSS, then homogenized in 2 ml HBSS/g wet weight in a Polytron (2 pulses of 15 sec at setting 6–7). The homogenates were centrifuged at 30,000g for 30 min at 4°C and the supernatants stored at −20°C. Prior to use, the preparations were centrifuged again at 38,000g for 20 min at 4°C, and the supernatants diluted as required with DMEM. Protein content of these extracts was measured by the method of Lowry et al [26].

RESULTS

Glycosaminoglycan Content of Tumors

The hyaluronate and chondroitin sulfate contents of tumor and normal tissue were determined by HPLC of unsaturated disaccharide products following chondroitinase ABC/AC digestion. These measurements showed that the sum of hyaluronate plus chondroitin sulfate contents was in all cases higher for the tumors than for the normal subcutaneous tissue (Table I). Hyaluronate was found to be present in all three types of tumors as well as in normal tissue. However, the A-10 tumors contained a 5–7 times higher concentration of hyaluronate than normal subcutaneous tissue and approximately 18 times more than muscle. The B-16 tumors contained similar levels

TABLE I. GAG Content of Tumors and Normal Tissues

	GAG (μg per g dry wt of tissue)[a]				
	Hyaluronate				
	Colorimetric	HPLC	C6S (HPLC)	C4S (HPLC)	Total[b] (HPLC)
A-10 carcinoma	3562 ± 934	3508 ± 1259	323 ± 111	867 ± 281	4698
B-16 melanoma	—[c]	538 ± 436	100[d]	724[d]	1362
S-180 sarcoma	329 ± 80	237 ± 2	98 ± 59	551 ± 155	886
Subcutaneous tissue	494 ± 40	612 ± 149	—[e]	—[e]	612
Muscle tissue	194 ± 19	ND[f]	ND[f]	ND[f]	—

[a]Values are means ± SEM. Hyaluronate was measured by two methods. The first involved degradation by streptococcal hyaluronidase followed by colorimetric assay of terminal N-acetylhexosamine; the second employed chondroitinase ABC digestion followed by HPLC of the resulting dissaccharides. C6S, chondroitin-6-sulfate; C4S, chondroitin-4-sulfate.

[b]Total GAG = hyaluronate plus C6S plus C4S as determined by HPLC.

[c]Reliable data could not be obtained by the colorimetric method owing to interference by melanin pigment.

[d]Data are given for one experiment only.

[e]Below the level of detection.

[f]ND, not determined.

of hyaluronate as subcutaneous tissue whereas the S-180 tumors had approximately 50% of this concentration (Table I). The levels of chondroitin-4 and -6-sulfates were too low to be detectable in normal tissue but were elevated in all three tumors. In each tumor, chondroitin-4-sulfate was present in considerably greater concentration than chondroitin-6-sulfate, and was the major GAG, of those measured, in the sarcoma S-180 and the B-16 melanoma. The hyaluronate levels in these tissues were also analyzed by digestion with streptococcal hyaluronidase and measurement of the increase in terminal acetylated hexosamine. As can be seen in Table I, similar levels of hyaluronate were obtained by using either method for estimation.

Glycosaminoglycan Production by Tumor Cells

We then compared the ability of normal mouse fibroblasts and tumor cells to produce GAG in cell culture. We chose to compare the tumor cells with fibroblasts, since the latter are known to produce GAG actively and since the tumors in vivo were implanted into subcutaneous tissue wherein the majority of cells are fibroblasts. Incorporation of [^{3}H]acetate into testicular hyaluronidase-sensitive GAG produced by A-10 tumor cells was found to be very low compared with normal fibroblasts (Fig. 1). This low level of incorporation was observed in both the medium and cell-associated compartments for all three tumor cell types (data not shown). For the fibroblasts and the three types of tumor cells, greater than 90% of the testicular hyaluronidase-sensitive GAG was hyaluronate. Only 10% of the total GAG produced by fibroblasts was testicular hyaluronidase-resistant, whereas 30% of that from A-10 cells, 53% from S-180, and 63% from B-16 was resistant.

Chemical measurements by HPLC of GAG synthesized in culture confirmed the above results. As shown in Table II, A-10 cells and B-16 cells synthesized less than

TABLE II. HPLC Analysis of GAG Synthesized by Tumor Cells and Fibroblasts*

Cell type	GAG ($\mu g/10^6$ cells)		
	HA	C6S	C4S
MF—medium	47.0	15.0	3.0
MF—cell layer	1.9	ND	ND
A-10—medium	1.7	2.0	2.0
A-10—cell layer	0.1	ND	ND
S-180—medium	1.1	3.6	1.4
S-180—cell layer	—[a]	ND	ND

*ND, not determined; MF, mouse fibroblasts; HA, hyaluronate; C6S, chondroitin-6-sulfate; C4S, chondroitin-4-sulfate.
[a]Below the level of detection.

5% as much hyaluronate as normal mouse fibroblasts over a period of 48 hr. Chondroitin-6-sulfate levels were also significantly lower in the tumor cells.

To ensure that the particular culture conditions used did not cause the low level of GAG synthesis by the tumor cells relative to normal fibroblasts, incorporation of [^{3}H]acetate was examined in cultures of A-10 cells and fibroblasts at different concentrations of serum and glucose (Table III). Varying the serum concentration had opposite effects on the fibroblasts and A-10 cells. Increasing the serum concentration stimulated GAG production by the former but caused a decrease in the latter. Increasing the glucose concentration increased incorporation in both cell types. However, the capacity of A-10 cells to synthesize GAG was consistently lower than fibroblasts under each of the conditions tested. In other experiments (data not shown), the influence of (a) increasing the concentration of insulin in the medium or (b) plating the A-10 cells on matrix ("substrate-attached material" [27]) produced by fibroblasts was tested. In neither case was there any significant effect on GAG production by the A-10 cells.

The low level of GAG production by the tumor cells in the above experiments could have resulted from changes owing to subculturing. Thus, 15th-passage A-10 cells were reinoculated into mice and cells were isolated from the resulting tumors. First-passage cultures of these cells were then compared with the 15th-passage cells and both were found to produce low amounts of GAG relative to fibroblasts (Table IV).

Thus, although the tumors in vivo accumulate amounts of GAG that are similar to or greater than the levels found in normal GAG-rich tissues, cultured cells derived from these tumors produce much less GAG than fibroblasts derived from normal tissue.

Glycosaminoglycan Production in Cocultures of Tumor and Normal Cells

Since isolated tumor cells were not found to synthesize GAG actively in comparison to fibroblasts, interaction in vivo between tumor cells and host fibroblasts may be necessary for the elevated accumulation of hyaluronate and chondroitin sulfate in the neoplasms, especially the A-10 adenocarcinoma. Consequently, we measured GAG production in cocultures of normal and tumor cells. As can be seen from Figure 2, less GAG was produced in the cocultures than in cultures of fibroblasts alone. This

TABLE III. Effect of Serum and Glucose Concentration on [^{3}H]Acetate Incorporation into GAG

Cell type	Serum %	Glucose (g/liter)	GAG[a] (cpm/24 hr)
MF	0	1.0	1,634
MF	1	1.0	2,560
MF	2	1.0	3,681
MF	3	1.0	3,664
MF	5	1.0	5,452
MF	5	4.5	8,428
A-10	0	1.0	1,137
A-10	5	1.0	660
A-10	10	1.0	520
A-10	5	4.5	1,248

[a]Expressed as mean cpm incorporated into testicular hyaluronidase-sensitive GAG in duplicate culture wells over a 24-hr period. No correction is made for different growth rates of the cells under the different conditions.

TABLE IV. Comparison of GAG Production by Early- and Late-Passage A-10 Cells

	GAG (cpm)[a]		
	A-10: 1st passage	A-10: 15th passage	Fibroblasts
With 5% FCS[b]	89 ± 18	143 ± 24	2366 ± 196
Without FCS	36 ± 7	84 ± 27	653 ± 86

[a]Incorporation into testicular hyaluronidase-sensitive, CPC-precipitable GAG during a 24-hr period. In each case >90% of these cpm was hyaluronate. For the fibroblasts, 83% of the total CPC-precipitable cpm was testicular hyaluronidase-sensitive; for the A-10 cells, 34–66% was sensitive depending on the experimental conditions.
[b]Fetal calf serum.

was the case with all three types of tumor cells. Since GAG synthesis may have been inhibited owing to the higher density of cells present in the cocultures than in the individual cultures, the cocultures of fibroblasts with A-10 or B-16 cells were repeated at much lower plating densities. GAG production was then measured while these cultures were still sparse (Fig. 3; 24 hr after plating) and after reaching confluence (Fig. 3; 72 hr after plating). Inhibition was only observed at confluence. Nevertheless, there was no stimulation of GAG production in the sparse cultures. The incorporation obtained was approximately equivalent to that for the fibroblasts alone (Fig. 3; 24 hr).

To ensure further that the lack of stimulation in cocultures was not due to cell contact, the influence of conditioned medium from tumor cell cultures on fibroblast GAG production was tested. These experiments were performed under four different conditions, namely, using dialyzed and nondialyzed, serum-containing and serum-free conditioned media. In none of these cases was any significant effect on GAG production observed (Table V).

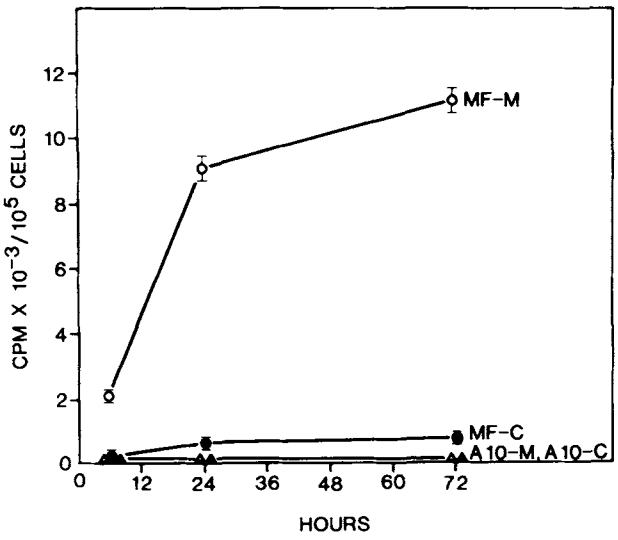

Fig. 1. Synthesis of GAG by tumor and normal cells in culture. Values represent the amount of [^{3}H] acetate incorporated into testicular hyaluronidase-sensitive GAG that were isolated from medium (M) and cell-associated (C) compartments. Mouse fibroblasts (MF), (○, ●); A-10 cells, (△, ▲). Each time point represents individual wells in which 10^5 cells were labeled with 20 μCi/ml [^{3}H] acetate in 1.0 ml DMEM containing 5% fetal calf serum and antibiotics. For the normal cells, approximately 10% of the [^{3}H]acetate incorporated into CPC-precipitable material was resistant to testicular hyaluronidase; for the A-10 cells, approximately 30% was resistant. Error bars represent SEM of values derived from two separate experiments.

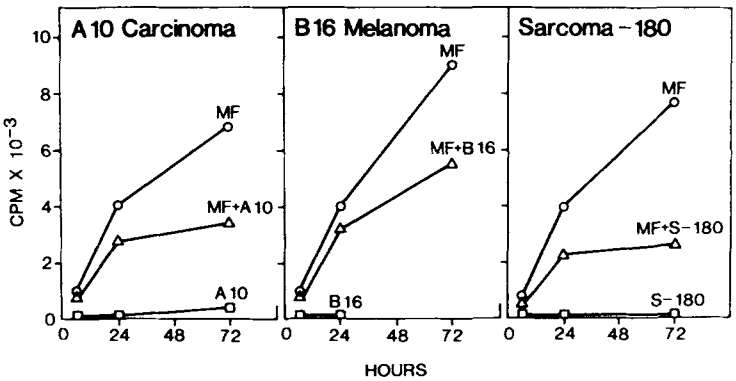

Fig. 2. GAG production in confluent cocultures of mouse fibroblasts (MF) with tumor cells. Values represent the amount of [^{3}H]acetate incorporated into testicular hyaluronidase-sensitive GAG that were isolated from the medium. Each time point represents individual wells in which 10^5 cells were plated individually or in coculture in a 1:1 ratio (ie, 2 × 10^5 cells/well). Each of the wells was labeled with 20 μCi/ml [^{3}H]acetate in 1.0 ml DMEM containing 5% fetal calf serum and antibiotics. For the fibroblasts alone and the cocultures, approximately 10% of the [^{3}H]acetate incorporated into CPC-precipitable material was resistant to testicular hyaluronidase; for the A-10, B-16, and S-180, approximately 30%, 63%, and 53% were resistant, respectively. Values represent mean of two experiments each assayed in duplicate.

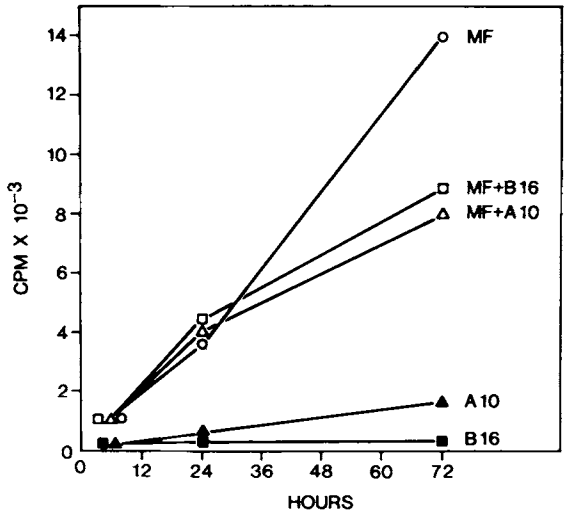

Fig. 3. GAG production in sparse cocultures of mouse fibroblasts (MF) with tumor cells. Values represent the amount of [^{3}H]acetate incorporated into testicular hyaluronidase-sensitive GAG that were isolated from the medium. Each time point represents individual 100-mm dishes in which 10^5 cells were plated individually or in coculture in a 1:1 ratio (ie, 2×10^5 cells/dish). Each of the dishes was labeled with 20 μCi/ml [^{3}H]acetate in 10.0 ml DMEM containing 5% fetal calf serum and antibiotics. For the fibroblasts alone and the cocultures, approximately 10% of the [^{3}H]acetate incorporated into CPC-precipitable material was resistant to testicular hyaluronidase; for the A-10 and B-16 cultures, approximately 42% and 64% were resistant, respectively. Values represent means of duplicates from a single experiment.

One possible reason for the low level of production of GAG by the tumor cells, or for the apparent lack of stimulation in cocultures, would be the presence of a neutral hyaluronidase in tumor cell media. Thus, labeled GAG produced by the fibroblasts was added to cultures of A-10 and S-180 cells. In neither case was significant degradation of the GAG observed (data not shown).

Recently, one of us [19] has shown that B-16 and A-10 cells do not secrete detectable levels of collagenase (against type I collagen) into their culture media, but do have the capacity to stimulate collagenase production by normal fibroblasts. Mouse fibroblasts, however, were not responsive in this system, whereas rabbit and rat fibroblasts were stimulated [19]. For this reason, GAG production was measured in cocultures of rabbit synovial fibroblasts and B-16 cells, one of the combinations which gives rise to a large stimulation in collagenase production. In similar fashion to the experiments above using mouse fibroblasts, GAG production was decreased rather than stimulated in the cocultures, as compared to fibroblasts alone (Fig. 4).

Influence of Tumor Extracts on Glycosaminoglycan Production by Fibroblasts

Since no stimulation of GAG synthesis was obtained in cocultures of tumor cells and fibroblasts, we examined the effect of tumor extracts on GAG production by cultured cells and found that they markedly stimulated GAG production by mouse

TABLE V. Effect of Conditioned Medium From Tumor Cells on GAG Production by Fibroblasts

	GAG (cpm)[a]	
	With FCS	Without FCS
Control medium	3,400 ± 266	1,019 ± 137
Dialyzed conditioned medium		
A-10	3,666 ± 501	1,162 ± 54
S-180	3,847 ± 292	1,216 ± 112
Nondialyzed conditioned medium		
A-10	2,992 ± 221	1,210 ± 105
S-180	3,051 ± 329	1,054 ± 39

[a]Incorporation into testicular hyaluronidase-sensitive GAG during a 24-hr incubation. An average of 88% of the total CPC-precipitable cpm was testicular hyaluronidase-sensitive.

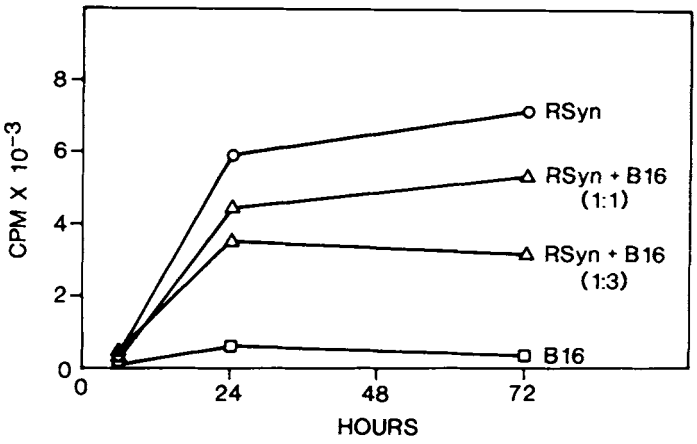

Fig. 4. GAG production in cocultures of rabbit synovial fibroblasts (RSyn) with B-16 melanoma cells. Values represent the amount of [^{3}H]acetate incorporated into testicular hyaluronidase-sensitive GAG that were isolated from the medium. Each time point represents individual wells in which 10^5 cells were plated individually or in coculture in a 1:1 or 3:1 ratio of B16: RSyn (ie, 2×10^5 and 4×10^5 cells/well, respectively). Each well was labeled with 20 μCi/ml [^{3}H]acetate in 1.0 ml DMEM containing 5% fetal calf serum and antibiotics. For the synovial fibroblasts and the cocultures, approximately 26% of the [^{3}H]acetate incorporated into CPC-precipitable material was resistant to testicular hyaluronidase; for the B-16 cells, approximately 76% was resistant. Values represent means of two experiments each assayed in duplicate.

fibroblasts. In Figure 5A, the potency of the A-10 tumor extracts in stimulating fibroblast GAG production is compared with that of fetal calf serum and AHe/J mouse serum over a wide concentration range. The tumor extract was most active in a concentration range of 0.2–1 mg/ml of protein (equivalent to a 1–5% dilution of the extract). Fetal calf serum gave a similar level of stimulation at 1–3 mg/ml (equivalent

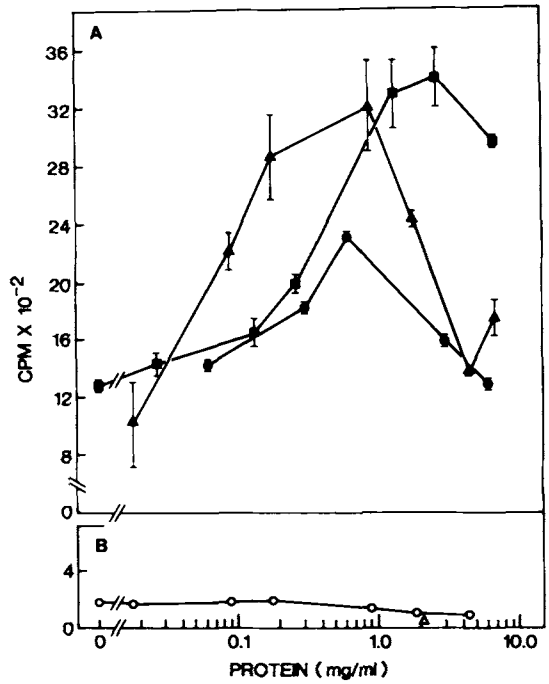

Fig. 5 Effect of A-10 tumor extract on GAG production by (A) mouse fibroblasts and (B) A-10 tumor cells. Values represent the amount of [^{3}H]acetate incorporated into testicular hyaluronidase-sensitive GAG which were isolated from the medium. Each time point represents individual wells in which 10^5 mouse fibroblasts or A-10 cells were plated. After 24 hr in culture, cells were incubated for another 24 hr with 20 μCi/ml [^{3}H]acetate in 1.0 ml DMEM containing various concentrations of A-10 tumor extract or serum. A) Mouse fibroblasts: ▲, A-10 tumor extract; ●, AHe/J mouse serum; ■, fetal calf serum. B) A-10 cells: ○, A-10 tumor extract; △, fetal calf serum. For the normal cells, approximately 10% of the [^{3}H]acetate incorporated into CPC-precipitable material was resistant to testicular hyaluronidase; for the A-10 cells, approximately 30% was resistant. Error bars represent SEM of four values derived from two experiments. Error for the values in B were less than 10%.

to 5–10% serum). The mouse serum was not as stimulatory as fetal calf serum or the A-10 tumor extract (Fig. 5A). In each case greater than 90% of the GAG produced in the stimulated cultures was hyaluronate.

Stimulation of fibroblast GAG production by the tumor extract and by serum was found to be additive. Figure 6 demonstrates the influence of increasing amounts of serum in the presence or absence of 5% tumor extract. At each serum concentration, the addition of tumor extract gave rise to a similar level of increase in incorporation into GAG to that obtained in the absence of serum.

Neither the A-10 extract nor fetal calf serum stimulated GAG production by A-10 cells in culture (Fig. 5B).

DISCUSSION

The major findings of this study are the following: (a) although the murine tumors studied accumulate amounts of GAG that are similar to or greater than the

levels found in normal GAG-rich tissues, the cells derived from these tumors produce much less GAG than fibroblasts derived from normal tissue; and (b) extracts of tumors stimulate hyaluronate production by normal fibroblasts. These two results strongly suggest involvement of host-tumor cell interactions in the regulation of hyaluronate production in the tumors.

Previous investigations have shown that many types of tumors are enriched in hyaluronate and/or chondroitin sulfate [12–17], but measurements of GAG production by tumor or transformed cells in culture have given contradictory results. For example, murine B-16 [28] and human [29] melanoma cells have been shown to produce small amounts of hyaluronate and chondroitin sulfate. We have shown (Table I), however, that tumors resulting from injection of B-16 cells into mice contain as much hyaluronate and considerably more chondroitin sulfate than subcutaneous tissue, one of the most GAG-rich normal tissues in the body. In addition, the A-10 carcinoma cells, which also produced low amounts of GAG in culture (Table II), had approximately six times more GAG than subcutaneous tissue in vivo (Table I). In the least dramatic case, the S-180 sarcoma, the tumor still contained much greater amounts of chondroitin sulfate than subcutaneous tissue in vivo and a somewhat lower level of hyaluronate (Table I). In culture, the S-180 cells produced only about 2% as much hyaluronate and about 25% as much chondroitin sulfate as the normal fibroblasts (Table II).

Many previous studies [30–33] have shown increased incorporation of isotopic precursors into GAG, especially hyaluronate, produced by transformed cells as compared to their parent counterparts. However, chemical measurements have in some cases given the opposite result owing to the fact that precursor uptake is far greater in the transformed cells [34]. Other isotope incorporation studies have suggested that murine mammary carcinoma cell variants that exhibit high efficiencies of metastasis (or colonization) in vivo produce more hyaluronate in culture than variants exhibiting lower efficiencies of metastasis [35,36]. However, no comparison was made with normal fibroblasts or other active GAG-producing cells. Thus, it is possible that all these sets of variants were producing low levels of GAG. In the present study we have shown that, with three types of murine tumor cells, the production of GAG is far less than for normal fibroblasts using both chemical and isotope incorporation methods; yet all these tumor types in vivo are rich in GAG. We conclude from the above that many types of tumor cells and transformed cells accumulate only small amounts of GAG, whereas the corresponding tumors are enriched in GAG, especially hyaluronate and chondroitin sulfates. This conclusion leads in turn to the postulate that host-tumor cell interactions are responsible for the elevated GAG levels in tumors.

Past studies indicate that host-tumor cell interactions are most likely also involved in the control of collagenase levels in certain tumors [19,20,37]. Cultured tumor cells have been shown to contain a factor that stimulates normal fibroblasts to produce very high levels of collagenase against type I collagen [19]. In the present study we could not obtain evidence in tumor cell cultures for a similar factor which stimulates GAG production, even in systems where collagenase stimulation was obtained (Fig. 4). However, tumor extracts do contain a factor that stimulates production of GAG, especially hyaluronate (Figs. 5,6). In the case of the A-10 carcinoma, which is especially enriched in hyaluronate in vivo, the extracts were found to be considerably more potent than fetal bovine serum or syngenic mouse

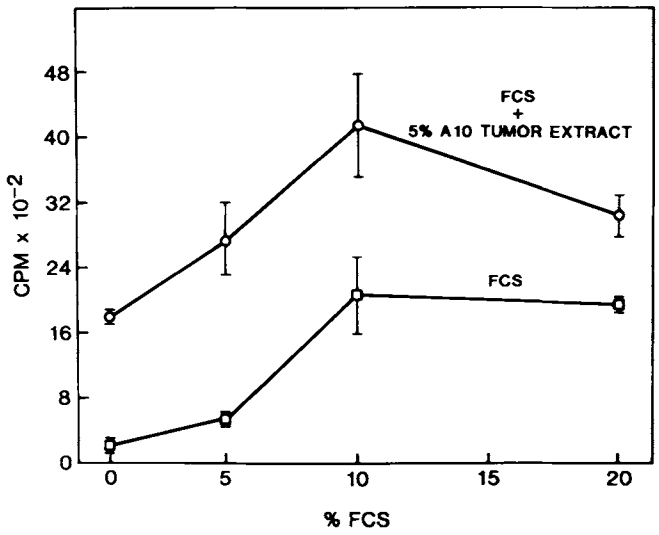

Fig. 6. Additive effects of A-10 tumor extract and fetal calf serum on GAG synthesis by mouse fibroblasts. Values represent the amount of [^{3}H]acetate incorporated into testicular hyaluronidase-sensitive GAG which were isolated from the medium. Each time point represents individual wells in which 10^5 mouse fibroblasts were plated. After 24 hr in culture, cells were incubated for another 24 hr with 20 μCi/ml [^{3}H]acetate in 1.0 ml DMEM containing various concentrations of fetal calf serum with or without a 5% dilution of A-10 tumor extract. Serum alone, □; serum plus A-10 extract, ○. Error bars represent SEM of four values.

serum and the effect of the extracts was additive to that of serum. Preliminary attempts to characterize the active factor in the A-10 tumor extracts have shown that it is nondialyzable, resistant to treatment with 250 μg/ml trypsin at 37°C for 30 min, and stable on heating at 70°C for 15 min. Further work is necessary to determine (a) the biochemical nature of the stimulatory factor, (b) whether potency of different tumor extracts matches the concentration of hyaluronate in the type of tumor from which the extract was made, and (c) whether the stimulatory factor in the extracts acts locally in the tumors or is a circulating factor.

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