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Interactions between human tumor cells and fibroblasts stimulate hyaluronate synthesis

(glycosaminoglycans/host–tumor cell interactions)

WARREN KNUDSON, CHITRA BISWAS, AND BRYAN P. TOOLE

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

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ABSTRACT **Several types of tumors contain high concentrations of hyaluronate, yet isolated tumor cells in culture often produce little glycosaminoglycan. To explore the possibility that interactions between tumor cells and host fibroblasts stimulate hyaluronate synthesis, human tumor cells were grown separately from and in coculture with normal human fibroblasts. Stimulation was observed with each of the three types of tumor cells used: LX-1 lung carcinoma, DAN pancreatic carcinoma, and TRIG melanoma. The interaction between LX-1 cells and fibroblasts was studied in detail. Under serum-free conditions, cocultures of LX-1 and fibroblasts synthesized 3-fold more hyaluronate than the sum of that produced by LX-1 and fibroblast cultures grown separately. This stimulation was linear over 72 hr and hyaluronate represented 80% of the glycosaminoglycan synthesized. Maximum stimulation occurred at a ratio of fibroblasts to LX-1 cells of 1–2:1. Quantitation of unlabeled glycosaminoglycans by HPLC analysis of disaccharides generated by digestion with chondroitin ABC and AC lyases (EC 4.2.2.4 and 4.2.2.5) demonstrated that net accumulation of hyaluronate increased 2-fold and that hyaluronate represented 80% of total chondroitinase-sensitive glycosaminoglycan produced by the cocultures. The disaccharide patterns obtained showed that accumulations of chondroitin-4- and chondroitin-6-sulfates were stimulated proportionately to that of hyaluronate in these cocultures. Similar levels of stimulation due to coculture were obtained in serum-containing and serum-free media. Stimulation was not effected by addition of LX-1-conditioned medium to fibroblast cultures or by culturing LX-1 and fibroblasts under conditions where they shared the same medium but were physically separated. Cell contact between LX-1 and fibroblasts thus appears to be necessary for the stimulation of hyaluronate synthesis.**

Hyaluronate is present in high concentrations in the extracellular matrix surrounding migrating and proliferating cells during development of embryonic tissues and organs and during regeneration or healing in adult tissues (1). Hyaluronate is also present in large amounts in several types of tumors—e.g., Wilm (2), mammary (3), hepatic (4), lung (5), and parotid gland (6) tumors in humans. In a previous study (7), we showed that a high concentration of hyaluronate accumulates in the V2-carcinoma grown in the rabbit, under conditions where it is highly invasive. In comparison, much lower amounts of hyaluronate were found when the same tumor was grown in the nude mouse, where it is noninvasive. These findings suggest that hyaluronate may be associated with the invasive behavior of malignant tumors. Thus we have begun to investigate the regulation of hyaluronate metabolism during tumorigenesis.

Hyaluronate is actively synthesized by some transformed and tumor cell lines in culture (8–10) but this property is not common to all tumorigenic cells. We have found that simian virus-transformed 3T3 cells and polyoma-transformed BHK cells produce far less hyaluronate than their nontransformed counterparts (11). Also, many epithelial tumor cell lines, such as murine B16 (12), human melanoma (13), murine A10 carcinoma (14), and the human tumor cell lines used in this study, produce little hyaluronate in culture. Interaction between tumor cells and host fibroblasts that stimulates hyaluronate synthesis by the fibroblasts is a possible mechanism that would explain the high concentrations of hyaluronate in tumors derived from these cells. Interaction between fibroblasts and epithelial cells (15, 16) or leukocytes (17–19) can lead to alterations in metabolism of connective tissue macromolecules. Recent evidence has shown that in certain cases tumor-derived factors stimulate fibroblast collagen (20) and proteoglycan synthesis (21). It has been shown that interactions between tumor cells and normal fibroblasts promote the production of collagenase (22, 23), an enzyme thought to be important in tumor invasion (24). Here we report that interactions between human tumor cells and normal human fibroblasts in coculture cause stimulation of hyaluronate production. However, whereas tumor cells shed or secrete a factor that stimulates fibroblast collagenase production (22), contact between the tumor cells and fibroblasts is required for stimulation of hyaluronate.

METHODS

Cell Cultures. Fibroblasts were grown from explants of human skin obtained through the courtesy of E. Schwartz. Human LX-1 lung carcinoma, DAN pancreatic carcinoma, and TRIG melanoma cells were isolated by sequential trypsin and collagenase treatment (25) of tumors grown in nude mice (Mason Research Institute, Worcester, MA). Cells were maintained in Dulbecco's modified Eagle's medium (DME medium)/10% fetal calf serum/1% penicillin/streptomycin solution at 37°C in humidified 5% CO_2/95% air. Experiments were done with human fibroblasts and tumor cells between 4th and 12th passage, using serum-free or serum-containing DME medium.

Analysis of Incorporation of Labeled Precursors into Glycosaminoglycans. Human fibroblasts and LX-1 cells were added to 16-mm^2 wells (Falcon) at a density of 10^5 cells of each type per well containing 1.0 ml DME medium/5% fetal calf serum/antibiotics. After allowing cells to attach for 24 hr, medium was replaced with 1 ml of fresh medium containing 20 μCi of [^{3}H]acetate (2.3 Ci/mmol, 1 Ci = 37 GBq; New England Nuclear) or 20 μCi of [^{35}S]sulfate (carrier-free, New England Nuclear). After appropriate incubation times, duplicate cultures were processed by a modification of previous methods (26). Medium plus cell wash were digested with protease (type XIV, Sigma) at 0.5 mg/ml in 0.1 M Tris, pH 8.0, for 24 hr at 37°C and then heated at 100°C for 10 min; 100-μl aliquots were then incubated in 0.1 M sodium acetate/0.05 M NaCl, pH 6.0 for 12 hr at 37°C with or without 2.5 units of *Streptomyces* hyaluronidase (EC 4.2.2.1, former-

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ly EC 4.2.99.1; Calbiochem). The samples were heated at 100°C for 10 min, carrier hyaluronate and chondroitin sulfate (12.5 μg of each) were added, and the glycosaminoglycans were precipitated with 10% cetylpyridinium chloride. Incorporation of [^{3}H]acetate into hyaluronate was measured as the difference in radioactivity of the precipitates from the hyaluronidase-treated and from control samples. Radioactivity in the control samples was used as a measure of incorporation into total glycosaminoglycan. Incorporation of [^{35}S]sulfate into chondroitin sulfate was measured in a similar fashion, with 38.5 turbidity units of testicular hyaluronidase (type I-S, Sigma) in 0.1 M sodium acetate/0.05 M NaCl, pH 5.0, replacing the *Streptomyces* hyaluronidase. 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.

Chemical Analysis of Glycosaminoglycans by HPLC. Fibroblasts and LX-1 cells were plated into 100-mm dishes (Falcon). When the cultures were confluent, the medium was replaced with fresh medium and the cultures were incubated for 48 hr. Medium and cell wash were combined, protease-digested as described in the previous section, and dialyzed. Aliquots (2 ml) of protease-digested samples were precipitated overnight with 3 volumes of 1.0% potassium acetate in ethanol at −20°C. Precipitates were washed twice in phosphate buffered saline, dissolved in 0.5 ml of H_2O, and divided into two equal portions; one was incubated with 0.25 unit each of chondroitin ABC and AC lyases (EC 4.2.2.4 and 4.2.2.5, Sigma) in Tris buffer, pH 7.4 (27), the other, with buffer only. After 12 hr at 37°C, both portions were heated at 100°C for 10 min. Unsaturated disaccharide products were then separated and quantitated by a modification of the HPLC method of Hjerpe *et al.* (28). 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-APS polar-bonded-phase column (Beckman) under isocratic conditions and at a constant flow rate of 1.0 ml/min. Effluent fractions were monitored for absorbance at 231 nm. Disaccharides were identified by their elution times relative to standards. Undigested samples were also chromatographed to ensure the absence of material in the region of the disaccharide peaks. Hyaluronate and chondroitin disaccharides eluted at 11 and 14 min, respectively, in 9 mM KH_2PO_4, pH 2.55. Chondroitin-6-sulfate and chondroitin-4-sulfate disaccharides eluted at 12.4 and 15.4 min, respectively, in 100 mM KH_2PO_4, pH 4.80. Comparison of sample peak areas to peak areas of reference standards was used for quantitation. This method has a detection limit of 500 ng of disaccharide.

RESULTS

Stimulation of Hyaluronate Synthesis in Cocultures of Human Tumor Cells and Fibroblasts. When human lung carcinoma cells (LX-1) and human fibroblasts were cultured together, there was a large increase in incorporation of [^{3}H]acetate into hyaluronate compared to the sum of the incorporations by each cell type cultured alone. Fig. 1 shows the time course of incorporation of [^{3}H]acetate into hyaluronate secreted into the medium of serum-free cultures of fibroblasts alone, of LX-1 alone, and of the two cell types in coculture at equal densities. These cocultures produced more than twice as much hyaluronate as the sum of the individual cultures at all times tested. However, the incorporation into hyaluronate in the individual cultures plateaued between 48 and 72 hr whereas in the cocultures it continued to increase linearly. Thus, the increase in the cocultures became more pronounced with increasing time of radiolabeling (Fig. 1). Hyaluronate was the major glycosaminoglycan secreted by both the cell types, representing >70% of the total glycosaminoglycan in the tumor cell cultures and >80% in the fibroblast cultures. The cocultures were similar to the latter indicating that, although other glycosaminoglycans were minor products relative to hyaluronate, they were stimulated proportionately (data not shown).

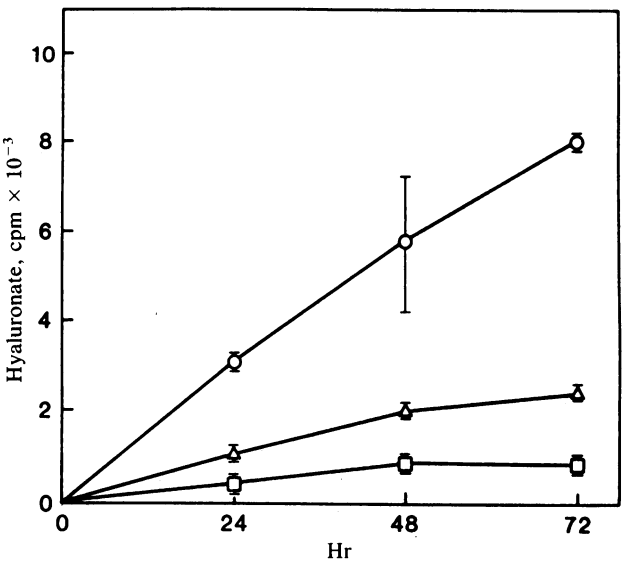

FIG. 1. Time course of [^{3}H]acetate incorporation into hyaluronate in the medium of LX-1 cultures (□), fibroblast cultures (△), and cocultures of LX-1 and fibroblasts (○). Each time point represents replicate experiments in which 10^5 cells were plated individually or in combined culture in a 1:1 ratio (i.e., final density: 2 × 10^5 cells per well) in 16-mm^2 wells of Falcon multiwell dishes. Each of the cultures was incubated with 20 μCi of [^{3}H]acetate in 1.0 ml of DME medium containing antibiotics. For the fibroblasts and combined cell cultures, ≈82% of the [^{3}H]acetate incorporated into total glycosaminoglycan was susceptible to *Streptomyces* hyaluronidase (and was, therefore, in hyaluronate); for the tumor cells, ≈72% was susceptible. Error bars represent the SEM for two separate experiments each assayed in duplicate.

The increase in hyaluronate in the cocultures was not due to a stimulation of cell proliferation since the number of cells in these cultures was equal to the sum of the cells in the individual cultures. In addition, protein synthesis, as measured by ^{3}H-labeled amino acid incorporation into trichloroacetic acid-precipitable material, did not increase in the cocultures relative to the sum of that of the individual cultures (data not shown).

Table 1 compares the incorporation into hyaluronate obtained in the cell-layer and medium compartments of serum-

Table 1. Effect of coculturing LX-1 cells and fibroblasts on hyaluronate synthesis in the presence and absence of serum

Cell type	[^{3}H]Acetate incorporated, cpm		
	Medium	Cell layer	Total
In serum-free medium			
Fibroblasts	921 ± 173	122 ± 17	1043
LX-1	442 ± 16	119 ± 23	561
Fibroblasts plus LX-1	3371 ± 36	461 ± 130	3832
In medium containing 5% fetal calf serum			
Fibroblasts	2370 ± 185	158 ± 29	2528
LX-1	389 ± 45	19 ± 19	408
Fibroblasts plus LX-1	4352 ± 352	263 ± 138	4615

Values represent radioactivity [mean ± SEM (n = 4)] incorporated into *Streptomyces* hyaluronidase-sensitive glycosaminoglycans that were isolated from the medium and cell layer after a 24 hr incubation with [^{3}H]acetate. Conditions were as described in the legend to Fig. 1.

free and serum-containing cultures. Five percent fetal calf serum caused an ≈2-fold increase in incorporation into hyaluronate in the fibroblast cultures but had little effect on the LX-1 cells. Fetal calf serum at 10% caused no further increment in hyaluronate synthesis. Both in the presence and absence of 5% serum, stimulation was observed in the medium and cell-layer compartments of cocultures as compared to the sum of the individual cultures. Thus, the stimulation by serum and the stimulation due to coculturing with tumor cells appear to be additive effects, presumably due to different mechanisms. It is also evident from these data that there is too little hyaluronate in the cell layer to account for the increased hyaluronate in the medium of the cocultures by an increased rate of shedding.

The stimulation of hyaluronate synthesis was dependent on the ratio of tumor cells to fibroblasts in the cocultures. Stimulation increased with the number of LX-1 cells when the number of fibroblasts was held constant (Fig. 2*A*) or with the number of fibroblasts when the number of LX-1 cells was constant (Fig. 2*B*). However, the increase occurred with smaller increments in cell number in the former case. Maximum stimulation was obtained at a ratio of fibroblasts to LX-1 cells of 1–2:1.

Markedly increased synthesis of hyaluronate was also observed in combined cultures of fibroblasts with tumor cells other than LX-1. Table 2 shows the increments obtained in

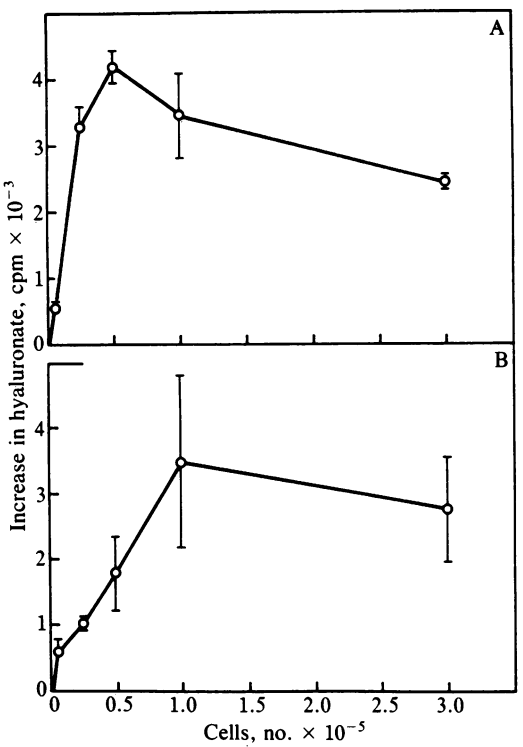

FIG. 2. Hyaluronate synthesis in cocultures with various LX-1/fibroblast ratios. Values represent the increment due to coculture in the amount of [^{3}H]acetate incorporated into *Streptomyces* hyaluronidase-sensitive glycosaminoglycan found in the medium. Thus, the sum of the incorporation for the individual cultures has been subtracted from the coculture values for each point. Each data point represents replicate experiments in which 10^5 fibroblasts were plated together with the various indicated numbers of LX-1 cells (*A*) or 10^5 LX-1 cells were plated together with the various indicated numbers of fibroblasts (*B*). Each of the cultures was incubated with 20 μCi of [^{3}H]acetate in 1.0 ml of DME medium containing antibiotics in 16-mm^2 wells of Falcon multiwell dishes. The percentage of [^{3}H]acetate incorporated into total glycosaminoglycan that was susceptible to *Streptomyces* hyaluronidase was the same as given in the legend to Fig. 1. Error bars represent the SEM for two separate experiments each assayed in duplicate.

Table 2. Stimulation of hyaluronate synthesis in cocultures of human fibroblasts and various human tumor cell types

Cell type	cpm
Fibroblasts	1624 ± 463
LX-1	186 ± 59
Fibroblasts plus LX-1	5015 ± 379
DAN	628 ± 15
Fibroblasts plus DAN	3812 ± 76
TRIG	<50
Fibroblasts plus TRIG	2841 ± 217

Values represent radioactivity [mean ± SEM (n = 4)] incorporated into *Streptomyces* hyaluronidase-sensitive glycosaminoglycans that were isolated from the medium after a 24-hr incubation with [^{3}H]acetate. Conditions were as described in the legend to Fig. 1. DAN, human pancreatic carcinoma; TRIG, human melanoma.

cocultures of human fibroblasts with human lung carcinoma (LX-1), human melanoma (TRIG), or human pancreatic carcinoma (DAN). In addition, coculture of LX-1 cells with the human fibroblast cell lines CCD28Sk and CCD48Sk (American Type Culture Collection) resulted in a 2- and 2.5-fold stimulation of hyaluronate production, respectively.

Chemical Analysis of Glycosaminoglycan Accumulation. The effect of combined culture on hyaluronate synthesis described above was further substantiated by the use of a chemical method to quantitate glycosaminoglycans. Glycosaminoglycans secreted by unlabeled cultures were analyzed by HPLC after digestion to disaccharides with a mixture of chondroitin ABC and AC lyases. This allowed simultaneous quantitation of unlabeled hyaluronate, chondroitin, chondroitin-4-sulfate (plus dermatan sulfate), and chondroitin-6-sulfate. These results were compared to parallel cultures labeled with [^{3}H]acetate and analyzed as above. The stimulation of hyaluronate synthesis as determined by [^{3}H]acetate incorporation was 3-fold. The increase in accumulation of hyaluronate as measured by HPLC was 2-fold. The HPLC data in Table 3 show that hyaluronate was the major glycosaminoglycan in the medium. No nonsulfated chondroitin was detected in any of these cultures and only the fibroblast-containing cultures showed detectable chondroitin sulfates. The ratio of hyaluronate to the chondroitin sulfates in the cocultures was similar to that in the cultures of fibroblasts alone, indicating that the synthesis of the chondroitin sulfates was stimulated proportionately to the synthesis of hyaluronate. This was confirmed using [^{35}S]sulfate as a precursor: a 3-fold increase was observed in incorporation into chondroitin sulfates in the combined cultures compared to the sum of the individual cultures (data not shown).

Requirement for Cell Contact. To begin investigating the mechanism of hyaluronate stimulation in these cultures, we determined whether actual contact between tumor cells and fibroblasts was required or whether the stimulation was induced via a soluble factor released into the culture medium. In initial experiments, various amounts of conditioned medium, prepared as described (22) from cultures of tumor cells alone or from cocultures of tumor cells and fibroblasts, were added to fibroblast cultures. Incorporation of [^{3}H]acetate into hyaluronate was unaffected by these supplements. The same result was obtained when medium conditioned by fibroblasts was added to tumor cells. It was possible that, in these experiments, the putative stimulatory factor(s) became inactive due to instability or to handling of the conditioned medium. Therefore, tumor cells and fibroblasts were plated into separated compartments yet allowed to share the same medium during incubation. A deeply notched 35-mm culture dish was attached to the inside of a 60-mm dish to provide the separated compartments. In this way, any factor released into the medium in one compartment could immediately diffuse to and act on cells in the adjacent compartment.

Table 3. HPLC analysis of glycosaminoglycans produced in cultures

Cell type	HA, cpm*	HPLC†				
		HA, μg	COS, μg	C4S, μg	C6S, μg	Total GAG
Fibroblasts	1713 ± 214	73.2 ± 15.0 (82%)	<0.5	13.7 ± 0.5 (15%)	2.5 ± 0.2 (3%)	89.4 (100%)
LX-1	987 ± 62	17.2 ± 2.2 (100%)	<0.5	<0.5	<0.5	17.2 (100%)
Fibroblasts plus LX-1	8305 ± 477	177.4 ± 8.5 (84%)	<0.5	23.7 ± 1.3 (11%)	10.6 ± 2.3 (5%)	211.7 (100%)

HA, hyaluronate; COS, chondroitin; C4S, chondroitin-4-sulfate; C6S, chondroitin-6-sulfate; GAG, glycosaminoglycan.
*Values represent radioactivity [mean ± SEM (n = 4)] from [^{3}H]acetate incorporated into *Streptomyces* hyaluronidase-sensitive GAGs that were isolated from the medium. Conditions were as described in the legend to Fig. 1.
†Values represent μg [mean ± SEM (n = 4)] of each GAG present in the culture medium from a 100-mm dish of cells. Percentage of the total GAG recovered in each fraction is shown in parentheses.

Even under these conditions, however, no effect on hyaluronate synthesis was observed (Table 4). When both cell types were plated together in either one of the compartments as a positive control, hyaluronate synthesis was again stimulated (Table 4). Therefore, cell contact between the tumor cells and fibroblasts appears to be required for the stimulatory effect on hyaluronate synthesis.

DISCUSSION

The results obtained in this study show that several human tumor cell lines interact with human fibroblasts in coculture to cause stimulation of hyaluronate production. Using the LX-1 cell line, we have shown that there is not only an increment in incorporation of isotopic precursor into hyaluronate but also an increased net accumulation of total unlabeled hyaluronate in the cocultures (as much as 2-fold that in the individual cultures over a 48 hr period, see Table 3). The stimulation of hyaluronate synthesis due to coculture is additive to that caused by serum (Table 1). These results provide an explanation for the observation that, although many types of tumors contain high concentrations of hyaluronate (2–7, 14), tumor or transformed cells in culture often produce only small amounts of this polysaccharide (11–14). In these cases, it would seem likely that interactions between tumor cells and fibroblasts of the host are responsible for the high hyaluronate concentrations in the tumors.

Many tumors also contain elevated concentrations of chondroitin sulfates (2–6, 29–32), presumably present as complex proteoglycans (31). Recent work by Iozzo (21) has shown that conditioned medium from cultures of colon carcinoma cells stimulates fibroblasts to produce increased amounts of chondroitin sulfate-proteoglycan. We have observed that the cocultures of LX-1 cells and fibroblasts produce higher amounts of chondroitin sulfates than the individual cultures, although at a much lower level than hyaluronate.

Table 4. Cell contact requirement for hyaluronate stimulation

Cell type	Compartment*	cpm
Fibroblasts	Inner	2703 ± 22
	Outer	2364 ± 109
LX-1	Inner	222 ± 31
	Outer	214 ± 59
Fibroblasts plus LX-1	Inner	4880 ± 67
	Outer	5174 ± 118
Fibroblasts/LX-1	Inner/Outer	3023 ± 160
	Outer/Inner	2886 ± 15

Values represent radioactivity [mean ± range (n = 2)] incorporated into *Streptomyces* hyaluronidase-sensitive glycosaminoglycans that were isolated from the medium of these cultures after a 24 hr incubation with [^{3}H]acetate.
*Cells were plated into a vessel made by attaching a deeply notched 35-mm tissue culture dish inside a 60-mm tissue culture dish; 10^5 cells of each specified type were plated into either the inner compartment (35-mm notched dish) or the outer compartment (space between the walls of the 60- and 35-mm dishes).

In other studies (22), one of us has shown that interactions between fibroblasts and tumor cells also give rise to high levels of collagenase production. The tumor cells secrete a factor into their culture medium that, on addition to the fibroblasts, causes equivalent production of collagenase to that found in the cocultures. Addition of fibroblast-conditioned medium to the tumor cells does not result in significant production of collagenase (22). However, the stimulation of hyaluronate synthesis in cocultures of human fibroblasts and tumor cells does not appear to be mediated by secreted factors since conditioned medium from either tumor cells or fibroblasts did not stimulate the other cell type to produce increased amounts of hyaluronate. In addition, there was no significant stimulation under conditions where the two cell types were cultured separately but shared the same medium (Table 4). Thus, cell contact would appear to be necessary for the stimulation of production of hyaluronate but not of collagenase, although these experiments do not completely rule out the possibility of short-range effects of very labile soluble mediators.

It is not yet certain which cell type is responsible for production of the additional hyaluronate in the cocultures. In other experiments using murine tumors (14), we have been able to show that extracts of these tumors stimulate hyaluronate production by fibroblasts but not by tumor cells. Also, the ratio of hyaluronate to chondroitin sulfate production in the human cocultures described herein is similar to that in the fibroblasts alone. Finally, we have shown that membranes isolated from sonicated human tumor cells stimulate hyaluronate production by fibroblasts whereas fibroblast membranes had no effect on hyaluronate production by fibroblasts or tumor cells (unpublished results). These three results strongly suggest that the source of elevated hyaluronate production is the fibroblasts.

It is obvious that tumor cell invasion is the result of numerous factors such as changes in cytoskeletal and cell surface characteristics, penetration of host tissues, and interactions with cells of the immune and vascular systems. Two important requirements for successful penetration of host tissues would appear to be (*i*) destruction of connective tissue barriers and (*ii*) reconstruction of an appropriate environment for cellular proliferation and movement. Both of these requirements are likely to be multifaceted; but important in the first phenomenon would be production of type I collagenase (24), and in the second, production of a hydrated, hyaluronate-rich pericellular milieu (1). The results of this and other studies (22, 23) suggest strongly that interac-

tions between host fibroblasts and invading tumor cells may be important in regulation of synthesis of both collagenase and hyaluronate, albeit by different mechanisms.

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