Hyaluronate Synthetase Inhibition by Normal and Transformed Human Fibroblasts during Growth Reduction

Koozi Matuoka,* Masayoshi Namba,‡ Youji Mitsui§

*Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Sakae-cho 35-2, Itabashi-ku, Tokyo 173, Japan;
‡Department of Pathology, Kawasaki Medical School, Matsushima 577, Kurashiki 701-01, Japan; and
§Cell Science and Technology Division, Fermentation Research Institute, Agency of Industrial Science and Technology, Yatabe-cho 1-1-3, Tsukuba, Ibaraki 305, Japan

Abstract. To establish the relation of glycosaminoglycan synthesis to cell proliferation, we investigated the synthesis of individual glycosaminoglycan species by intact cells and in a cell-free system, using normal and transformed human fibroblasts under differing culture conditions. Reducing serum concentration brought about a marked decline in the synthesis of hyaluronate (HA) as well as cell proliferation on both normal and transformed cells. Both HA synthesis and proliferation decreased with increasing cell densities markedly (in inverse proportion to cell density) in normal cells but gradually in transformed cells. This noticeable congruity of the changes in HA synthesis and proliferation indicates that the change in HA synthesis is related primarily to cell proliferation rather than to cell density or cellular transformation. Examination of HA synthesis in a cell-free system demonstrated that the activity of HA synthetase also fluctuated in conjunction with cell proliferation. Furthermore, growth-reduced cells (except crowded transformed cells) inhibited cell-free HA synthesis and this inhibition was induced coincidentally with a decrease in both HA synthetase activity and proliferation. These findings suggest that the change in HA synthesis is significant in the regulation of cell proliferation.

Most animal cells in vivo and in vitro produce the polyanionic macromolecules generically termed glycosaminoglycans (GAG's)[1] and proteoglycans, which are distributed on the cell surface and in subcellular constituents as well as the extracellular matrix and connective tissues (19, 22, 47). GAG's consist of a variety of subspecies such as hyaluronate (HA), heparan sulfate (HS), and galactosaminoglycans (GalAG's), which include chondroitin sulfates and dermatan sulfate. The GAG species, with the exception of HA, are originally synthesized on protein cores to form proteoglycans. Recent studies on GAG's and proteoglycans suggest that these substances are not only the substrate materials of connective tissues but that they also play diverse roles in the regulation of cellular functions (15, 19, 22, 47). It has been demonstrated that treatment with exogenous GAG's and proteoglycans, especially HS, affects cell proliferation (11, 23, 32, 34, 57), suggesting that GAG's and proteoglycans are involved in the regulation of cell proliferation such as density-dependent growth inhibition (30).

In this connection, there have been a number of studies on the synthesis and composition of GAG's and proteoglycans in relation to the cell cycle (25, 46), cell density (7), transformation (20), aging (1, 31, 52), and other factors related to cell proliferation (for reviews see references 19, 22, 47). In a general sense these studies show that cells actively synthesize HA and chondroitin sulfates in the growing phase but tend to synthesize HS and dermatan sulfate in the stationary phase of cell proliferation. However, the general features of GAG's in relation to cell proliferation remain to be established, because a complicated concomitance of the above factors interferes with a decisive attribution of the observed changes in GAG's to any one factor. Concerning this issue, we recently found that the changes in cell-associated HA and HS relate mainly to the state of cell growth (33). This motivated us to further investigate the relationship between GAG's and cell proliferation.

Here we describe a detailed analysis of growth-related changes in GAG synthesis using normal and transformed human fibroblasts under various culture conditions, the results establishing a tight coupling of HA synthesis with cell proliferation. Furthermore, we report that various growth-reduced cells (except crowded transformed cells) inhibited HA synthesis in a cell-free system and that this inhibition was

1. *Abbreviations used in this paper*: GAG, glycosaminoglycan; GalAG, galactosaminoglycan; HA, hyaluronate; HAS, hyaluronate synthetase; HAS inhibition, inhibition of cell-free hyaluronate synthesis; HexUA, hexuronic acid; HS, heparan sulfate; UDP-GlcUA, uridine 5'-diphosphoglucuronic acid.

© The Rockefeller University Press, 0021-9525/87/04/1105/11 $1.00

induced coincidentally with the decrease in both the activity of HA synthetase (HAS) and cell proliferation. These findings suggest that the change in HA synthesis is significant in the regulation of cell proliferation.

Materials and Methods

Cell Cultures and Materials

The following cell strains were used: WI-38 (human embryonic lung fibroblasts, ATCC CCL 75; middle passage cells at a population doubling level of 34–40), WI-38 CT-1 (^{60}Co-transformed WI-38 cells [42, 43]), and BALB/3T3 (mouse embryonic cells, ATCC CCL 163). Cells were supplied from a cell depository in liquid nitrogen and subcultured at least twice before use. Unless otherwise specified, cells were cultured in DME (No. 430-2100, 4.5 g/liter of D-glucose; Gibco, Grand Island, NY) supplemented with 10% FBS (No. 210471, Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany) and 100 μg/ml of kanamycin sulfate throughout maintenance and experiments as previously described (31).

Radioisotope-labeled compounds were purchased from the Radiochemical Centre (Amersham, England); GAG lyases and authentic GAG's (except HS prepared from bovine kidney [32]) were from Seikagaku Kogyo Co. (Tokyo, Japan); and commercial grade chemicals were from Wako Pure Chemical Industries (Osaka, Japan) unless otherwise indicated.

Assay of GAG Synthesis by Intact Cells

Cells were inoculated into 90-mm dishes (45 cm^2/dish, No. 3003, Falcon Labwares, Oxnard, CA) at various cell densities and cultured for 2 d (or for 5 d to obtain dense cultures, which received fresh medium 2 d before labeling). In subsequent sections, the term "serum" means the above FBS and the terms "sparse" and "dense" (or "crowded") indicate cell densities of $0.5–2.0 \times 10^4$ cells/cm^2 and those $>5 \times 10^4$ cells/cm^2, respectively, at harvest. Cells received 6 ml/dish of a medium containing 5 μCi/ml of [^{3}H]-glucosamine (22.6 Ci/mmol, TRK.398) and were incubated for 24 h under the culture conditions. A set of cultures was preincubated in a medium supplemented with 0.2% serum for 24 h before labeling with [^{3}H]glucosamine in 6 ml/dish of a medium supplemented with 0.2% serum. GAG's from extracellular (medium-secreted and trypsin-removed) and intracellular (cell-associated after trypsinization) pools were isolated by alkali treatment, protease digestion, and precipitation with cetylpyridinium chloride as previously described (31). The content of hexuronic acid (HexUA) in GAG preparations was determined by the method of Bitter and Muir (4) and, if necessary, was corrected by subtracting the amount of serum-derived HexUA using the value of 55.6 μM HexUA in serum.

At the onset and the end of glucosamine labeling, cells were washed twice with Dulbecco's Ca^{2+}- and Mg^{2+}-free PBS and harvested by trypsinization (followed by counting the number of cells with a hemocytometer) or by dissolving in an appropriate volume of 0.3 N KOH at 37°C for 1 h. The lysate of the KOH treatment was neutralized with HCl and assayed with a protein assay kit (Bio-Rad Laboratories, Richmond, CA). An aliquot of the lysate from the initial cell layer was processed for isolation of GAG's as described above to determine the initial GAG content in the cell layer.

GAG preparations were dissolved in an appropriate volume of water and, with or without addition of authentic GAG's, their aliquots containing ~1 nmol HexUA equivalent were subjected to electrophoresis on a cellulose acetate membrane in 0.1 N H_2SO_4 at 1 mA/cm for 120 min (33). The membrane was stained in 0.1% Alcian Blue 8GX in 0.1% acetic acid for 10 min, destained in 0.1% acetic acid, lightly blotted with filter papers, and air dried (17). For the samples mixed with authentic GAG's, spots of individual GAG's were cut off and directly transferred to a vial to measure radioactivity by liquid scintillation counting. The samples without authentic GAG's were analyzed by densitometry as follows. A dried membrane was immersed in liquid paraffin, put between glass plates, and analyzed with a TLC scanner (CS-910 and C-R3A Chromatopac, Shimadzu Corp., Kyoto, Japan). Authentic GAG's (porcine skin HA, whale cartilage chondroitin 4-sulfate, shark cartilage chondroitin 6-sulfate, porcine skin dermatan sulfate, and bovine kidney HS) were used to calibrate absorbance at 595 nm, which was linear with concentration of HA, HS, and chondroitin 4-sulfate up to 1.3, 1.1, and 0.8 nmol, respectively, as HexUA equivalent per spot. As the specific absorbance of chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, and HS was 1.87, 1.88, 1.85, and 1.42, respectively, taking that of HA as 1.00, chondroitin 4-sulfate was used as a standard for GalAG's. Contents of individual GAG's (as HexUA equivalent) in serum were determined by the same method (HA, 13.8 μM; HS, 30.4 μM; GalAG's, 16.2 μM) and, if necessary, GAG contents in the samples were corrected by subtracting the values corresponding to the amount of each GAG derived from serum. In an experiment, the cellulose acetate membrane was removed from glass plates, washed in benzene, and air dried. Spots of the samples (corresponding to authentic GAG's on a separate lane) were cut off for liquid scintillation counting.

Assay of GAG Synthesis in a Cell-free System

The activity of the enzyme systems synthesizing individual GAG species was assayed as follows: sparse, dense, serum-starved sparse, and serum-starved dense cultures were prepared in 4–16 dishes in a fashion similar to the assay of GAG synthesis by intact cells. Cell layers were washed twice with 0.15 M NaCl, scraped in 0.15 M NaCl with a piece of Teflon sheet, pelleted by centrifugation (1,400 *g* for 5 min), and immediately stored at −80°C. Aliquots were directly subjected to the assay of protein content as described above. The cell pellets were thawed in an appropriate volume of 0.25 M sucrose to obtain cell suspensions containing 2–5 mg/ml of cellular proteins. These were stored at −80°C and thawed just before assay to use as enzyme sources for cell-free GAG synthesis.

The reaction mixture for cell-free GAG synthesis in a final volume of 100 μl contained 50 μl of enzyme source in 0.25 M sucrose, 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 4 mM $MnCl_2$, 5 mM $CaCl_2$, 0.2 mM uridine 5'-diphospho-*N*-acetylglucosamine (Sigma Chemical Co., St. Louis, MO), 0.2 mM uridine 5'-diphospho-*N*-acetyl-galactosamine (Sigma Chemical Co.), 0.1 mM adenosine 3'-phosphate 5'-phosphosulfate (Sigma Chemical Co.), 10 mM ATP (Sigma Chemical Co.), and 4.6 μM uridine 5'-diphospho-[^{14}C]glucuronic acid (UDP-[^{14}C]GlcUA; 272 mCi/mmol, CFB.106) (cf. reference 49). Cell-free GAG synthesis was performed at 37°C for 1 h with an enzyme source containing ~100 μg of cellular proteins in 100 μl of the above reaction mixture, unless otherwise indicated. In all subsequent sections, the name of sugars combined with UDP is abbreviated as UDP-sugar.

After the reaction, the mixture was immediately cooled on ice, mixed with 10 μl of 5 N NaOH, and incubated overnight at 4°C. Then 10 μl of acetic acid was added and the sample was lyophilized and dissolved in an appropriate volume of water. Its aliquots (50 μl each) were incubated at 37°C for 4 h with 50 μl of either water (control), hyaluronidase solution (*Streptomyces* hyaluronidase [44], EC 4.2.2.1; 20 turbidity reducing units (TRU)/ml in 100 mM Na acetate buffer, pH 5.0) or heparanase solution (*Flavobacterium* heparan sulfate lyase [21], EC 4.2.2.8; 10 U/ml in 50 mM Tris-HCl, 50 mM Na acetate, 1.25 mM $CaCl_2$, and 0.25 mg/ml of BSA, pH 7.5). Another aliquot was incubated at 37°C for 4 h with 50 μl of chondroitinase solution (*Proteus* chondroitinase ABC [59], EC 4.2.2.4; 1 U/ml in the same buffer as heparanase), followed by an additional treatment with hyaluronidase solution (50 μl) at 37°C for 4 h.

Each of the digested samples was lyophilized, dissolved in 25 μl of 1 N NaOH, and spotted onto a filter paper (No. 50, Toyo Roshi, Tokyo, Japan) with 40 μl of a mixture of propanoic acid and water (1:1, vol/vol) (or 1% SDS), which was used to wash the sample tube. Descending paper chromatography was carried out overnight with the first solvent (1-butanol/ethanol/water [52:32:16, vol/vol/vol]) to remove salts in the sample (18) and then again overnight with the second solvent (1-butanol/propanoic acid/1 N NH_4OH (1:2:2, vol/vol/vol]). After air drying the paper, the spot at the origin was cut off for liquid scintillation counting. The amounts of GAG species synthesized in the cell-free system were calculated from the differences in the radioactivity between the samples treated or untreated with GAG lyases.

Assay of Inhibition of Cell-free HA Synthesis (HAS Inhibition)

Combinations of enzyme sources were used in the above assay of HA synthesis in a cell-free system to examine the presence of HAS modulator. A source from sparse WI-38 CT-1 cells (25 μl; used as the target enzyme system) was mixed with 25 μl of another source (with a comparable protein content) or 0.25 M sucrose (control). Then substrate solutions were added to a final volume of 100 μl to obtain a cell-free HA synthesis. The amount of HA synthesized by each combination was determined as described above and the change in cell-free HA synthesis due to mixing the cells was estimated as described in the footnote to Table V.

Analysis of Cell Proliferation

Cells were inoculated at various cell densities into 6-well plates (8 cm^2/well, No. 3506; Costar, Cambridge, MA) containing 15-mm glass cover-

slips, and cultured in a medium supplemented with 0.1% serum for 2 d. Cells were then stimulated to proliferate by feeding 2 ml/well of a medium containing 1–10% serum and 0.1 μCi/ml of [^{3}H]thymidine (21.0 Ci/mmol, TRK. 296). At intervals coverslips were removed, washed three times with PBS, and fixed in 60% aqueous methanol at −20°C overnight. Then they were washed twice with 60% methanol and 100% ethanol, air dried, and mounted on a slide glass. The samples were processed for autoradiography using NR-M2 emulsion (Konishiroku Photo Ind. Co., Tokyo, Japan) according to the manufacturer's instruction (with exposure for 7 d at 4°C) and stained with a 20-fold dilution of Giemsa solution (No. 9204, E. Merck, Darmstadt, Federal Republic of Germany). About 200 cells on each coverslip from triplicate samples were examined under a microscope to determine the percentage of nuclei labeled with silver grains.

Induction of HAS Inhibition

Cells were inoculated at a split ratio of 1:8 into 90-mm dishes or into 6-well plates (containing coverslips or not). 2 d later the cells received a medium containing 0.2% serum (6 ml/dish or 1 ml/well) with or without 1 μg/ml of cycloheximide (C6255; Sigma Chemical Co.) or 0.2 μg/ml of actinomycin D (A4262; Sigma Chemical Co.). At intervals, cells in 90-mm dishes were harvested for assay of HAS activity and HAS inhibition as described earlier.

Parallel cultures in 6-well plates were starved of serum as described above and incubated for 6 h with 0.1 μCi/ml of ^{14}C-amino acid mixture (55 Ci/g atom carbon, CFB. 104) and 1 μCi/ml of [^{3}H]uridine (40.0 Ci/mmol, TRK. 410). The cells were washed twice with PBS, fixed in 7% (wt/vol) TCA on ice for 30 min, washed twice with ice-cold 7% TCA, and dissolved in 0.5 ml of 0.3 N KOH at 37°C for 1 h. Each cell lysate, combined with 0.5 ml of 0.3 N HCl and 1 ml of PBS to wash the well, was transferred to a vial for liquid scintillation counting.

At intervals after the onset of serum starvation, cells cultured on coverslips in 6-well plates received 10 μl of [^{3}H]thymidine in PBS to a final concentration of 1 μCi/ml and incubated for 1 h. The coverslips were then sampled and processed for autoradiography as described above.

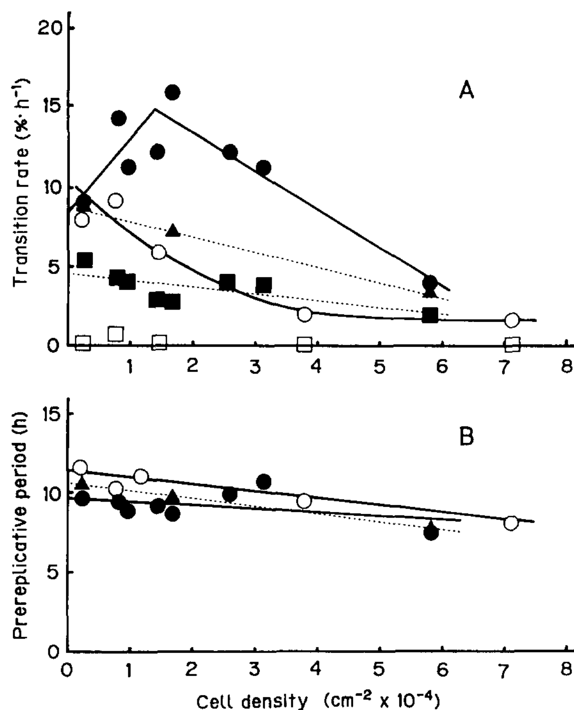

Figure 2. Changes in transition rate (*A*) and prereplicative period (*B*) of normal (WI-38, *open symbols*) and transformed (WI-38 CT-1, *closed symbols*) human fibroblasts with different cell densities and serum concentrations (○ and ●, 10%; ▲, 1%; □ and ■, 0.1%). Transition rate and prereplicative period were obtained from such experiments as that shown in Fig. 1 and were plotted as a function of cell density at 0 time.

Results

Changes in Cell Proliferation with Different Cell Densities and Serum Concentrations

We have reported that the growth of WI-38 CT-1 cells (gamma ray-transformed human fibroblasts) is relatively insusceptible to cell crowding, whereas the growth of WI-38 cells (normal human fibroblasts) markedly decreases with increasing cell density, and that serum starvation brings about growth reduction for both (34). To investigate the above observation in detail, transition rate and prereplicative period, which are parameters of a cell's proliferative activity, were determined at various cell densities and serum concentrations. Fig. 1 shows an example of the diagrams used to calculate these parameters. Transition rates and prereplicative periods obtained from such diagrams for other experiments are presented in Fig. 2.

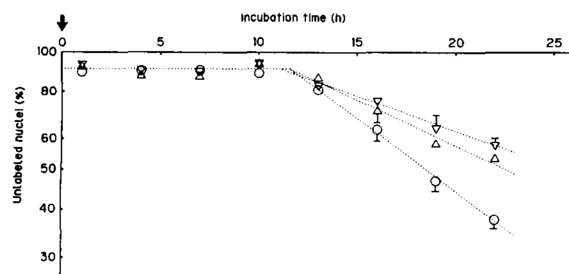

Figure 1. Changes in transition rate and prereplicative period of normal (WI-38) human fibroblasts with different serum concentrations. Sparse WI-38 cells cultured in a medium containing 0.1% serum for 2 d were stimulated to proliferate by elevating serum concentration to 1 (▽), 2 (△), or 10% (○) at 0 time (indicated by an arrow) and continuously incubated in the presence of [^{3}H]thymidine. At intervals, cells were sampled to determine the percentage of unlabeled nuclei as described in Materials and Methods ($n = 3$; short bars indicate SEM's). According to the model of Smith and Martin (53), transition rate at which cells emerge from the G_0 phase to enter the S phase is presented as a slope of the logarithm of the percentage of unlabeled nuclei and prereplicative period as an interval from 0 time to the time at which the slope changed.

Reducing serum concentration led to a decrease in transition rate of transformed cells (Fig. 2) as well as normal cells (Fig. 1). Increasing cell density brought about a marked decrease in transition rate of normal cells virtually in inverse proportion to cell density (the coefficient of correlation of the reciprocal of transition rate to cell density was +0.957). Concerning 10% serum-supplied transformed cells, there appears to be an increase in transition rate with increasing cell densities followed by a gradual decline in transition rate at densities above 1.3 × 10^4 cells/cm^2 (for the latter change, the coefficient of correlation of transition rate to cell density was −0.931). As transition rate of transformed cells at densities up to 3.1 × 10^4 cells/cm^2 was higher than or comparable to that of rapidly growing normal cells, transformed cells were very proliferative in the presence of 10% serum until their density became so high that they piled up in a multilayer (5.8 × 10^4 cells/cm^2). Interestingly, it appears that at low serum concentrations such as 1 and 0.1%, transformed cells continuously decreased in transition rate with increasing cell densities from 0.25 × 10^4 cells/cm^2 to 5.8 × 10^4 cells/cm^2.

Table I. Differences in Specific Radioactivity of GAG's Synthesized in the Presence of [^{3}H]Glucosamine among Various Cell Cultures

Cell strain	Culture condition	GAG content (nmol HexUA/dish) Initial	Final	Synthesized	Radioactivity incorporated	Specific radioactivity
					dpm/dish	*dpm/nmol HexUA*
WI-38	Sparse	7.9	95.9*	54.6	545,000*	9,980
	Crowded	20.8	203.9	149.7	1,164,000	7,780
WI-38 CT-1	Sparse	1.7	50.6	15.5	438,000	28,300
	Crowded	9.6	106.0	63.0	1,813,000	28,800
WI-38	Sparse	4.5	81.5‡	43.6	503,000‡	11,560
	Serum-starved sparse	12.2	31.4	18.5	170,000	9,210
WI-38 CT-1	Sparse	3.2	77.0	40.4	1,530,000	37,900
	Serum-starved sparse	4.5	20.1	14.9	387,000	26,000

Cells were cultured in the presence of [^{3}H]glucosamine for 24 h under various culture conditions. GAG's were isolated from the cell layer harvested at the onset of labeling and from the whole culture harvested at the end of labeling as described in Materials and Methods. HexUA content and radioactivity in the GAG preparations were determined by the carbazole method and liquid scintillation counting, respectively. As the GAG preparation from the final whole culture includes preexistent GAG's (derived from serum and the initial cell layer), the amount of "synthesized" GAG's was obtained by subtracting both the "initial" GAG content and the amount of serum-derived GAG's (33.4 nmol, but for serum-starved cultures 0.7 nmol) from the "final" GAG content. For details see Materials and Methods.
* Mean of duplicate determination (standard errors for GAG content and radioactivity were <20 and 19% of the mean, respectively).
‡ Mean of triplicate determination (standard errors for GAG content and radioactivity were <11 and 6% of the mean, respectively).

These changes in transition rate are consistent with those in the growth rate based on the net cell number (34).

On the other hand, prereplicative period of both normal and transformed cells was slightly shortened by increasing cell density and was virtually unaffected by changing serum concentration. These results indicate that the rate of cell growth is reflected by transition rate rather than prereplicative period in the present experiments.

Estimation of GAG Synthesis by Precursor Incorporation

In this study we measured the incorporation of labeled glucosamine into GAG's to estimate the amount of synthesized GAG's. Under the present conditions, incorporation of labeled glucosamine into each GAG species (on the basis of cell number) increased linearly up to at least 24 h of incubation and radioactivity in each GAG species in the cell layer reached a plateau by 7 h (for HA and GalAG's) or 24 h (for HS) after the onset of incubation (data not shown). When cells were cultured for 24 h in the presence of [^{3}H]glucosamine, washed, and then chased in an isotope-free medium for 24 h, the radioactivity in GAG's preexistent in the cell layer was completely recovered in the GAG preparation after chasing (e.g., 106% of the total; 26% in cells and 80% in medium) (data not shown), indicating that there was little degradation of GAG's in the cell cultures examined. These observations suggest that the rate of glucosamine incorporation parallels the rate of GAG synthesis. However, it has been demonstrated that the specific radioactivity of UDP-sugars, the final precursors for GAG synthesis, differs between culture conditions (24, 60), leading to a dissociation of the apparent isotope incorporation from the net amount of synthesized GAG's.

To examine this issue, the net content of GAG's was determined as HexUA equivalent by chemical analysis. The results shown in Table I indicate that the specific radioactiv-

Table II. Comparison of GAG Composition Determined by Densitometry with Distribution of Incorporated Radioactivity to GAG Species

Cell culture	GAG species	GAG content (nmol HexUA/dish) Initial	Final	Synthesized	Radioactivity incorporated	Specific radioactivity
					dpm/dish	*dpm/nmol HexUA*
Sparse WI-38 cells						
	HA	3.0	64.0 ± 5.0*	52.7 (87.7)‡	510,000 ± 7,100* (89.0)‡	9,680
	HS	<1.0	22.9 ± 2.7	4.2§ (7.0)	36,000 ± 1,200 (6.3)	8,570
	GalAG's	<1.0	13.4 ± 1.7	3.2§ (5.3)	27,300 ± 1,100 (4.8)	8,530
Crowded WI-38 cells						
	HA	6.3	115.4 ± 5.3	100.8 (63.5)	781,600 ± 46,200 (62.7)	7,750
	HS	5.2	58.9 ± 2.0	35.5 (22.4)	306,100 ± 10,700 (24.6)	8.620
	GalAG's	3.0	35.2 ± 0.7	22.5 (14.2)	158,000 ± 5,600 (12.7)	7,020

GAG's were isolated from the cell culture incubated with [^{3}H]glucosamine as described in the footnote to Table I. The amount of individual GAG species was determined by densitometry after electrophoresis on a cellulose acetate membrane. Then the spot of each GAG on a membrane was cut off to measure radioactivity. The amount of "synthesized" GAG species was obtained by subtracting both the "initial" content and the amount of serum-derived GAG (HA, 8.3 nmol; HS, 18.2 nmol; and GalAG's, 9.7 nmol) from the "final" content. For details see Materials and Methods.
* Mean ± SEM (n = 2).
‡ Percentage of the total GAG's.
§ Calculated taking "initial" as 0.5 nmol.

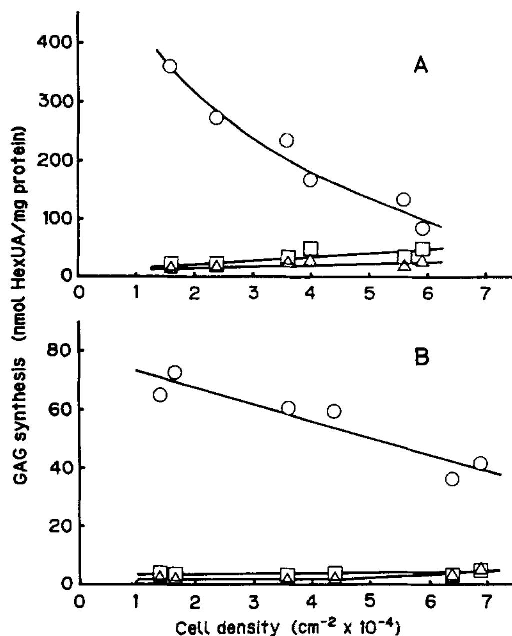

Figure 3. Changes in GAG synthesis by normal (*A*, WI-38) and transformed (*B*, WI-38 CT-1) human fibroblasts with different cell densities. Cells at various cell densities were cultured for 24 h in the presence of 10% serum and [^{3}H]glucosamine. GAG's were isolated from the cell cultures and analyzed as described in the footnote to Table III. The amount of individual GAG species synthesized was plotted as a function of cell density at harvest (n = 2; standard errors for the whole GAG's were <28% of the mean). ○, HA; □, HS; and △, GalAG's.

ity of newly synthesized GAG's varies with cell strains, serum concentrations, and cell densities. On the other hand, when the percentages of individual GAG species to the total amount of synthesized GAG's were compared with the percentages to the total radioactivity incorporated into GAG's, they were found to be virtually identical (Table II).

Although direct determination of the amount of individual GAG species would most convincingly indicate the net synthesis of GAG species, we could not always obtain a reliable value for the amount of individual GAG's preexistent in the cell layer (which should be subtracted as background) because of a methodological limit of sensitivity. Moreover, it appears that densitometric analysis failed to give correct results when the amount of synthesized GAG's was noticeably less the amount of serum-derived GAG's. Therefore, in the following experiments, the synthesis of individual GAG species was estimated from the net increase in HexUA content during glucosamine labeling (corresponding to the total amount of synthesized GAG's) and the percentage of radioactivity incorporated into each GAG species.

Changes in GAG Synthesis with Different Cell Densities and Serum Concentrations

The synthesis of individual GAG species was investigated in relation to the changes in cell proliferation. Fig. 3 and Table III show the changes in GAG synthesis on the basis of cellular protein content.

Increasing cell density from 1.4 × 10^4 cells/cm^2 to 6.9 × 10^4 cells/cm^2 brought about a marked decrease in HA synthesis by normal cells in inverse proportion to cell density (the coefficient of correlation of the reciprocal of the synthesized amount to cell density was +0.968), whereas there was a gradual decrease in HA synthesis by transformed cells (the coefficient of correlation of the synthesized amount to cell density was −0.939). Concerning lower cell densities, we could not obtain a reliable value for the net amount of synthesized HA, although it was observed that incorporation of labeled glucosamine into HA by transformed cells at a cell density of 0.34 × 10^4 cells/cm^2 was slightly less (by 19%) than at 1.4 × 10^4 cells/cm^2. These changes in HA synthesis at different cell densities, at least higher than 1.4 × 10^4 cells/cm^2, closely resemble the change in transition rate shown in Fig. 2. Synthesis of HS and GalAG's by normal cells and synthesis of GalAG's by transformed cells became almost twice with increasing cell density, but changing cell density did not affect HS synthesis by transformed cells.

On the other hand, serum starvation decreased HA synthesis by normal and transformed cells to about a half, while

Table III. Changes in GAG Synthesis by Normal (WI-38) and Transformed (WI-38 CT-1) Human Fibroblasts Due to Serum Starvation

Cell strain	GAG species	GAG synthesized (nmol HexUA/mg cellular protein)			
		10% serum		0.2% serum	
WI-38					
	Whole GAG's	468 ± 89*		475 ± 35*	
	HA	405	(86.5 ± 1.1)*‡	193	(40.8 ± 2.9)*‡
	HS	20	(4.3 ± 0.4)	156	(33.0 ± 1.9)
	GalAG's	43	(9.1 ± 0.9)	125	(26.3 ± 3.5)
WI-38 CT-1					
	Whole GAG's	245 ± 19		196 ± 18	
	HA	188	(76.9 ± 1.2)	95	(48.6 ± 1.4)
	HS	17	(7.1 ± 0.4)	48	(24.7 ± 5.1)
	GAlAG's	40	(16.1 ± 0.8)	52	(26.6 ± 3.7)

Cells were cultured for 24 h in the presence of [^{3}H]glucosamine at a serum concentration of 10 or 0.2%. The total amount of GAG's synthesized during labeling period (shown as "whole GAG's" in this Table) was determined chemically as HexUA equivalent (see Table I) and the percentage of radioactivity incorporated into each GAG (shown in parentheses in this Table) was determined by liquid scintillation counting after electrophoretic separation (see Table II). The amount of individual GAG species synthesized was obtained by dividing the total amount according to the percentage of each GAG.
* Mean ± SEM (n = 3).
‡ Percentage of radioactivity incorporated into each GAG to the total radioactivity in GAG's.

Table IV. Changes in Synthetic Activity of GAG's in Normal (WI-38) and Transformed (WI-38 CT-1) Human Fibroblasts with Different Cell Densities and Serum Concentrations

Cell strain	GAG species	UDP-[^{14}C]GlcUA incorporated (pmol/h per mg)			
		Sparse cells		Crowded cells	
		10% serum	0.2% serum	10% serum	0.2% serum
WI-38					
	HA	24.5 ± 3.5*	10.3 ± 2.0*	14.6 ± 3.0*	Not done
	HS	14.6 ± 2.5	26.7 ± 0.6	17.7 ± 1.0	Not done
	GalAG's	11.5 ± 2.8	31.4 ± 2.8	13.9 ± 2.8	Not done
WI-38 CT-1					
	HA	84.1 ± 2.4	20.1 ± 6.6	35.9 ± 1.4	16.6 ± 1.2*
	HS	21.5 ± 2.1	26.2 ± 1.0	27.1 ± 1.6	24.4 ± 0.7
	GalAG's	14.5 ± 2.3	32.4 ± 3.8	13.9 ± 1.1	21.4 ± 1.0

Sparse and crowded cells cultured in a medium containing 10 or 0.2% serum were scraped and disrupted by freezing and thawing to obtain enzyme sources for the assay of cell-free GAG synthesis as described in Materials and Methods.
* Mean ± SEM (n = 3).

HS synthesis by both the cells and synthesis of GalAG's by normal cells were much enhanced (Table III). As transition rate of both normal and transformed cells decreased with reducing serum concentrations (Figs. 1 and 2), these observations indicate that serum starvation as well as increasing cell density brought about a change in HA synthesis parallel to that in transition rate. It is also of note that the percentages of both HS and GalAG's to the whole GAG's increased with a reduction in cell growth (the percentage of HA accordingly decreased).

Expressing the above results on the basis of cell number, however, produces a somewhat different relation of the synthesis of HS and GalAG's: the amounts of synthesized HS and GalAG's on a per-cell basis, in some cases, decreased with a reduction in cell proliferation, while there was little change on a per-protein basis. It may not be valid to describe on such results how the synthesis of HS and GalAG's changed. Nevertheless, the percentages of individual species to the total GAG's are the same, regardless of which basis is used. Therefore, here we state that a decrease in HA synthesis and an increase in the percentages of both HS and GalAG's to the whole GAG's synthesized accompanied a growth reduction in normal and transformed human fibroblasts.

Changes in Synthetic Activity of GAG's with Different Cell Densities and Serum Concentrations

As a modulation of the enzyme systems for GAG synthesis is the most probable mechanism for the changes in GAG synthesis, we examined the synthetic activity of individual GAG species, using a cell-free system with cells disrupted by freezing and thawing as enzyme sources. The synthesis of individual GAG species in this system increased linearly up to at least 1 h of incubation and up to at least 200 μg/100 μl of a final protein concentration (data not shown). Concerning the concentration of the substrate UDP-[^{14}C]GlcUA, doubling substrate concentration to 9.2 μM increased the cell-free synthesis of each GAG only slightly (e.g., HA synthesis by sparse and serum-starved sparse transformed cells increased by 8 and 10%, respectively) (data not shown), indicating that GAG-synthesizing enzymes were subsaturated with the substrate at a concentration of 4.6 μM. In addition, the amount of UDP-[^{14}C]GlcUA incorporated into GAG's (and any other macromolecules) was small (e.g., at most 5.3% of the total in the experiment shown in Table IV) and the remainder of radioactivity was recovered as a single peak at the same position as authentic UDP-GlcUA by paper chromatography.

As shown in Table IV, serum starvation brought about a marked reduction in cell-free HA synthesis by both normal and transformed cells. Although increasing cell density also resulted in a reduction in HA synthesis by both types of cells, it failed to reduce HA synthesis by transformed cells to the lowest level. These changes in cell-free HA synthesis correlated, if not always quantitatively, with the changes in HA synthesis by intact cells. As HA synthesis in the present cell-free system is thought to reflect the activity of HAS (see Discussion), the results of the cell-free HA synthesis indicate

Table V. Inhibition of Cell-free HA Synthesis (HAS Inhibition) by Serum-starved Cells

Source (protein content)	Cell-free HA synthesis	Apparent change	HAS-Inhibition
	pmol/h	%	%
(1) Sparse cells alone (78 μg)	9.70 ± 0.10*(A)		
(2) Serum-starved cells alone (70 μg)	1.22 ± 0.06 (B)		
(3) Sparse cells plus serum-starved cells (78 + 70 μg)	7.42 ± 0.08 (C)	−32.1(D)	40.2 (E)

Cell-free HA synthesis by the following combinations of enzyme sources from WI-38 CT-1 cells and 0.25 M sucrose (25 μl each) was assayed as described in Materials and Methods: (*1*) sparse cells plus 0.25 M sucrose; (*2*) serum-starved sparse cells plus 0.25 M sucrose; and (*3*) sparse cells plus serum-starved sparse cells. "Apparent change" (*D*) and "inhibition" (*E*) were calculated as $D = 100 \times (C-[A+B])/(A+B)$ and $E = 100 \times ([A+B]-C)/A \times$ (protein content of [*1*])/(protein content of [2]), respectively. HA synthesis by the mixed source (*C*) was less than the sum of HA synthesis of the two separate sources ($A + B$).
* Mean ± SEM (n = 3 or 4).

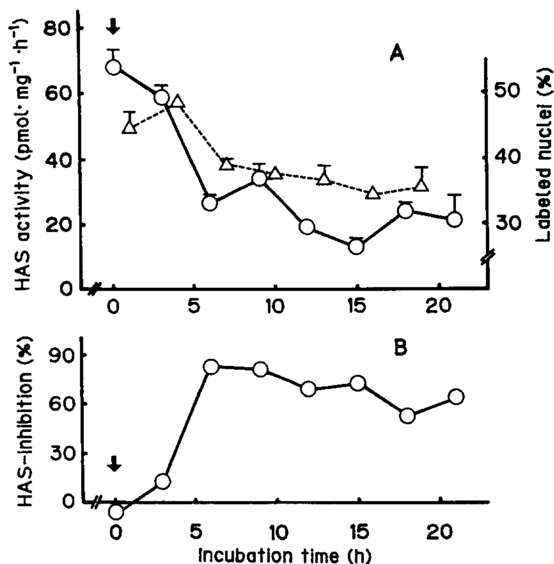

Figure 4. Induction of HAS inhibition in transformed (WI-38 CT-1) human fibroblasts by serum starvation. Sparse transformed cells under the normal culture conditions (with 10% serum) were fed with a medium containing 0.2% serum at 0 time (indicated by arrows). At intervals the cells were harvested to determine HAS activity (*A*, ○; $n = 2$) and HAS inhibition (*B*, ○) as described in Materials and Methods, and simultaneously parallel cultures were pulse-labeled with [^{3}H]thymidine for 1 h to measure the percentage of labeled nuclei (*A*, △; $n = 3$) by autoradiography (short bars indicate SEM's). To examine HAS inhibition, the cells harvested at the indicated time were subjected to the assay of cell-free HA synthesis in combination with sparse WI-38 CT-1 cells (the target) as described in the footnote to Table V.

that HAS activity as well as HA synthesis by intact cells fluctuated in conjunction with cell proliferation.

Presence of HAS Inhibition in Serum-starved Cells

To investigate the mechanism of the growth-related change in HAS activity, we performed cell-free HA synthesis with a mixture of enzyme sources from sparse (rapidly growing) and serum-starved sparse (growth-reduced) transformed cells. Table V shows that the mixture synthesized a lesser amount of HA than the sum of that synthesized by each enzyme source alone (32% in this experiment). This inhibition was specific to HA synthesis, as cell-free synthesis of HS and GalAG's by the mixed cells was virtually the sum of their separate syntheses (104 and 106% of the sum, respectively, in the above experiment) (data not shown). This suggests the presence of a HA synthesis inhibitor(s) in the serum-starved cells.

Induction of HAS Inhibition by Serum Starvation

We thought that the above HAS inhibition might be related to growth reduction, as proliferation was low in the serum-starved transformed cells. Therefore we investigated the occurrence of HAS inhibition in relation to the change in cell proliferation.

Growing WI-38 CT-1 cells were starved of serum and cell proliferation, HAS activity, and HAS inhibition were examined as a function of incubation time (Fig. 4). Here we expressed cell proliferation as the percentage of labeled nuclei after incubation with [^{3}H]thymidine for 1 h, which indicates the percentage of cells in the S phase. The percentage of labeled nuclei began to decrease 7 h after the cells were starved of serum and stayed at a low level up to at least 19 h. HAS activity also markedly decreased by 6 h after the onset of serum starvation. On the other hand, HAS inhibition was absent initially in the rapidly proliferating cells, but serum starvation of the cells led to a high level of HAS inhibition 6 h later.

In addition, we found that treatment of the cells with actinomycin D (at a dose to inhibit RNA synthesis by 87%) or cycloheximide (at a dose to inhibit protein synthesis by 85%) suppressed the induction of HAS inhibition to 26 or 35% of the control, respectively (Table VI). In this experiment, however, treatment with these drugs failed to prevent the decrease in HAS activity, as both treated and untreated cells decreased in HAS activity to a similar level 6 h after the onset of serum starvation.

Differences in HAS Inhibition between Cell Strains and Culture Conditions

As the foregoing indicates a close relation between HAS inhibition and the reduction in cell proliferation, we examined HAS inhibition in various cell cultures (Table VII). Normal human fibroblasts, whose growth was suppressed by cell crowding or serum starvation, were high in HAS inhibition. Also, BALB/3T3 cells were high in HAS inhibition when

Table VI. Effects of Actinomycin D and Cycloheximide on the Induction of HAS Inhibition in WI-38 CT-1 Cells

Time after serum starvation	Treatment	HAS activity	HAS inhibition*	Incorporation	
				[^{3}H]Uridine	^{14}C-Amino acids
h		*pmol/h per mg*	*%*	*dpm/well*	*dpm/well*
0	None	59.8	−9.7		
3	None	54.5 ± 2.0‡	−2.3		
6	None	35.0 ± 0.6	42.3	58,485 ± 744‡	1,680 ± 49‡
6	Actinomycin D	41.9 ± 0.9	10.9	8,029 ± 339	1,327 ± 92
6	Cycloheximide	25.4 ± 1.2	15.0	40,698 ± 207	254 ± 5

Sparse WI-38 CT-1 cells were starved of serum at 0 time and were incubated in the presence or absence of 0.2 μg/ml of actinomycin D or 1 μg/ml of cycloheximide for the indicated periods and harvested to determine both HAS activity and HAS inhibition. Parallel cultures were identically treated with the drugs in the presence of 1 μCi/ml of [^{3}H]uridine and 0.1 μCi/ml of ^{14}C-amino acid mixture for 6 h after the onset of serum starvation to measure the synthesis of RNAs and proteins. For details see Materials and Methods and the footnote to Table V.

* Effect on cell-free HA synthesis by sparse WI-38 CT-1 cells, calculated from duplicate assay.

‡ Mean ± standard error ($n = 2$ for "HAS activity" and $n = 3$ for "incorporation").

Table VII. Changes in HAS Inhibition in Normal and Transformed Cells under Different Culture Conditions

Cell strain	Culture condition	HAS inhibition*
		%
WI-38	Sparse	11.3
	Serum-starved sparse	62.2
	Crowded	56.8
WI-38 CT-1	Sparse	2.2
	Serum-starved sparse	45.8
	Crowded	5.3
BALB/3T3	Sparse	19.7
	Crowded	47.2

Sources from various cell cultures were assayed for HAS inhibition by mixing them with sparse WI-38 CT-1 cells (the target enzyme system) as described in the footnote to Table V and in Materials and Methods.

* Effect on cell-free HA synthesis by sparse WI-38 CT-1 cells, calculated from triplicate assay.

their growth was density inhibited (percent increase in cell number during 24 h was 128 and 14% for sparse and crowded cells, respectively). Interestingly, there was little HAS inhibition in crowded WI-38 CT-1 cells.

Discussion

In the present study to examine the relation of GAG synthesis to cell proliferation, we estimated the synthesis of individual GAG species by measuring both the total amount of GAG's synthesized by cultured cells during incubation with labeled glucosamine (determined chemically as HexUA equivalent) and the percentage of radioactivity incorporated into each GAG species (determined by liquid scintillation counting after electrophoretic separation). It was found that there was little degradation of GAG's in the cell cultures examined (hence an increase in the total amount of GAG's during the labeling period was attributable to the amount of newly synthesized GAG's) and that the distribution of incorporated radioactivity to individual GAG species was parallel to the composition of the newly synthesized GAG's (i.e., all the GAG species synthesized during the labeling period were similar in their specific radioactivity). We consider that these facts validate the present estimation of the synthesis of individual GAG species.

Such a determination of GAG species synthesized under varying culture conditions demonstrated that changes in HA synthesis by both normal and transformed cells in conjunction with the alteration of cell density and serum concentration noticeably resembled the changes in the proliferation of these cells. We consider this to have established the coupling of the change in HA synthesis with cell proliferation rather than with cell density or cellular transformation in the human fibroblasts examined. Concerning HS and GalAG's, however, we could draw no conclusion about the relation of their synthesis to cell proliferation as interpretation depended on whether their synthesis was estimated on the basis of cell number or cellular protein content. Nevertheless, the percentages of both HS and GalAG's to the total amount of synthesized GAG's were found to be elevated by growth reduction. This growth-related change in the balance in GAG synthesis resulted, at least in part, from the decrease in HA synthesis by growth-reduced cells.

It appears that the subcellular composition of GAG's also fluctuated with differing culture conditions: as judged from the radioactivity incorporated into GAG species in intracellular and nuclear fractions after labeling with [^{3}H]glucosamine for 24 h, a reduction in cell growth was accompanied by a decrease in the percentage of HA to the whole GAG's and an increase in the percentage of HS (data not shown). To establish the relation of subcellular GAG composition to cell proliferation, however, the net amounts of GAG species in the subcellular pools should be determined, because there were differences in the turnover rate between GAG species and also in the specific radioactivity of the precursors for GAG synthesis between cells under different culture conditions.

In this connection, the cell nuclei have been found to contain GAG's (2, 14, 28, 54) and recently the presence of a uniquely structured HS in the nuclei was demonstrated (10). Although the role of GAG's in the nucleus is not clear, HS, but not HA or GalAG's, has been revealed to exert a potent inhibition on DNA synthesis in isolated nuclei and in cell-free systems (12, 13, 58). Thus, it is conceivable that increasing the predominance of HS in the cell nucleus leads to the inhibition of DNA synthesis, presenting the possibility that the regulation of cell proliferation involves a change in nuclear GAG composition. In this case, HA synthesis, which we found to fluctuate with cell proliferation, may be of primary significance in the modulation of subcellular GAG composition, especially in fibroblasts that mainly synthesize HA. Investigation of the change in HA synthesis would then provide a clue to the regulation of cell proliferation.

There may be several possible mechanisms for the growth-coupled modulation of HA synthesis. Altering the supply of the substrates for GAG synthesis may change the synthesis of individual GAG species. However, HA synthesis fluctuated quite differently from HS synthesis, in spite of the fact that both HA and HS are synthesized of the same substrates, UDP-GlcUA and UDP-*N*-acetylglucosamine (19, 40, 49). Thus, this presumption is unlikely. Another possibility is a growth-related modulation of intracellular pH (5, 37), which may affect the activity of the enzyme system for HA synthesis (conventionally termed "hyaluronate synthetase" [HAS]). According to our examination, however, intracellular pH in both WI-38 and WI-38 CT-1 cells was, irrespective of cell densities, 7.20–7.24 in the presence of 10% serum and 7.10–7.13 in the presence of 0.2% serum (our unpublished data). This could not explain the density-related change in HA synthesis. Furthermore, judging from cell-free HA synthesis at varying pH's (data not shown), such a difference in pH may have little effect on HAS activity.

On the other hand, analysis of GAG synthesis in a cell-free system demonstrated that cell-free HA synthesis by both normal and transformed cells exhibits a growth-related change similar to HA synthesis by intact cells, suggesting that the growth-related change in HA synthesis is mainly attributable to a change in the activity of HAS. However, this presumption needs to be tested, as a deficit in acceptors (including primers) for HA synthesis would also affect incorporation of the substrate (UDP-[^{14}C]GlcUA) into HA chains. In addition, a decrease in the concentration of the labeled substrate, preexistence or formation of unlabeled UDP-GlcUA, or deg-

radation of HA chains in the cell-free system would result in an apparent change in HA synthesis.

Concerning the acceptors, the presumed limitation of HA synthesis due to a deficit of the acceptors is unlikely, as addition of boiled cell lysate to the reaction mixture did not affect the cell-free synthesis of any GAG species. Furthermore, the result of experimentation mixing different cells shown in Table V contradicts the above presumption: if both sparse and serum-starved cells had an insufficient amount of receptors relative to HAS, there could be no acceptors free of HAS in both cells. Mixing these cells would not result in a change in HA synthesis because of the lack of acceptors. If only serum-starved cells were insufficient in acceptors (HAS free of acceptors in serum-starved cells and acceptors free of HAS in sparse cells), mixing these would increase HA synthesis due to a coupling of free HAS with free acceptors. However, neither was the case. These results are acceptable, as there has been no demonstration that HA synthesis involves acceptors other than nascent HA chains (50) or one of the substrates (UDP-GlcUA or UDP-*N*-acetylglucosamine) (36, 48).

On the other hand, the concentration of UDP-[^{14}C]GlcUA decreased little during the reaction as described in Results. Preexistence of unlabeled UDP-GlcUA in cell lysates is considered to have barely affected HA synthesis, as the activity of HA synthesis was determined at such a concentration of cell lysates that the rate of HA synthesis was linear; and furthermore, a fractionation of cells to prepare a crude plasma membrane fraction (45) (hence enriched with HAS and free of cytosolic substances) did not change the ratio in HA-synthesizing activity between the cells (data not shown). Activities converting UDP-glucose to UDP-GlcUA and degrading HA were not detected in the present cell-free system (data not shown). Consistently, addition of D-saccharic acid 1,4-lactone (inhibiting β-glucuronidase [27]) or β-NADH (inhibiting UDP-glucose dehydrogenase [61]) to the reaction mixture had no effect.

In this connection, there are other enzymes that could affect cell-free GAG synthesis. However, it is conceivable that nucleotide pyrophosphatase (3) and 5′-nucleotidase (9) (both suggested to be involved in the termination of HA chain elongation [48]) may be inhibited in the presence of 10 mM ATP and that UDP-GlcUA decarboxylase (16) and UDP-GlcUA 5-epimerase (8) (both converting UDP-GlcUA) may not function in the absence of β-NAD$^+$.

In conclusion, the differences in the cell-free HA synthesis between the cells examined were attributable to differences in HAS activity and, consequently, it is suggested that the growth-related change in HA synthesis by intact cells resulted from a change in HAS activity.

Concerning HAS, it has been reported that treatment of cultured cells with growth stimulators such as chicken and calf sera (38, 55), epidermal growth factor (26), prostaglandin $F_{2\alpha}$ (39), and CTAP-I and III (51) and with a promoter tetradecanoylphorbol acetate (56) brings about an elevation of HAS activity, which is suppressed by treatment with actinomycin D (39, 55) or cycloheximide (51, 55). These observations led one to think that the change in HA synthesis was the result of HAS modulation such as formation and turnover of the enzyme system. Although the recent study of Mian (35) demonstrated a difference in phosphorylation of a protein complex synthesizing HA oligosaccharides between growing and density-inhibited human fibroblasts, the process of HAS modulation has not been established.

To examine the possibility of this modulation, we investigated cell-free HA synthesis using a mixture of enzyme sources from various cells. Mechanisms for the change in HAS activity might be a modulation of HAS itself, modulation by a factor(s) inhibiting or stimulating the enzyme system, or both of these. In the first case, a combination of different enzyme sources would synthesize an amount of HA comparable to the sum of that synthesized by each enzyme source alone. The result, however, demonstrated that the cell-free HA synthesis was considerably reduced by mixing enzyme sources from sparse (rapidly growing) and serum-starved sparse (slowly growing) WI-38 CT-1 cells.

The above reduction in cell-free HA synthesis was not due to a dilution of some indispensable factor(s) in sparse cells, as all the samples were adjusted to the same final volume and sucrose concentration. Neither a change in the substrate concentration nor degradation of HA in the cell-free system could account for HAS inhibition as discussed earlier. It is noteworthy that the inhibition was specific to HA synthesis, as this eliminates a possible attribution of HAS inhibition to the proteolysis of HAS.

Consequently, it is likely that HAS inhibition resulted from the presence of a factor(s) inhibiting HAS activity. We are now examining HAS inhibition in detail, the results so far confirming the existence of a HAS inhibitor. This discovery of a HAS inhibitor would introduce a novel mechanism in the growth-related modulation of HA synthesis. Indeed, the present examination of normal and transformed human fibroblasts under varying culture conditions revealed that the cells high in HAS inhibition were also inactive in HA synthesis. Furthermore, serum starvation of transformed cells brought about induction of HAS inhibition coincidentally with a decrease in HAS activity. These findings suggest that HAS inhibition accounts for most, if not all, of the change in HA synthesis. Although the suppression of induction of HAS inhibition by either actinomycin D or cycloheximide failed to prevent cells from decreasing in HAS activity, this might result from the drugs' interference with the formation or activation of HAS (55).

It is worth mentioning again that cell proliferation decreased coincidentally with the induction of HAS inhibition and the decrease in HAS activity, emphasizing their tight coupling. In this connection, it is most interesting that HAS inhibition was absent in crowded WI-38 CT-1 cells. The proliferation of the transformed cells was relatively insusceptible to (or, at very low densities, rather stimulated by) an increase in cell density until cells became so crowded that they pile up. This suggests that the proliferation of transformed and tumor cells, which are insensitive to cell-to-cell contact (41), is suppressed at very high cell densities by other factors such as spatial and nutritional limitation (6). It is also suggestive that WI-38 CT-1 cells cultured at a low serum concentration exhibited HAS inhibition as well as a continuous decrease in proliferation with increasing cell densities. These facts imply that the "normal" regulation of cell proliferation involves HAS inhibition. In addition, high HAS inhibition was found in crowded BALB/3T3 cells, whose growth was density inhibited. Thus, HAS inhibition may occur not only in human fibroblasts but in cells under the "normal" regulation of cell proliferation. This seems relevant to the view that

some function in the normal cell contributes to the switching of cell proliferation to cause the cell to enter the G_0 phase (29).

Received for publication 19 May 1986, and in revised form 24 November 1986.

References

1. Bartold, P. M., R. R. Boyd, and R. C. Page. 1986. Proteoglycans synthesized by gingival fibroblasts derived from human donors of different ages. *J. Cell. Physiol.* 126:37–46.
2. Bhavanandan, V. P., and E. A. Davidson. 1975. Mucopolysaccharides associated with nuclei of cultured mammalian cells. *Proc. Natl. Acad. Sci. USA.* 72:2032–2036.
3. Bischoff, E., T. Tran-Thi, and K. F. A. Decker. 1975. Nucleotide pyrophosphatase of rat liver. A comparative study on the enzymes solubilized and purified from plasma membrane and endoplasmic reticulum. *Eur. J. Biochem.* 51:353–361.
4. Bitter, T., and H. M. Muir. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4:330–334.
5. Busa, W. B., and R. Nuccitelli. 1984. Metabolic regulation via intracellular pH. *Am. J. Physiol.* 246:R409–R438.
6. Castor, L. N. 1972. Contact inhibitions of cell division and cell movement. *J. Invest. Dermatol.* 59:27–32.
7. Cohn, R. H., J.-J. Cassiman, and M. R. Bernfield. 1976. Relationship of transformation, cell density, and growth control to the cellular distribution of newly synthesized glycosaminoglycan. *J. Cell Biol.* 71:280–294.
8. Davidson, E. A. 1966. UDP-glucuronic acid 5-epimerase from rabbit skin. *Methods Enzymol.* 8:281–284.
9. Evans, W. H., and J. W. Gurd. 1973. Properties of a 5'-nucleotidase purified from mouse liver plasma membranes. *Biochem. J.* 133:189–199.
10. Fedarko, N. S., and H. E. Conrad. 1986. A unique heparan sulfate in the nuclei of hepatocytes: structural changes with the growth state of the cells. *J. Cell Biol.* 102:587–599.
11. Fritze, L. M. S., C. F. Reilly, and R. D. Rosenberg. 1985. An antiproliferative heparan sulfate species produced by post-confluent smooth muscle cells. *J. Cell Biol.* 100:1041–1049.
12. Furukawa, K., and V. P. Bhavanandan. 1982. Influence of glycosaminoglycans on endogenous DNA synthesis in isolated normal and cancer cell nuclei. *Biochim. Biophys. Acta.* 697:344–352.
13. Furukawa, K., and V. P. Bhavanandan. 1983. Influences of anionic polysaccharides on DNA synthesis in isolated nuclei and by DNA polymerase α: correlation of observed effects with properties of the polysaccharides. *Biochim. Biophys. Acta.* 740:466–475.
14. Furukawa, K., and H. Terayama. 1977. Isolation and identification of glycosaminoglycans associated with purified nuclei from rat liver. *Biochim. Biophys. Acta.* 499:278–289.
15. Gallagher, J. T., M. Lyon, and W. P. Steward. 1986. Structure and function of heparan sulfate proteoglycans. *Biochem. J.* 236:313–325.
16. Gebb, C., D. Baron, and H. Grisebach. 1975. Spectroscopic evidence for the formation of a 4-keto intermediate in the UDP-apiose/UDP-xylose synthase reaction. *Eur. J. Biochem.* 54:493–498.
17. Hata, R., and Y. Nagai. 1973. A micro colorimetric determination of acidic glycosaminoglycans by two dimensional electrophoresis on a cellulose acetate strip. *Anal. Biochem.* 52:652–656.
18. Honda, A., M. Iwata, T. Umeda, and Y. Mori. 1982. The teratogenic mechanism of 6-aminonicotinamide on limb formation of chick embryos: abnormalities in the biosynthesis of glycosaminoglycans and proteoglycans in micromelia. *J. Biochem.* 91:1959–1970.
19. Höök, M. 1984. Cell-surface glycosaminoglycans. *Annu. Rev. Biochem.* 53:847–869.
20. Hopwood, J. J., and A. Dorfman. 1977. Glycosaminoglycan synthesis by cultured human skin fibroblasts after transformation with simian virus 40. *J. Biol. Chem.* 252:4777–4785.
21. Hovingh, P., and A. Linker. 1970. The enzymatic degradation of heparin and heparitin sulfate. III. Purification of a heparitinase and a heparinase from Flavobacteria. *J. Biol. Chem.* 245:6170–6175.
22. Iozzo, R. V. 1985. Biology of disease. Proteoglycans: structure, function, and role in neoplasia. *Lab. Invest.* 53:373–396.
23. Kawakami, H., and H. Terayama. 1981. Liver plasma membranes and proteoglycan prepared therefrom inhibit the growth of hepatoma cells in vitro. *Biochim. Biophys. Acta.* 646:161–168.
24. Kim, J. J., and H. E. Conrad. 1976. Kinetics of mucopolysaccharide and glycoprotein synthesis by chick embryo chondrocytes. Effect of D-glucose concentration in the culture medium. *J. Biol. Chem.* 251:6210–6217.
25. Kraemer, P. M., and R. A. Tobey. 1972. Cell-cycle dependent desquamation of heparan sulfate from the cell surface. *J. Cell Biol.* 55:713–717.
26. Lembach, K. J. 1976. Enhanced synthesis and extracellular accumulation of hyaluronic acid during stimulation of quiescent human fibroblasts by mouse epidermal growth factor. *J. Cell. Physiol.* 89:277–288.
27. Levvy, G. A., and J. Conchie. 1966. Mammalian glycosidases and their inhibition by aldonolactones. *Methods Enzymol.* 8:571–584.
28. Margolis, R. K., C. P. Crockett, W.-L. Kiang, and R. Margolis. 1976. Glycosaminoglycans and glycoproteins associated with rat brain nuclei. *Biochim. Biophys. Acta.* 451:465–469.
29. Marin, G., D. Wynford-Thomas, A. Lamontagne, and D. M. Prescott. 1984. Density-dependent regulation of growth in somatic hybrids between normal chinese hamster fibroblasts and V79-8 ($G1^-$) cells. *Exp. Cell Res.* 155:575–582.
30. Matuoka, K., and Y. Mitsui. 1981. Involvement of cell surface heparan sulfate in the density-dependent inhibition of cell proliferation. *Cell Struct. Funct.* 6:23–33.
31. Matuoka, K., and Y. Mitsui. 1981. Changes in cell-surface glycosaminoglycans in human diploid fibroblasts during *in vitro* aging. *Mech. Ageing Dev.* 15:153–163.
32. Matuoka, K., Y. Mitsui, and S. Murota. 1984. Heparan sulfate enhances growth of transformed human cells. *Cell Struct. Funct.* 9:357–367.
33. Matuoka, K., Y. Mitsui, and S. Murota. 1985. Growth-coupled changes in glucosaminoglycans (heparan sulfate and hyaluronic acid) in normal and transformed human fibroblasts. *Cell Biol. Int. Rep.* 9:577–586.
34. Matuoka, K., Y. Mitsui, S. Murota, and M. Namba. 1985. Actions of exogenous heparan sulfate and hyaluronic acid on growth and thymidine incorporation of normal and transformed human fibroblasts. *Cell Biol. Int. Rep.* 9:815–824.
35. Mian, N. 1986. Analysis of cell-growth-phase-related variations in hyaluronate synthase activity of isolated plasma membrane fractions of cultured human skin fibroblasts. *Biochem. J.* 237:333–342.
36. Mian, N. 1986. Characterization of a high-M_r plasma membrane-bound protein and assessment of its role as a constituent of hyaluronate synthase complex. *Cell Biol. Int. Rep.* 237:343–357.
37. Moolenaar, W. H., L. G. J. Tertoolen, and S. W. de Laat. 1984. Phorbol ester and diacylglycerol mimic growth factors in raising cytoplasmic pH. *Nature (Lond.).* 312:371–374.
38. Moscatelli, D., and H. Rubin. 1977. Hormonal control of hyaluronic acid production in fibroblasts and its relation to nucleic acid and protein synthesis. *J. Cell. Physiol.* 91:79–88.
39. Murota, S., M. Abe, and K. Otsuka. 1977. Stimulatory effect of prostaglandins on the production of hexosamine-containing substances by cultured fibroblasts (3). Induction of hyaluronic acid synthetase by prostaglandin $F_{2\alpha}$. *Prostaglandins.* 14:983–991.
40. Nagai, Y. 1981. Proteoglycans and ageing. *Metabol. Dis.* 18:649–654.
41. Nakamura, T., Y. Nakayama, H. Teramoto, K. Nawa, and A. Ichihara. 1984. Loss of reciprocal modulations of growth and liver function of hepatoma cells in culture by contact with cells or cell membranes. *Proc. Natl. Acad. Sci. USA.* 81:6398–6402.
42. Namba, M., K. Nishitani, and T. Kimoto. 1980. Characteristics of WI-38 cells (WI-38 CT-1) transformed by treatment with Co-60 gamma rays. *Gann (Japanese Journal of Cancer Research).* 71:300–307.
43. Namba, M., T. Kusaka, F. Fukushima, and T. Kimoto. 1984. Differential growth response of normal human diploid fibroblasts and *in vitro* transformed human fibroblasts in serum-free defined culture medium. *Int. J. Cancer.* 34:645–649.
44. Ohya, T., and Y. Kaneko. 1970. Hyaluronidase from *Streptomyces. Biochim. Biophys. Acta.* 198:607–609.
45. Philipson, L. H., and N. B. Schwartz. 1984. Subcellular localization of hyaluronate synthetase in oligodendroglioma cells. *J. Biol. Chem.* 259:5017–5023.
46. Piepkorn, M., H. Carney, and A. Linker. 1985. Growth-related variations in the glycosaminoglycan synthesis of ultraviolet light-induced murine cutaneous fibrosarcoma cells. *Lab. Invest.* 53:187–193.
47. Poole, A. R. 1986. Proteoglycans in health and disease: structures and functions. *Biochem. J.* 236:1–14.
48. Prehm, P. 1983. Synthesis of hyaluronate in differentiated teratocarcinoma cells. Mechanism of chain growth. *Biochem. J.* 211:191–198.
49. Riesenfeld, J., M. Höök, and U. Lindahl. 1982. Biosynthesis of heparan sulfate in rat liver. Characterization of polysaccharides obtained with intact cells and with a cell-free system. *J. Biol. Chem.* 257:7050–7055.
50. Rodén, L., T. Koerner, C. Olson, and N. B. Schwartz. 1985. Mechanisms of chain initiation in the biosynthesis of connective tissue polysaccharides. *Fed. Proc.* 44:373–380.
51. Sisson, J. C., C. W. Castor, and J. A. Klavons. 1980. Connective tissue activation. XVIII. Stimulation of hyaluronic acid synthetase activity. *J. Lab. Clin. Med.* 96:189–197.
52. Sluke, G., D. O. Schachtschabel, and J. Wever. 1981. Age-related changes in the distribution pattern of glycosaminoglycans synthesized by cultured human diploid fibroblasts (WI-38). *Mech. Ageing Dev.* 16:19–27.
53. Smith, J. A., and L. Martin. 1973. Do cells cycle? *Proc. Natl. Acad. Sci. USA.* 70:1263–1267.
54. Stein, G. S., R. M. Roberts, J. L. Davis, W. J. Head, J. L. Stein, C. L. Thrall, J. V. Veen, and D. W. Welch. 1975. Are glycoproteins and glycosaminoglycans components of the eukaryotic genome? *Nature (Lond.).* 258:639–641.
55. Tomida, M., H. Koyama, and T. Ono. 1975. Induction of hyaluronic acid synthetase activity in rat fibroblasts by medium change of confluent cul-

tures. *J. Cell. Physiol.* 86:121–130.

56. Ullrich, S. J., and S. P. Hawkes. 1983. The effect of the tumor promoter, phorbol myristate acetate (PMA), on hyaluronic acid (HA) synthesis by chicken embryo fibroblasts. *Exp. Cell Res.* 148:377–386.
57. Wever, J., D. O. Schachtschabel, G. Sluke, and G. Wever. 1980. Effect of short- or long-term treatment with exogenous glycosaminoglycans on growth and glycosaminoglycan synthesis of human fibroblasts (WI-38) in culture. *Mech. Ageing Dev.* 14:89–99.
58. Winterbourne, D. J., and J. G. Salisbury. 1981. Heparan sulfate is a potent inhibitor of DNA synthesis *in vitro*. *Biochem. Biophys. Res. Commun.* 101:30–37.
59. Yamagata, T., H. Saito, O. Habuchi, and S. Suzuki. 1968. Purification and properties of bacterial chondroitinases and chondrosufatases. *J. Biol. Chem.* 243:1523–1535.
60. Yurchenco, P. D., C. Ceccarini, and P. H. Atkinson. 1978. Labeling complex carbohydrates of animal cells with monosaccharides. *Methods Enzymol.* 50:175–204.
61. Zalitis, J., M. Uram, A. M. Bowser, and D. S. Feingold. 1972. UDP-glucose dehydrogenase from beef liver. *Methods Enzymol.* 28:430–435.