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HYALURONIC ACID SYNTHESIS AND SECRETION BY RAT LIVER FAT STORING CELLS (PERISINUSOIDAL LIPOCYTES) IN CULTURE

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SUMMARY: The ability of rat liver fat storing cells to synthesize and to secrete hyaluronic acid was examined in monolayer cultures. The cells produce [^{3}H] glucosamine-labeled hyaluronic acid, of which about 80 % are secreted into the medium. The synthesis rate per cell (mg DNA) of labeled total glycosaminoglycans and hyaluronic acid in the medium increases significantly with culture time, but hyaluronic acid expressed as fraction of total glycosaminoglycans declines from about 0.70 in early cultures (up to the 4th day) down to 0.20 in advanced cultures. Cycloheximide increases and ß-D-xylopyranoside decreases significantly the fraction of hyaluronic acid in the medium, colchicine up to 5 uM was without effect. The synthesis of hyaluronic acid is a newly recognized function of this special type of sinusoidal liver cells. The results suggest that fat storing cells are likely to be a major source of hyaluronic acid in normal and probably also in injured liver.

Liver fat storing cells (FSC), also known as vitamin A storing cells, perisinusoidal lipocytes and Ito cells (1, 2), are a highly specialized type of nonparenchymal, sinusoidal liver cells devoted primarily to the storage of vitamin A (3, 4). In addition, these cells have been identified by immunofluorescence and -peroxidase staining, light- and electronmicroscopic autoradiography and by metabolic studies in cell culture to be capable of synthesizing a complex spectrum of connective tissue molecules including serveral types of collagens (5-9), laminin (8), and fibronectin (10). Recently, we have established in monolayers of rat liver FSC an active and predominant synthesis and secretion of proteodermatan- and -chondroitin sulfate whereas heparan sulfate is a mainly cell-associated type of sulfated proteoglycans (11).These data together with preliminary results obtained by others (12) support the notion that FSC might be a major source of sulfated GAG in liver. Until now it is not known whether the lipocytes produce HA as well, the only type of GAG not covalently linked to a protein moiety (13) and lacking sulfate groups. HA is present as a minor GAG component in normal liver but occurs in highly elevated concentrations in fibrotic liver tissue (14). This study describes the synthesis and secretion of HA by primary cultures of FSC established from normal rat liver.

Abbreviations: GAG, glycosaminoglycans; HA, hyaluronic acid; FSC, fat storing cells; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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MATERIALS AND METHODS

Isolation and Culture of FSC: The cells were isolated from one year old male Sprague Dawley rats (body weight 500 - 650 g, Lippische Versuchstierzucht, Extertal, FRG) which had free access to a standard laboratory chow diet containing 1500 U vitamin A pro kg and tap water. Nonparenchymal liver cells were isolated by the pronase-collagenase method (6). FSC were purified from the nonparenchymal liver cell suspension by a single-step density gradient centrifugation with Nycodenz^{R} (analytical grade, Nyegaard and Co. AS, Oslo, Norway). The details of the procedure have been described elsewhere (11). Some criteria of isolated FSC are summarized in table 1. FSC were identified by their typical light microscopic appearance, transmission electron microscopy (11), positive indirect immunofluorescence staining for desmin (15, 16), and by vitamin A-specific autofluorescence (11). Viability was tested by trypan blue exclusion. The cells were seeded with a mean plating efficiency of 70 % at a starting density of 0.4 x 10^6 cells/ 2 cm^2 and maintained as monolayers in 24-well culture plates (Falcon, Becton Dickinson, Oxnard, USA) in 1 ml/ 2 cm^2 well of Dulbecco's modification of Eagles medium (Flow Laboratories GmbH, Bonn, FRG) containing 4 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and 10 % (v/v) fetal calf serum (Boehringer GmbH, Mannheim, FRG) in a humidified atmosphere of 5 % CO_2 and 95 % air as described (11). The first medium change was made 24 h after seeding, the following changes of medium were performed routinely in 48 h intervals. Modifications of this procedure are described in the legends of appropriate figures and tables.

Determination of total GAG synthesis: Cells were incubated for 24 h with either D-[6-^{3}H] glucosamine hydrochloride (specific activity 1.3 TBq/mmol) or D-[1-^{14}C] glucosamine hydrochloride (specific activity 2.18 GBq/mmol, Amersham Buchler, FRG). Labeled GAG were measured separately in the medium and cell layer of the cultures. After sucking off the medium the cell layer was washed 3 times with phosphate buffered saline and detached from the well by three cycles of freezing (with liquid nitrogen) and thawing as described (11). Cells were rinsed from the well with 1 ml water, of which a 300 µl fraction was separated for fluorometric determination of DNA. The remaining portion of the cell-free medium (after centrifugation for 6 min, 1 000 g, 4 °C) was proteolysed with papain (EC 3.4.22.2, Boehringer GmbH, Mannheim, FRG) for 48 h at 60 °C in 0.1 M sodium acetate, pH 6.2 (11). Proteolysis was terminated by boiling the samples for 5 min. The supernatant obtained after centrifugation (4 500 g, 10 min, 4 °C) was mixed with unlabeled GAG (HA, heparin, chondroitin 4-sulfate, chondroitin 6-sulfate, all from Sigma Chem. Comp., St. Louis, USA) used as carrier (each at a final concentration of 0.12 mg) and total GAG were bound during an incubation of 45 min to a batch of DEAE-sephacel (Pharmacia Fine Chem., Uppsala, Sweden) equilibrated with 0.1 M sodium acetate, pH 6.2 (17). After washing the resin 7 times with the same buffer total GAG were eluted with 2 ml of 2.2 M NaCl and precipitated for 12 h at room temperature with 4 ml absolute ethanol. After centrifugation (4500 g, 20 min) the sediment was washed 2 times each with 3 ml of absolute ethanol to remove NaCl. The final sediment was dissolved in 1 ml water, of which a 100 µl aliqot was counted for total GAG radioactivity.

Determination of HA synthesis: The remaining 900 µl of total GAG were subjected to enzymatic digestion with hyaluronate lyase (EC 4.2.2.1, from streptomyces hyalurolyticus, Seikagaku Co., Tokyo, Japan) (18). Portions of 200 µl were incubated for 3 h at 60 °C (alternatively for 24 h at 37 °C) in 33 mM sodium acetate, pH 5.0 with 5 TRU hyaluronate lyase (dissolved in 0.1 M NaCl, 12.5 TRU/ml) in a total reaction volume of 1 ml. Control incubations were done with a similar volume of 0.1 M NaCl instead of enzyme. All incubations were done in duplicates. After termination of the reaction by cooling in ice those GAG not degraded by hyaluronate lyase were precipitated for 16 h at room temperature with 4 volumes of sodium acetate saturated ethanol and centrifuged (4 500 g, 10 min). The sediment was dried, dissolved in 250 µl water, of which 200 ul were counted for radioactivity. The amount of labeled HA was calculated from the difference between control and enzyme incubation. In some experiments HA in the medium was isolated and characterized by chromatography on DE-52 cellulose (Whatman Chem. Sep., England) and sepharose Cl-6B (19) as described in detail in figure 1.

General techniques: Cells were quantitated either by counting in a hemocytometer or by fluorometric determination of DNA (20). Hexuronic acid was measured by the carbazole reaction (21), amino sugars were determined after acid hydrolysis of GAG with an automated liquid chromatography system (Biotronic LC 5001, Munich, FRG).

Statistical analysis: Differences of $n \geq 4$ independent determinations were tested with the Scheffé multiple range test for pairwise comparisons of means; $p < 0.05$ was accepted to be statistically significant (22).

Table 1. Some criteria of fat storing cells isolated from 1-year old male Sprague-Dawley rats

		mean values ± S.D.	range
cells x 10^6/liver	(n = 42)	46.8 ± 25.2	0.5 - 91
viability (%)	(n = 25)	91.0 ± 6.2	84 - 96
purity (%)	(n = 42)	92.0 ± 7.0	65 - 100

RESULTS AND DISCUSSION

Characterization of FSC cultures: The criteria of freshly isolated cells are summarized in table 1. Some cell types (endothelial and Kupffer cells, damaged hepatocytes, lymphocytes) contaminating fresh isolations were removed with the first change of medium one day after seeding. The cultures used for determination of HA had a purity of at least 97 % as determined by the proportion of desmin-positive cells. FSC were in a preconfluent state if not otherwise stated. The cells showed the typical morphological characteristics like numerous lipid droplets around the nucleus and irregular cellular extensions (2).

Identification of HA in the medium and cell layer of FSC: The medium of cells incubated with [^{3}H] glucosamine was proteolysed and subjected to DE-52 cellulose and sepharose Cl-6B chromatography (19). The labeled material was eluted from the ion exchange column with peak fractions at 0.11 and 0.25 M NaCl (Fig. 1A). The latter fraction applied to the sepharose column was eluted near the void volume (K_{av} : 0.01) (Fig. 1B). When this fraction was digested with hyaluronate lyase prior to gel chromatography the large molecular weight peak disappeared and radioactivity was now eluted in two low molecular weight portions with K_{av} of 0.59 and 0.76, respectively (Fig. 1B). Peak 1 of the DE-52 column was eluted at the total volume (V_t) from the sepharose column and represents free rest radioactivity. [^{35}S] sulfate labeled medium GAG showed clearly distinct elution profiles on both columns, which were insensitive to degradation with hyaluronate lyase (Fig. 1). Very similar results were obtained with proteolysates of the cell layer (not shown). These results together with the analytical criteria of the enzymatic assay showing high specificity and efficiency for HA let us conclude that FSC are synthesizing and secreting hyaluronate (Table 2).

Synthesis and secretion of HA by FSC: Cells incubated up to 36 h incorporated linearly [^{3}H] glucosamine into total medium GAG. The fraction of HA rised sharply at 6 h and declined continuously thereafter. The amount of labeled HA increased up to 24 h (Fig. 2). At 24 h the cell/medium ratios of total GAG and HA were 0.24 ± 0.03 (n = 8) and 0.20 ± 0.05, respectively. The rate of [^{3}H] glucosamine incorporation by FSC into total medium GAG and HA increased significantly with culture time (Fig. 3). This is also observed if the incorporation is corrected for the increase in total cellular DNA (Fig. 3B and Fig. 4). The production of HA expressed as fraction of total GAG declined between the 2nd and 9th culture day by about 80 % (from 0.73 to 0.16) and , therefore, the maximum increase of total hyaluronate production during this time is smaller (2.3 fold) than that of total GAG (5.9 fold) (Fig. 4). It can be seen from fig. 3 and 4 that HA production is dependent on the addition of fresh fetal calf serum since a strong rise was observed after each medium change and the synthesis rate of HA is very low in the absence of fetal calf serum (not shown). Independent on the culture day about 80 % of newly formed HA were secreted into the

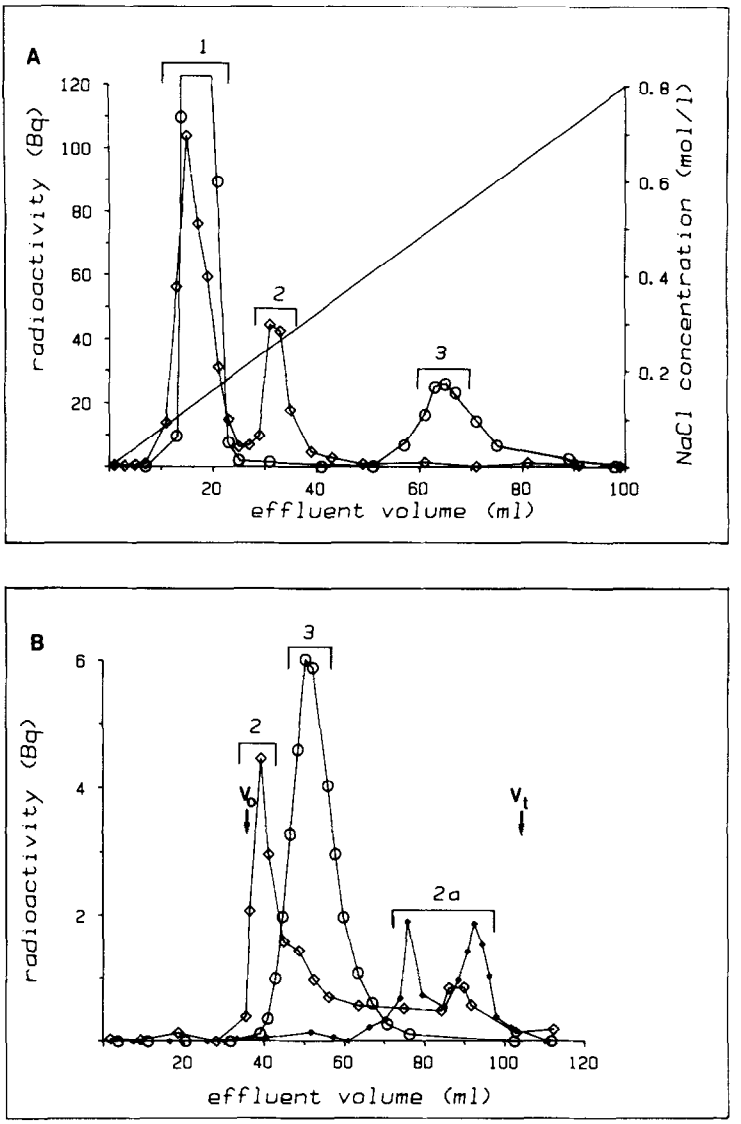
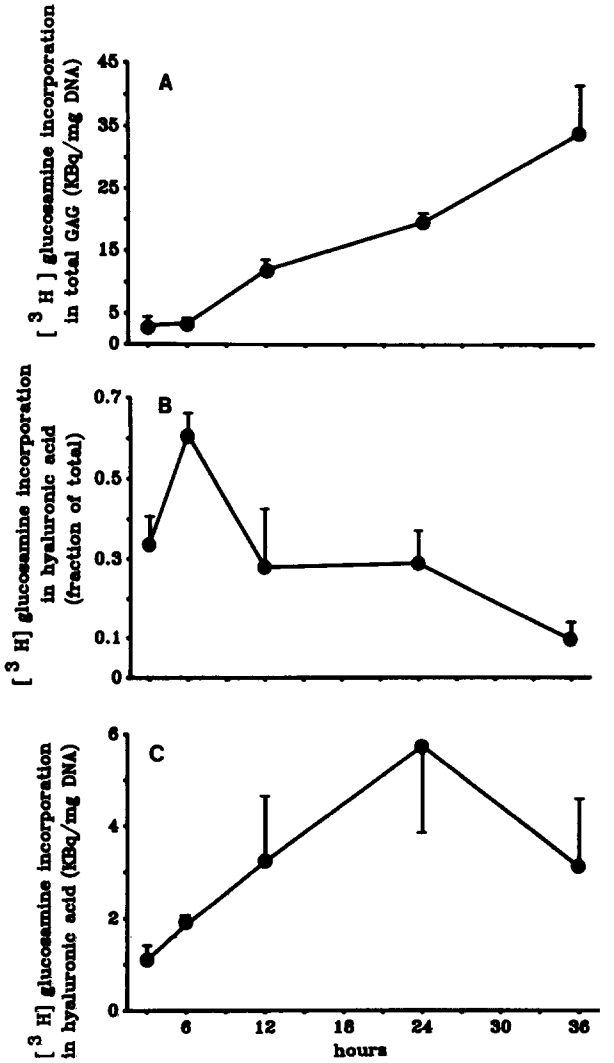


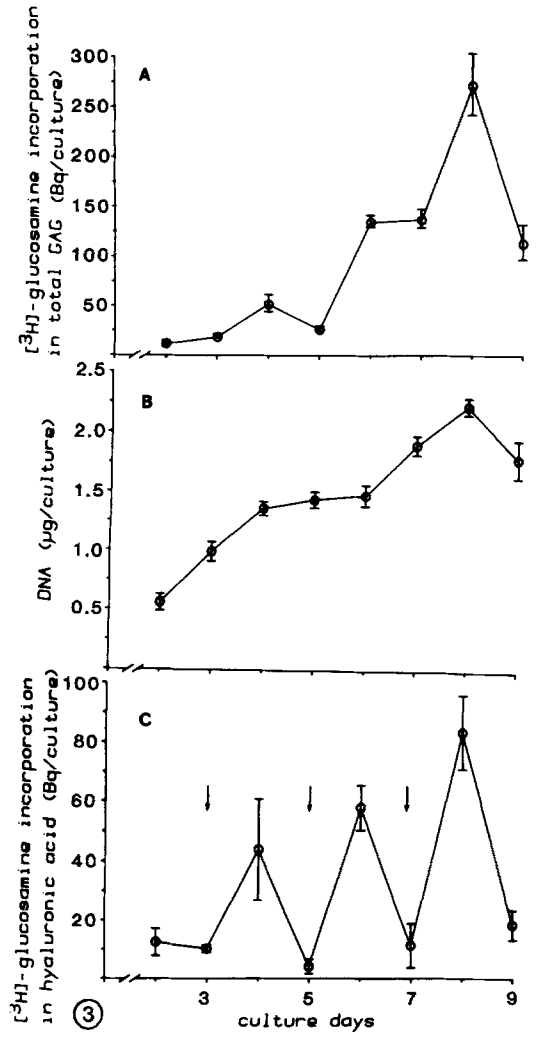
Figure 1. Ion exchange (A) and molecular sieve (B) chromatography of medium GAG of FSC. Cells seeded at a density of 0.5 x 10^6 cells/ 2 cm^2 were maintained under standard conditions for 4 days. Between the 3rd and 4th day GAG were labeled for 24 h with 15 µCi [^{3}H] glucosamine per well. Medium was harvested, digested with papain and dialysed for 20 h at 4 °C against 10 volumes of 10 mM Tris-HCl, pH 7.8, 1.5 mM $CaCl_2$. The material was applied to a DE-52 cellulose column (A, 0.8 x 70 cm), washed with 10 volumes of 10 mM Tris-HCl, pH 8.4 and eluted with a linear 0 - 0.8 M NaCl gradient. The radioactivity in the 1 ml fractions was counted. [^{35}S] sulfate labeled medium GAG obtained from another FSC incubation was processed in a similar way. [^{3}H] glucosamine labeled material eluted with two peak fractions (1 and 2) at 0.11 and 0.25 M NaCl, respectively; [^{35}S] sulfate labeled GAG eluted with two peaks (1 and 3) at 0.14 and 0.57 M NaCl, respectively. The fractions of peak 2 were pooled, dialysed against water and divided in 2 aliqots. Portion 1 was lyophylised, dissolved in column buffer (0.13 M Tris-HCl, pH 7.2, 1 mM EDTA, 1 mM PMSF, 0.1 % SDS) and applied to a sepharose Cl-6B-column (B, 1.6 x 51 cm). The column was eluted with the same buffer (flow rate 12 ml/h) and the radioactivity in 1 ml fractions was monitored (◇—◇). Portion 2 of peak 2 was lyophylised, dissolved in 500 µl water, digested with hyaluronate lyase, precipitated with sodium acetate saturated ethanol, centrifuged and washed twice with absolute ethanol. The dried sediment was dissolved in column buffer and subjected to gel permeation chromatography as described above (◆—◆). The [^{35}S] labeled peak 3 of DE-52 cellulose chromatography was subjected to sepharose Cl-6B chromatography in a way similar to that of portion 1 of peak 2. The radioactivity in the effluent fractions was counted (O—O). The K_{av} of the peak fractions was calculated.

Table 2. Analytical criteria of the enzymatic assay of hyaluronic acid (HA)

	input fraction (nmol uronic acid)	recovered fraction of GAG (nmol uronic acid)	(% of input)
recovery of HA	42.2	39.6	94
degradation efficiency of HA and precision	39.6	5.2 ± 0.46 (8.8 %)	13
specificity			
chondroitin 4-sulfate	38.7	37.3	96
chondroitin 6-sulfate	47.1	43.5	92
dermatan sulfate	44.2	44.2	100
heparin	72.1	61.1	85

Defined amounts of HA and chondroitin sulfates (all from Sigma Chem. Comp.) corrected for slight impurities detected by amino sugar analysis were subjected to the enzymatic assay of HA described in Materials and Methods. Possible cross impurities of heparin and HA were not tested and, therefore, can not be excluded. The precision is given as ± S.D. and in parenthesis as coefficient of variation.





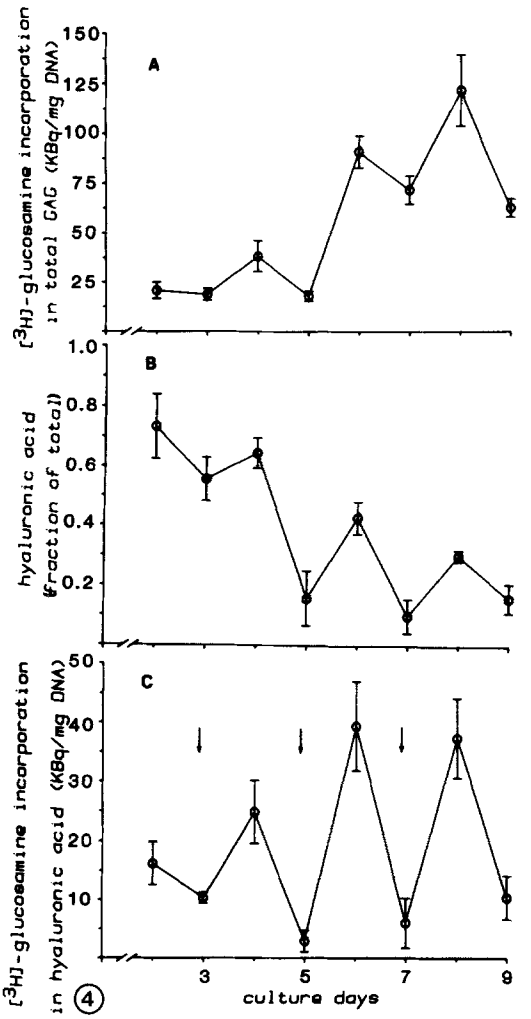


Figure 3. Rates of incorporation per culture well of [^{3}H] glucosamine into total GAG (A) and HA (C) of medium by monolayers of FSC in relation to culture time. Cells were seeded at a density of 0.4 x 10^6 cells/ 2 cm^2 well and maintained under standard culture conditions. Medium was changed in 48 h intervals at the days indicated by arrows. Beginning with the 1st day the cultures were incubated at each day for always 24 h with 5 µCi [^{3}H] glucosamine per ml medium. The medium was harvested, proteolysed and subjected to the isolation of total GAG (A) and HA (C), respectively. The amount of HA expressed as fraction of total GAG is shown in B. Mean values ± S.D. of 4 experiments are shown.

Figure 4. Rates of incorporation per mg cellular DNA of [^{3}H] glucosamine into total GAG (A) and HA (C) of medium by monolayers of FSC in relation to culture time. The culture conditions are identical with those described in figure 3. The changes of the DNA content of the cultures are shown in B.

Figure 2. Time course of [^{3}H] glucosamine incorporation into total GAG (A) and HA (C) of medium by monolayers of FSC. The amount of HA expressed as fraction of total labeled GAG is shown in B. The cells were seeded at a density of 0.4 x 10^6 cells/ 2 cm^2 well and kept under standard incubation conditions. The medium was changed at the 3rd day and 24 h later the cells were exposed for various periods with 5 µCi [^{3}H] glucosamine per ml medium. At the times indicated medium was harvested, proteolysed and subjected to the isolation of total GAG (A) and HA (C), respectively and the DNA content of the cultures was measured. Mean values ± S.D. of 4 experiments are shown.

Table 3. Effects of colchicine, cycloheximide, and ß-D-xylopyranoside on the synthesis of total GAG and HA in the medium of fat storing cells

Treatment of fat storing cells	[^{3}H] glucosamine incorporation into medium glycosaminoglycans (KBq/mg DNA)		
	total glycosaminoglycans	hyaluronic acid	hyaluronic acid (fraction of total)
none	350 ± 107	115 ± 32	0.35 ± 0.06
colchicine			
2 µM	350 ± 139	80 ± 56	0.23 ± 0.06
5 µM	350 ± 47	81 ± 26	0.23 ± 0.05
cycloheximide			
0.5 µg/ml	168 ± 33*	136 ± 13	0.82 ± 0.08*
ß-xylopyranoside			
0.5 mM	1080 ± 132*	54 ± 22	0.05 ± 0.02*

Cells were seeded at a density of 0.25 x 10^6 cells/ 2 cm^2 and maintained under standard conditions. At the 5th day cells were exposed for 24 h with the respective compounds and simultaneously labeled with [^{3}H] glucosamine. Total labeled GAG and HA in the medium were determined and refered to the DNA content of the culture. Mean values ± S.D. of 4 determinations are shown. Statistically significant changes in comparison with untreated cultures are indicated by asterisks.

medium. Cycloheximide reduced significantly the incorporation of [^{3}H] glucosamine into total GAG, which are synthesized as complexe proteoglycans (11), but did not alter the incorporation into HA, which is not covalently linked to a carbohydrate chain acceptor protein (13) and, therefore, its synthesis is not dependent on a functioning (core) protein synthesis machinery (23, 24). Thus, the fraction of HA was significantly elevated from 0.36 in control incubations to 0.82 in cycloheximide treated cultures (Table 3). The reverse effect was obtained by ß-xyloside, which acts as an artifical initiator of polysaccharide chain elongation of proteoglycans in competition with the endogenous, xylosylated core protein (25). The compound stimulated greatly the formation of GAG chains and reduced significantly (down to 0.05) the fraction of HA (Table 3). These results obtained with FSC confirm the proposed biosynthetic mechanism of HA synthesis, which does not involve initiation on a core protein (26). Colchicine up to 5 µM did not hinder the secretion of total GAG and HA, respectively (Table 3).

The data reported here present evidence that FSC in culture synthesize and secrete HA as a substantial fraction of total GAG, which suggests that these cells have the potential to be an important source of HA in liver. Production of HA in liver parenchymal cells has been claimed but in early cultures of these cells HA is synthesized only as a very minor fraction (27). The time-dependent increase in the synthesis of HA in hepatocyte cultures (27) may in fact be caused by the presence of a small fraction of contaminating, rapidly proliferating fat storing cells. This pitfall has been demonstrated recently in studies on collagen synthesis in hepatocyte cultures (28). HA synthesis in liver endothelial cells and Kupffer cells has been not demonstrated so far but circulating HA is efficiently taken up by receptor-mediated endocytosis and metabolized by sinusoidal endothelial cells (29, 30). Therefore, FSC may be the only cell type in liver capable of synthesizing and secreting significant quantities of HA. The significance of HA formation by FSC, either for their metabolic functions of for some properties of normal liver, is not known. In injured

liver tissue, in which FSC strongly proliferate and transform into fibroblast (-like cells) (31, 32) this cell type might contribute a major fraction to the largely increased amount of HA found in the experimental and human fibrotic liver extracellular matrix (14).

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