The synthesis of total and specific glycosaminoglycans during development of experimental liver cirrhosis

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Summary. During hepatic fibrogenesis induced by long-term administration of thioacetamide, the synthesis of chondroitin 4,6-sulfate and hyaluronic acid was strongly enhanced; the formation of heparan sulfate comprising at least 70% of total liver GAG synthesis and of a keratan-sulfate-like fraction was stimulated 1.7fold. Formation of dermatan-sulfate in liver could not be detected.

Chronic liver injury is associated with a progressive accumulation of the components of hepatic connective tissue including collagen, glycosaminoglycans (GAG) and structural glycoproteins[2–4]. The excessive deposition of collagen is attributed to both an increased synthesis[5,6] and a diminished rate of degradation[7]. Although early autoradiographic and biochemical studies demonstrated that the in vivo incorporation of $(^{35}SO_4)^{2-}$ into GAG is stimulated also during development of liver fibrosis[8,9], it is, however, very hard to quantitate rates of synthesis of GAG in the intact animal and by the use of ^{35}S as tracer as discussed before[10]. For these reasons we studied the synthesis of total and specific types of GAG in explants from chronically injured liver at various stages of fibrogenesis.

The proportional rates of synthesis of specific types of hepatic GAG during the development of liver cirrhosis

Duration of liver injury (months)	Percentage of total GAG synthesis: Heparan sulfate + heparin	Chondroitin 4-sulfate + chondroitin 6-sulfate	Hyaluronic acid	'Keratan sulfate'
0	90.0	9.5	0.2	0.3
1	86.2	12.8	0.7	0.4
2.5	87.6	10.4	1.6	0.3
5	87.7	10.8	1.2	0.3
7	85.3	12.0	2.3	0.4

Liver slices were incubated as described in figure 1. The rates of incorporation of (^{14}C)-glucosamine into individual GAG were assayed (figure 2) and expressed as percentage of the incorporation into total GAG.

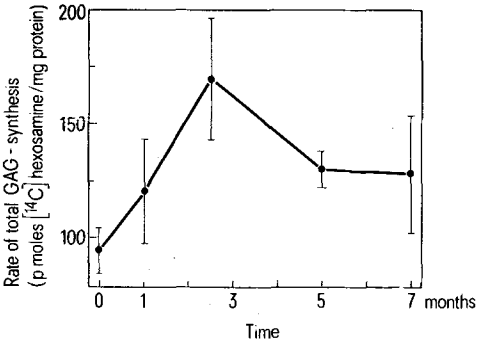

Fig. 1. Synthesis of total hepatic GAG during the development of liver cirrhosis. Male rats received water containing 0.03% (w/v) of thioacetamide for a total of 7 months. Control rats were housed similarly but thioacetamide was omitted from the drinking-water. 1, 2.5, 5 and 7 months after the beginning of treatment, the animals were sacrificed and slices of their livers incubated for 6 h at 37 °C in medium containing 2.5 μCi of D-(1-^{14}C) glucosamine. Thereafter the slices were washed in cold acetone and total GAG isolated from the proteolysed material and its radioactivity determined. Together with the treated rats, one control rat liver was analyzed identically for the synthesis of GAG. Each point represents the mean ± SD of 3–6 experiments. 0 months gives the mean ± SD of all control experiments performed together with the treated livers.

Methods. Male Sprague-Dawley rats (initial b.wt 180 to 200 g) were fed with a standard rat diet and tap water containing 0.03% (w/v) of thioacetamide[11]. Control rats received water without thioacetamide. 1, 2.5, 5 and 7 months after the beginning of the treatment 3–6 rats were sacrificed, 5 slices of 0.5 mm thickness (80–120 mg wet wt) prepared from each liver and incubated separately for 6 h at 37 °C in 3 ml of Dulbeccos modification of Eagles medium containing 2.5 μCi (275 nmoles) of D-(1-^{14}C)glucosamine-HCl under 95% O_2–5% CO_2 as described in detail previously[10]. At the end of incubation, the slices of each liver were pooled, washed in cold acetone, proteolysed with papain and total (^{14}C)-labeled GAG were isolated. The following has been described before[10]: The source of the materials; the quantitation of the synthesis of total GAG; the identification of specific types of GAG and quantitation of their rates of synthesis by a combination of chromatography on Dowex 1 × 2 and cetylpyridinium chloride (CPC)-cellulose, enzymatic digestion, degradation by nitrous acid and determination of (^{14}C)-amino-sugars; the isolation, characterization and quantitation of a keratan sulfate-like fraction from the CPC-soluble material and the measurement of the uptake of (^{14}C)-glucosamine by cells in chronically injured liver slices. The state of hepatic fibrosis and cirrhosis was examined by routine histological procedures.

Results. The incorporation of (^{14}C)-glucosamine into total liver GAG was increased by 30% 1 month after the beginning of the treatment with thioacetamide, reached its maximum after 2.5 months (167 pmoles [^{14}C]-hexosamine

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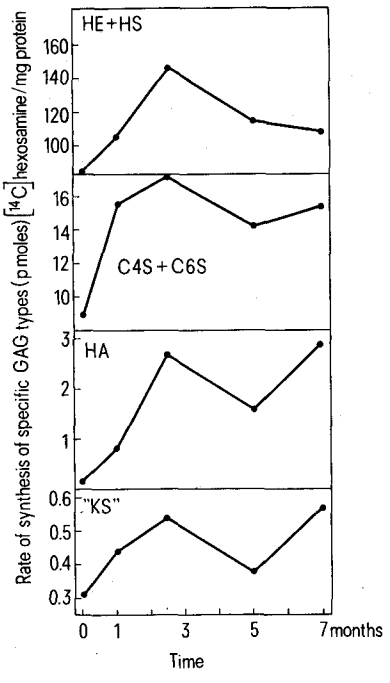

Fig. 2. Synthesis of specific types of hepatic GAG during the development of liver cirrhosis. Slices from chronically injured liver were incubated as described in figure 1. Specific GAG were identified and their rates of synthesis quantitated by a combination of chromatography on Dowex 1X2, enzymatic digestion, degradation by nitrous acid and detection of (^{14}C)-aminosugars. Each point represents the mean ± SD of 3–6 separate experiments, 0 months shows the incorporation into GAGs from control rat liver as described in figure 1. HE + HS, Heparin and heparan sulfate; C4S + C6S, chondroitin 4- and 6-sulfate; HA, hyaluronic acid; 'KS', keratan-sulfate-like fraction.

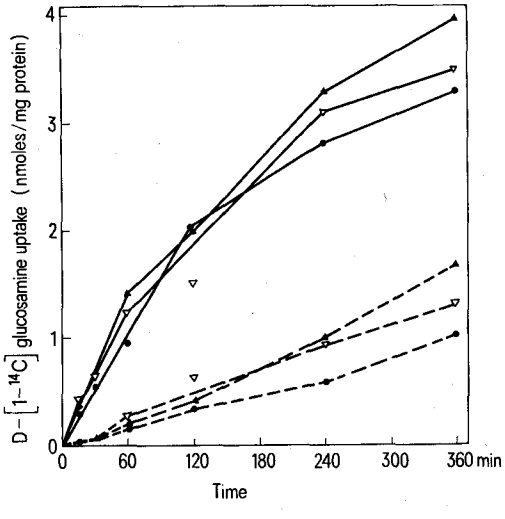

Fig. 3. Time-course of the uptake and incorporation into acid-insoluble material of (^{14}C)-glucosamine by cells in normal and chronically injured rat liver explants. Slices were incubated as described in figure 1. At the times indicated, the explants were removed, washed free of extracellular (^{14}C)-glucosamine, homogenized and the activity in the trichloroacetic acid-soluble (—) and insoluble (---) material determined. The points are the mean of duplicate experiments. Normal liver (▽—▽, ▽---▽); liver treated for 2 months (●—●, ●---●) and 5 months (▲—▲, ▲---▲) with thioacetamide.

incorporated/mg protein × 6 h, stimulation by 77%) and continued thereafter at a level elevated by about 40% (figure 1).

The rate of synthesis of individual GAG was very heterogeneous. Nearly 90% of the label incorporated into total GAG was found in a chondroitinase ABC-resistent but nitrous acid-labile fraction which contained predominantly heparan sulfate (at least 70%) and far less, if any, heparin[10]. The time-course and degree of stimulation of the synthesis of these types of GAG resembled closely that of total GAG synthesis (figure 2). The formation of chondroitin 4- and 6-sulfate comprised 10% of total GAG synthesis and was enhanced by 70% and nearly 100% 1 and 2.5 months, respectively, after onset of chronic liver injury. Thereafter synthesis remained elevated by about 70%. A strong and time-dependent 5- to 20fold increase in the synthesis of hyaluronic acid became evident during the development of liver cirrhosis (0.2–2.3% of total GAG synthesis). The incorporation of (^{14}C)-glucosamine into the keratan sulfate-like fraction was low (0.3% of total GAG synthesis) and increased 1.7fold during chronic hepatic damage (figure 2). Incorporation of the isotope into dermatan sulfate was not detectable. The relative rates of synthesis of specific types of GAG and their alteration during progression of liver fibrosis are shown in the table.

The time-course of the uptake of (^{14}C)-glucosamine by cells in slices from normal liver and liver injured for 2 and 5 months with thioacetamide was not different (figure 3). The most characteristic histological signs were a marked inflammation and hepatocellular necrosis during the first 2 months of treatment with a progressive fibrosis thereafter which resulted after 5–7 months in to a fully developed liver cirrhosis.

Discussion. The rate of synthesis of individual GAG in normal and diseased liver was found to be very unequal. The strong prevalence of the synthesis of heparan sulfate which is not only the major component of rat and human liver GAG[4,12] but also present at the surface of liver cells[13] and within liver mitochondria[14] might be connected with some of its proposed functions[15], e.g. in the regulation of cell proliferation[16]. Our failure to show any incorporation of (^{14}C)-hexosamine into dermatan sulfate might be due to the inappropriate conditions used, or it might reflect an exceptional metabolism of this type of GAG. Dermatan sulfate cannot be degraded by rat liver lysosomes[17]; therefore its accumulation in cirrhotic liver tissues[12] indicates the transition from the reversible to the irreversible phase[18]. Furthermore, recent studies on the microheterogeneity of dermatan sulfate showing a disproportionate change of its various subfractions in response to liver injury suggest that the metabolism of liver dermatan sulfate must be under different enzymatic control[19]. Therefore it seems reasonable to assume that

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the synthesis of this type of hepatic GAG is unmeasurably low, but its quantity gradually increases in liver fibrosis as a consequence of catabolic inertness and extreme reduced turnover rate. Although the existence of keratan sulfate in liver has not been proved so far[3], our data suggest that normal and diseased liver is capable of synthesizing this special type of GAG.

During chronic liver injury the synthesis of specific GAG increased disproportionately (figure 2, table). However, the degree of stimulation was much lower than demonstrated previously by the in vivo incorporation of $(^{35}SO_4)^{2-}$ [20]. The factors controlling the formation of GAG in chronically injured liver are not known. The changing population of cell types during fibrogenesis[21] in particular the increased number of mast cells[22] and the ability of the hepatocyte to synthesize collagen[23] necessitate cell-type differentiated studies of GAG synthesis in liver. Furthermore, it remains to be established whether the augmentation of GAG synthesis is a feature of parenchymal regeneration during chronic injury rather than a disease specific phenomenon[10].

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Mn^{2+} electron spin resonance studies on ATP phosphoribosyltransferase from *E. coli*[1]

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Summary. ESR binding studies of Mn^{2+} with each of the substrates and products suggests that substrate bridge complexes are formed in the reaction. This prediction is confirmed, comparing Mn^{2+} + ADP and Mn^{2+} + ADP + enzyme spectra.

ATP phosphoribosyltransferase (EC. 2.2.2.17) is the first enzyme of the histidine biosynthetic pathway, and it is allosterically inhibited by the end product, histidine[2]. It catalyses the reversible reaction of ATP and phosphoribosylpyrophosphate (PRibPP) to yield phosphoribosyl-ATP (PRibATP) and pyrophosphate[2]. The reaction requires Mg^{2+}[2], but as in most processes with participation of nucleotides, Mn^{2+} can substitute for Mg^{2+}[3]. Studies in our laboratory with the E. coli enzyme have dealt with conformational changes and association-dissociation processes effected by substrates and other ligands. We have reported on fluorescence data[4], association-dissociation[5], steady state kinetics[6] and nitroxide spin labelling of the enzymes[7]. In this paper, an ESR study has been carried out, in which use is made of the Mn^{2+} cation to obtain information on the environment of the metal during the catalytic reaction.

ESR parameters of aqueous Mn^{2+} solutions*

Ligand (mM)	$\frac{\Sigma H_i}{6}$ (G)	Relative intensity $\frac{\Sigma Y_i}{6}$	$\frac{\Sigma a_i}{6}$ (G)
–	29	346	96
ADP (0.3)	35	63	97
ADP (0.15) Enzyme (9 mg/ml)	40	54	97
ADP (0.15) Enzyme (9 mg/ml) Histidine (1)	38	68	94
ADP (0.3) PRibPP (0.3)	–	< 5	–
ATP (0.3)	52	21	97
ATP (0.15) PRibPP (0.15) Enzyme (9 mg/ml)	42	62	97

* The $MnCl_2$ concentration was 0.1 mM: in spectra in the presence of enzyme it was 0.05 mM. At these levels, the relative intensity of the signal is a function of the manganous ion concentration. ΔH_i is the peak-to-peak width of the first derivative ESR signal, Y_i is the peak height, a_i is the hyperfine splitting constant.

Materials and methods. ATP phosphoribosyltransferase was purified basically according to the method of Parsons and Koshland[8], as previously described[5], including a Sephadex G-200 step. Protein was determined by the method of Lowry et al.[9], with insulin standards. PRibPP (from Sigma) was used without further purification. PRibATP was prepared according to Klungsoyr and Kryvi[10], including a Sephadex G-10 final step; the purity was tested with ATP phosphoribosyltransferase, using a value of 3100 M^{-1} cm^{-1} for the extinction coefficient of PRibATP at pH 8.0 and 290 nm[11].

ESR measurements were carried out in a JEOL JM-PE-3 spectrometer, working at 23 °C and at X band (9.53 GHz), using a modulation amplitude of 4 G and microwave power of 6 mW. The solutions were contained in a quartz cylindrical cell, standard for the JEOL equipment. The solvent was in all cases 50 mM Tris-HCl buffer, pH 8.0. Analytical grade manganous chloride was obtained from Merck.

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