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INFLUENCE OF GLYCOSAMINOGLYCANS ISOLATED FROM THE RAT LIVER WITH VARIOUS STAGES OF CARBON TETRACHLORIDE-INDUCED FIBROSIS UPON COLLAGEN FIBRILLOGENESIS *IN VITRO*

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ABSTRACT

The influence of glycosaminoglycans (GAG) on collagen fibrillogenesis *in vitro* was investigated. GAG and collagen were isolated from the liver of rats treated with carbon tetrachloride at various time intervals (3-16 weeks). It was found that GAG obtained from the fibrotic liver accelerated collagen fibril formation. This effect was more evident in a solution of type I collagen than type III collagen. Studies on the liver samples showed that collagen was relatively slowly liberated in a solution of acid from the fibrotic liver as compared to normal tissue. The observed phenomena were related to the stage of fibrosis, i.e., to the duration of carbon tetrachloride treatment. Therefore, a role for GAG alteration in the development of hepatic fibrosis is suggested.

Keywords: Hepatic fibrosis, collagen, fibrillogenesis, glycosaminoglycan.
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INTRODUCTION

Connective tissue is a complex dynamic unit of cells and extracellular matrix, and these components constantly interact with one another. The extracellular matrix contains a number of macromolecules, including the collagens and proteoglycans. The latter consist of nonbranched chains of repeating disaccharides, known as glycosaminoglycans (GAG), bound to a protein core.[1-5] Proteoglycans and GAG influence the fibrillogenesis of collagen, i.e., control the size, orientation and rate of formation of collagen fibers.[6-22] This phenomenon has been investigated mostly *in vitro*, although it is believed that similar interactions exist in tissue.

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It is generally accepted that accumulation of collagen in the liver is a key phenomenon in chronic liver disorders. Hepatic fibrosis is widely used in animal models for investigations concerning the mechanism of connective tissue deposition in the liver. Collagen is a heterogenous class of proteins, and accumulation of an excess of collagen in the liver is accompanied by evident quantitative changes in the collagens.[23-29] Changes in GAG content and composition were also found in the liver of humans with cirrhosis and animals with experimental hepatic fibrosis.[30-42] It was found that collagen-GAG interactions depend on collagen type and the presence of individual GAGs.[8,23,43] Thus it is possible that some changes of the hepatic connective tissue can be elucidated by disturbed collagen-GAG interactions.

In the present study, GAG and collagen were isolated from the liver of rats with various stages of hepatic fibrosis and the effect of GAG on collagen fibrillogenesis *in vitro* was investigated. Additionally, the kinetics of collagen solubilisation in the tissue were reported as a relative

Table I. Effect of glycosaminoglycans on collagen fibrillogenesis–absorbance of the forming gel opacity after 21 min.

Period of treatment (weeks)	Collagen type I		Collagen type III		Natural mixture of collagen types	
	control group	CCl_4-treated group	control group	CCl_4-treated group	Control group	CCl_4-treated group
0	0.68 ± 0.02		0.15 ± 0.01		0.55 ± 0.02	
3	0.71±0.02	0.74±0.03	0.16±0.01	0.20±0.02**	0.55±0.02	0.59±0.02
6	0.70±0.02	0.79±0.03*	0.18±0.01	0.24±0.02*	0.54±0.03	0.63±0.02*
9	0.71±0.02	0.82±0.03*	0.18±0.02	0.29±0.02*	0.56±0.02	0.66±0.02*
12	0.73±0.02	0.84±0.02*	0.20±0.02	0.34±0.02*	0.56±0.02	0.70±0.04*
15	0.73±0.02	0.85±0.02*	0.20±0.02	0.40±0.02*	0.57±0.02	0.73±0.03*
18	0.73±0.02	0.87±0.02*	0.21±0.02	0.45±0.02*	0.57±0.02	0.78±0.04*

Statistical significance of the differences relative to the corresponding controls: *P < 0.001
**P < 0.01

measure of collagen-amorphous ground interactions.

MATERIALS AND METHODS

Animals and induction of fibrosis

Male rats of Wistar strain, obtained from the Central Experimental Animal House of the Silesian University School of Medicine were used. Three month old animals, weighing 210g were divided into experimental and control groups. Animals were fed with standard pellet chow and had free access to tap water. Rats of the experimental group were given carbon tetrachloride in olive oil solution (1:1 v/v) in a dose of 1 cm^3/kg of body weight. Injections were given twice a week subcutaneously. Control animals were given injections of olive oil only. Rats were killed by decapitation after 3-18 weeks of treatment at three week intervals. Liver samples were obtained at autopsy, and were used for isolation of collagen and GAG as well as for kinetic studies of collagen solubilisation.

Isolation of collagen

Collagen was isolated by using the method described by Grasedyck et al.[44] Type I and type III collagen were obtained from the liver tissue as described by Madri and Furtmayr.[45] Collagen preparations were purified and lyophilized.

Isolation of glycosaminoglycans

GAGs were isolated from the liver via the methods of Scott[46] and Svejcar and van Robertson[47] with some further modification.[48]

Collagen fibril formation after addition of GAG *in vitro*

The method described by Nemeth-Csóka[13] was used. A solution of collagen (0.2%) in 0.1M Tris-HCl buffer (pH= 7.4) supplemented with 0.15M potassium chloride was incubated at 32°C and after 10 min of incubation the glycosaminoglycan solution was added.

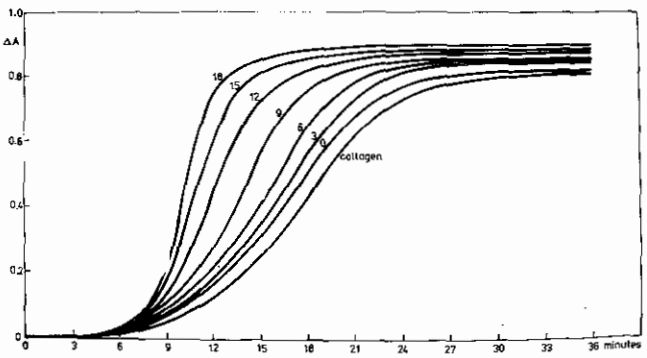

Fig. 1. Kinetics of type I collagen fibrillogenesis in the presence of GAG from livers with various stages of fibrosis.

Solubilisation of collagen

The kinetics of collagen solubilisation were measured as described by Dumitru and Garrett,[49] and Butzow and Eichhorn.[50]

Hydroxyproline

The collagen content of extracted fractions and purified preparations was measured as hydroxyproline content. Hydroxyproline was determined by the method described by Drozdz et al.[51] based on the reaction of Stagemann.[52]

Uronic acids

The colorimetric method of Dische,[53] modified by Bitter and Muir[54] was used for determination of uronic acids.

Table II. Collagen solubilisation rate –relative values expressed as percent of the amount of collagen solubilised after 48 hr.

Period of treatment (weeks)	After 2hr of solubilisation		After 8hr of solubilisation	
	control group	CCl_4-treated group	control group	CCl_4-treated group
0	48.276±3.827		96.552±2.413	
3	49.206±3.746	36.000±3.672****	95.238±2.746	82.667±2.725***
6	48.611±3.896	27.586±3.812**	94.444±3.166	71.264±3.008*
9	45.569±4.112	23.636±4.127**	96.202±4.567	64.545±4.276*
12	47.191±3.562	18.750±3.542*	93.258±4.982	56.250±3.907*
15	46.316±4.572	10.828±4.056*	93.684±5.025	42.038±5.545*
18	45.872±5.233	8.205±4.453*	92.661±6.458	31.795±7.623*

Statistical significance of the differences relative to the corresponding controls: *P < 0.001
**P < 0.005
***P < 0.01
****P < 0.05

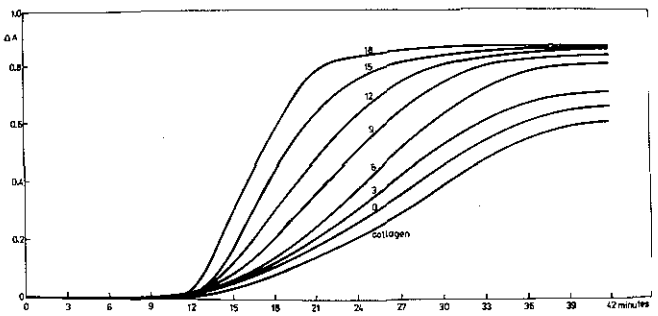

Fig. 2. Kinetics of type III collagen fibrillogenesis in the presence of GAG from livers with various stages of fibrosis.

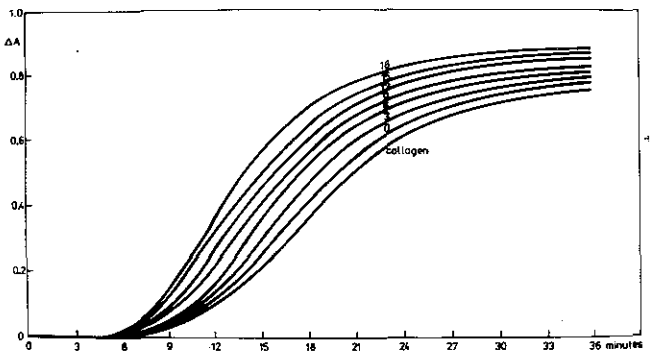

Fig. 3. Kinetics of the fibrillogenesis of collagen isolated from the fibrotic liver in the presence of GAG isolated from a liver with the same stage of fibrosis.

RESULTS

Effect of GAG on collagen fibril formation

The effect of GAGs isolated from livers with various stages of fibrosis upon collagen fibrillogenesis was studied *in vitro*. Preparations of collagen and GAG were isolated from the same group of animals, i.e., after the same period of carbon tetrachloride treatment. The fibrillogenesis of collagen type I and collagen type III was investigated.

It was found that GAG obtained from the fibrotic liver accelerated fibril formation. The rate of acceleration depended on the stage of fibrosis development. Kinetics of collagen type I fibrillogenesis are shown in Fig. 1. It was found that physiological aging did not influence the rate of fibril formation and all results obtained in the control group were similar to those obtained before treatment with carbon tetrachloride (Fig. 1).

The effect of GAG upon fibrillogenesis of type III collagen is shown in Fig. 2. It was found thatthe rateof fibril formation of collagen type III was slower than that of collagen type I. Progressive enhancement related to the stage of hepatic fibrosis was observed (Fig. 2).

Collagen in the fibrotic liver was found to be a mixture of various types of these proteins, predominantly type I and type III. The influence of GAG on the fibrillogenesis of collagen isolated from fibrotic livers without differentiation of the types is shown in Fig. 3. The results were similar to those of type I and type III collagen and reflected the ratio of type I to type III in the injured liver (Fig. 3).

Values of absorbance reflecting the forming gel opacity after 21 min of fibrillogenesis are summarized in Table I.

Solubilisation of collagen

Evidentchangesin the kinetics of collagen solubilisation in samples of the fibrotic liver were found. Liberation of collagen from the fibrotic tissue was significantly slower than that from control animals. This phenomenon depended

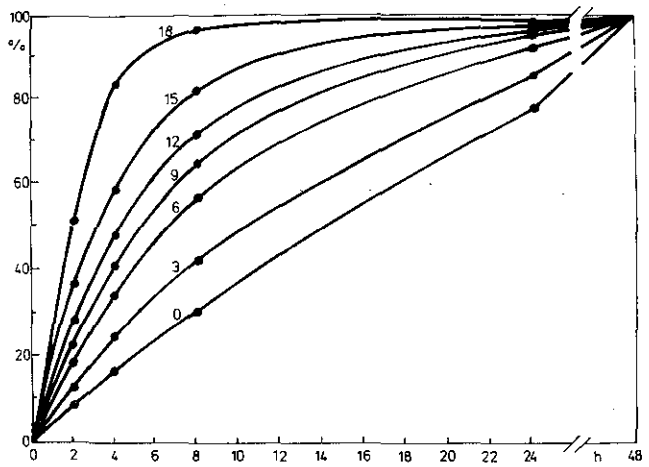

Fig. 4. Collagen solubilisation from the fibrotic liver tissue.

on the period of treatment with carbon tetrachloride. No influence of physiological aging was observed. Kinetics of collagen liberation are shown in Fig. 4 and relative values of collagen solubilised after 2hr and 8hr (expressed as percentage of the amount of total collagen solubilised after 48hr) are summarized in Table II.

DISCUSSION

The process of collagen fibrillogenesis consists of two independent stages, a nucleation phase and a growth phase.[23,55-7] During the nucleation phase no increase in turbidity of collagen solutions takes place, while during the growth phase a rapid increase in turbidity occurs. This phenomenon is connected with the linear and lateral growth of collagen aggregates.[58] The interaction between collagens and proteoglycans is complex, factors such as ionic strength, molecular composition of macromolecules and others influencing this process.

The obtained results indicate that GAG isolated from the fibrotic liver accelerates the fibrillogenesis of collagen. This acceleration is more evident in collagen type I fibrillogenesis than collagen type III fibril formation. The effect of GAG on the natural mixture of collagen types–because it has been isolated from the fibrotic liver–reflects the ratio of type I to type III collagen. Investigations of collagen heterogeneity in the fibrotic liver indicated changes in type I and type III content.

In a previous paper it was shown that the kinetics of collagen type III accumulation were more rapid than those of collagen type I.[59] On the other hand, studies by Rojkind et al.[29] showed that collagen in the fibrotic liver consisted predominantly of type I. Changes of other types of collagen were also described.[29]

It is possible that changes in GAG composition and structure influence fibril formation under conditions such as fibrosis. The fibers are more stable and resistant to solubilisation as has been shown in the present paper.

The nature of the changes in GAG is unclear. In the author's previous studies, a relative increase in the content of hyaluronic acid and chondroitin-6-sulphate, as well as a relative decrease in the content of keratan sulphate, heparan sulphate and dermatan sulphate were described.[38] It has been suggested that hyaluronic acid and chondroitin sulphates increase collagen fibril formation and, possibly, an elevation of these compounds is responsible for the observed phenomena. The structural changes of GAGs could also influence the process of collagen fibril formation, but changes in the molecular structure of GAGs were not investigated in the fibrotic liver. The extracellular biomatrix of the liver consists of collagen fiber bound to proteoglycans and structural glycoproteins. In the presented investigations isolated GAGs instead of proteoglycans were studied *in vitro*. It is possible that fibrillogenesis *in vitro* is more complex; however, a trend similar to that observed *in vitro* is suggested.

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