Serum concentrations of the *N*-terminal propeptide of type III procollagen and two type IV collagen fragments and gene expression of the respective collagen types in liver in rats with dimethylnitrosamine-induced hepatic fibrosis

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Dimethylnitrosamine (DMN)-induced liver fibrosis was used as an experimental model to study the relationship between serum concentrations of the *N*-terminal propeptide of type III procollagen [S-Pro(III)-N-P] and the *N*-terminal (S-7S) and *C*-terminal (S-NC1) domains of type IV collagen and hepatic concentrations of type III and IV collagen mRNAs. Increases in S-Pro(III)-N-P, and especially in the two type IV collagen-related antigens, were found to be early events in the formation of DMN-induced hepatic fibrosis. The mean concentration of S-Pro(III)-N-P was 120 % of the control mean on day 7 of DMN treatment, 230 % on day 14 and 250 % on day 21. The corresponding values for S-7S were 260, 950 and 1100 % and, for S-NC1, 310, 820 and 1000 %. All these changes were very similar to those found in the hepatic concentrations of the respective mRNAs. These data support a previous suggestion that an enhanced production of basement-membrane (type IV) collagen is an early event in the development of the DMN-induced hepatic fibrosis. The results also indicate that S-7S and S-NC1 are very sensitive indicators of changes in type IV collagen metabolism. Data obtained in gel-filtration experiments for these three serum antigens were consistent with the suggestion that all three antigens are mainly derived from the synthesis of the respective collagens.

INTRODUCTION

Hepatic fibrosis is a widespread alteration in the liver that consists primarily of increased collagen deposition in the tissue (for reviews, see [1–3]). The extent of liver fibrosis can be determined by histological examination of a biopsy specimen. Assessment of hepatic fibrogenesis, i.e. the actual rate of the connective-tissue formation, would, however, give more important information of the ongoing process in the liver, especially at the early stages of the disease and in the follow-up.

During recent years, several serum assays have been developed for the measurement of ongoing collagen metabolism. These include radioimmunoassays for the *N*-terminal propeptide of type III procollagen [S-Pro(III)-N-P] [4] and for two fragments of type IV collagen, namely the 7S [5] and NC1 domains [6–8] (see also [2,3,9–11]). A number of studies have indicated increases in S-Pro(III)-N-P in patients with various liver diseases (see [2,3,10]), and two recent reports also demonstrate such increases in S-7S [11] and S-NC1 [6]. Several problems have arisen, however, in the interpretation of the values obtained in these three assays. One problem is that type III collagen fibres in tissues contain, in part, molecules with an uncleaved *N*-terminal propeptide [12], the concentration of such molecules being currently

unknown. If the proportion of uncleaved molecules is high, a significant part of the serum propeptide may be derived from the degradation of molecules already incorporated into fibres instead of being derived from the synthesis of new collagen. The 7S and NC1 domains of type IV collagen are integral parts of the basement-membrane network (see [13]), and therefore serum antigens related to these two domains may be derived either from type IV collagen molecules that have escaped the basement-membrane network upon their synthesis or from type IV collagen fragments released during degradation of basement membranes. Data on S-7S and S-NC1 in patients with liver diseases have been interpreted to suggest that increases in the former serum antigen may mainly reflect increases in the rate of type IV collagen synthesis [11] and increases in the latter may reflect an enhanced degradation of the type IV collagen network [6]. Further studies, however, are clearly needed to verify these hypotheses.

Recently two studies have reported data on S-Pro(III)-N-P [14], S-7S and S-NC1 [15] in rats with carbon tetrachloride-induced hepatic injury. Increases in these three serum antigens were found already after 2 weeks of the injury [14,15]. We here report data on S-Pro(III)-N-P, S-7S and S-NC1 in rats with dimethylnitrosamine (DMN)-induced liver injury. In this model, hepatic

Abbreviations used: DMN, dimethylnitrosamine; Pro(III)-N-P, *N*-terminal propeptide of type III procollagen; 7S, 7S *N*-terminal domain of type IV collagen; NC1, *C*-terminal non-collagenous domain of type IV collagen; S-Pro(III)-N-P, S-7S and S-NC1, the corresponding proteins measured in serum.

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fibrosis is preceded by early increases in the hepatic concentration of the mRNAs for types I, III and especially type IV collagen [16]. Therefore this model would seem particularly suitable for a study of possible early changes in S-7S and S-NC1. We also measured hepatic concentrations of type III and type IV collagen mRNAs and studied the molecular sizes of the serum type III and type IV collagen-related antigens. The aim of these studies was to obtain experimental data on a possible relationship between S-Pro(III)-N-P, S-7S and S-NC1 and an increase in hepatic collagen synthesis and to learn whether these assays show changes that precede any significant collagen accumulation.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (10 weeks old) were used for the induction of liver injury. The animals were maintained under 12 h-light/12 h-dark cycles with food and water available *ad libitum*. Hepatic fibrosis was induced by intraperitoneal injections of DMN in doses of 1 μ l (diluted 1:100 with 0.15 M-NaCl)/100 g body wt. The injections were given on the first 3 consecutive days of each week over a period of 21 days. Some of the control animals ($n = 26$) were killed at the beginning of the experiment, and some, together with the treated animals, on days 7 ($n = 18$), 14 ($n = 11$) and 21 ($n = 20$).

Rats were anaesthetized with diethyl ether. Blood was collected by heart puncture, and after 1.5 h clotting at room temperature the sera were separated by centrifugation and stored at -70°C until analysed. The livers were rapidly removed, immediately frozen in liquid N_2 and weighed in the frozen state. The livers were then stored for up to 2 weeks at -70°C until analysed.

Radioimmunoassays

Procollagen type III antigens in serum were determined radioimmunologically [4] by using antibodies which were raised against the 14-amino-acid-long C-terminal part of the N-terminal propeptide of type III procollagen (full details are available from E.-R.S. on request).

7S and NC1 antigens of type IV collagen in serum were determined radioimmunologically as described previously [8].

Serum chromatography

The M_r distribution of serum antigens was determined by gel filtration on a Sephacryl S-300 or S-400 column (Pharmacia, Freiburg, Germany; 1.6 cm $\times$ 130 cm) equilibrated with phosphate-buffered saline (PM16; Serva, Heidelberg, Germany), containing 0.04% Tween 20, and by subsequent radioimmunological analysis. Serum samples (0.5–1.5 ml) were applied to the columns, and fractions (2.8 ml each) were collected. The columns were calibrated with globular-protein standards (Bio-Rad, Munich, Germany).

Some of the serum samples were exposed to bacterial collagenase (CLSPA; Worthington, Freehold, NJ, U.S.A.; 0.5 mg/ml, added directly to serum for a 24 h incubation at room temperature) before gel-filtration chromatography. Collagenase activity was stopped by the addition of EDTA (0.5 mM final concn.). It has been shown previously that this treatment does not degrade non-collagenous proteins [8].

RNA extraction and analysis

The total hepatic RNA was purified from 30–200 mg samples of liver as described by Ala-Kokko *et al.* [16] and analysed with slot-blot-hybridization techniques. Three dilutions of RNA (1–10 μ g) were dotted on to nitro-cellulose paper with a vacuum manifold (Minifold II; Schleicher and Schuell, Dassel, Germany). The filters were baked, prehybridized and hybridized in the presence of labelled cDNA probes for type III and IV collagen mRNAs [16]. Isolation and characterization of the cDNA clones for $\alpha 1(\text{III})$ [17] and for $\alpha 1(\text{IV})$ [18] chains have been described previously. After washing, the blots were exposed to Kodak X-Omat films, and the mRNA was quantified by using a Kontes K495000 densitometer connected to a Spectra-Physics SP4100 computing integrator [16].

Statistical analysis

One-way analysis of variance with the Bonferroni modification (see [16]) was used to compare changes in the parameters analysed between days 0, 7, 14 and 21 of liver injury.

RESULTS

General effects of DMN treatment

The general effects of DMN treatment were similar to those reported in our previous studies on this experimental model [16,19]. The body weight of the rats was not influenced by the DMN administration during the first 14 days, but was decreased to about 90% of the control value on day 21 (results not shown). No change was found in the mean collagen content of the liver, expressed as μ g of hydroxyproline per g of liver protein, on day 7 of the treatment, whereas this value had increased to about 150% of the control mean on day 14 and 360% on day 21. No significant changes were found in the concentration of liver DNA, expressed as mg per g of liver, at any of the time points.

Changes in the concentration of S-Pro(III)-N-P and liver type III collagen mRNA

The mean concentration of S-Pro(III)-N-P increased to about 230% of the control value on day 14 and to 250% on day 21 in the DMN-treated animals (Table 1). A slight increasing tendency could already be seen by day 7 of the treatment. These changes resembled those occurring in the hepatic concentration of the respective mRNA, except that the magnitude of the increase in the mRNA concentration was even greater at all the time points studied (Table 1).

Changes in the concentration of S-7S and S-NC1 and liver type IV collagen mRNA

The mean concentration of S-7S was 34.4 ± 10.2 (S.D.) μ g/l and of S-NC1 29.0 ± 8.7 μ g/l, in the control rats. Significant increases were already found in both these type IV collagen antigens by day 7 of DMN treatment, the values on day 21 being about 1100% of the control mean for S-7S and 1000% for S-NC1 (Table 1). At all time points these increases were considerably larger than those found for S-Pro(III)-N-P (see above). The changes in both serum type IV collagen antigens were very similar to those found in the hepatic concentration of the mRNA coding for the $\alpha 1$ chain of type IV collagen

Table 1. S-Pro(III)-N-P, S-7S and S-NCI and hepatic pro α 1(III) and pro α 1(IV) collagen mRNA levels in DMN-treated rats

Serum samples, together with liver specimens, were collected at each experimental time point. Serum samples were analysed for the collagen-fragment antigenicities by using the specific radioimmunoassays mentioned in the Materials and methods section. Total hepatic RNA was extracted and analysed by slot-blot hybridization, using specific cDNA probes as described in the Materials and methods section, and results are expressed as arbitrary absorbance units obtained after densitometric scanning. Values are given as means $\pm$ s.d. Significant differences compared with the control values: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Group	Type III collagen		Type IV collagen		
	S-Pro(III)-N-P ($\mu\text{g/l}$)	Pro α 1(III)-chain mRNA (arbitrary units)	S-7S ($\mu\text{g/ml}$)	S-NC1 ($\mu\text{g/ml}$)	Pro α 1(IV)-chain mRNA (arbitrary units)
Control DMN	8.5 $\pm$ 3.5	100	34.4 $\pm$ 10.2	29.0 $\pm$ 8.7	100
7 days	10.1 $\pm$ 3.0	217 $\pm$ 112	89.8 $\pm$ 49.6**	91.2 $\pm$ 35.2***	497 $\pm$ 90**
14 days	19.9 $\pm$ 6.6***	370 $\pm$ 125*	338.6 $\pm$ 44.6***	237.5 $\pm$ 27.0***	639 $\pm$ 115***
21 days	21.5 $\pm$ 9.8***	483 $\pm$ 225**	385.4 $\pm$ 101.9***	289.6 $\pm$ 64.3***	866 $\pm$ 134***

Fig. 1. S-Pro(III)-N-P antigenicity after gel filtration on a Sephacryl S-300 column

A 0.8 ml serum sample from a normal rat that had received DMN for 14 days was fractionated by gel filtration, and Pro(III)-N-P antigenicity was analysed by a radioimmunoassay. V_t corresponded to fraction 88 (not shown).

Fig. 2. S-7S and S-NC1 antigenicities after gel filtration on a Sephacryl S-400 column

A 0.6 ml serum sample from a rat that had received DMN for 21 days fractionated by gel filtration, and 7S (○) and NC1 (●) antigenicities were analysed by radioimmunoassay.

(Table 1). As no change was seen in the mean collagen content of the liver on day 7 of DMN treatment (see above), these increases in the serum concentrations of the two type IV collagen antigens and in the hepatic concentration of type IV collagen mRNA preceded any detectable increase in the liver collagen content.

Characterization of S-Pro(III)-N-P, S-7S and S-NC1 by gel filtration

Serum antigens related to the N-terminal propeptide of type III procollagen were eluted from a Sephacryl S-300 column as two major forms (Fig. 1). One of them

(fractions 32–37) was eluted close to the void volume of the column, probably representing either intact procollagen molecules or partially processed procollagen molecules lacking the C-terminal propeptide. The second major form (fractions 43–51) had an elution volume close to that of the N-terminal propeptide standard, but with slightly higher M_r , as has also been found in normal young rats [14]. Owing to the specificity of the antibodies for the C-terminal part of the propeptide, no significant amounts of low- M_r material corresponding to the N-

Fig. 3. S-NC1 antigenicity after gel filtration on a Sephacryl S-300 column

A 0.8 ml serum sample from a rat that had received DMN for 14 days was fractionated by gel filtration, and NC1 antigenicity was analysed by a radioimmunoassay. V_0 corresponded to fraction 88 (not shown).

terminal portion of the propeptide [2,3,10,14,20] were detected.

Serum antigens related to the 7S domain of type IV collagen were eluted from a Sephacryl S-400 column in two main peaks, one shortly after the void volume and the other slightly ahead of the elution position of a standard consisting of the 7S domains of four molecules bound together (Fig. 2). NC1 antigenicity was recovered from this column mainly as one peak, which was co-eluted with the high- M_r form of the 7S antigen, although small amounts of antigenicity were detected in lower- M_r materials (Fig. 2). To characterize further the small amounts of the lower- M_r NC1 antigens (Fig. 2), additional gel-filtration experiments were performed with a Sephacryl S-300 column (Fig. 3). In this column a distinct second peak, containing about 20% of the total NC1 antigenicity, was found with an elution position corresponding to an M_r of about 80 000–90 000 (Fig. 3), i.e. the M_r of the NC1 domain of one molecule [21]. In addition, small amounts of NC1 antigenicity were eluted in other peaks corresponding to various M_r values (Fig. 3).

When the serum was incubated with bacterial collagenase before gel filtration, neither the 7S nor the NC1 antigenicity was found in the elution position of the high- M_r form. After treatment with collagenase, the 7S antigen was eluted from the column as a heterogeneous material with an apparent M_r range from 15 000 up to the size of the tetrameric 7S domain. The NC1 antigen was found as a broad peak with an elution position corresponding to the M_r of the mono- and di-meric NC1 domain [21] (results not shown).

Analysis of the various forms of the serum 7S and NC1 antigens during the development of DMN-induced liver fibrosis indicated that marked increases occurred in all

the forms when expressed as $\mu\text{g/l}$ of serum (detailed results not shown). No significant changes were found in the relative distribution of the two forms of the S-7S antigen or the various forms of the S-NC1 antigen.

DISCUSSION

Increases in S-Pro(III)-N-P, and especially S-7S and S-NC1, were found here to be early events in the formation of DMN-induced hepatic fibrosis. These three serum antigens increased very similarly to their respective mRNAs in liver tissue, suggesting that the changes in S-Pro(III)-N-P, S-7S and S-NC1 reflected those occurring in the synthesis of the respective collagen types.

The serum concentrations measured here for the three antigens in control rats are in a good agreement with those reported previously [8,14,15]. The changes in S-Pro(III)-N-P during the development of DMN-induced hepatic injury were also very similar to those occurring in carbon tetrachloride-induced injury [14]. In contrast, distinct differences were found between the two liver-fibrosis models in the serum type IV collagen-related antigens. In the carbon tetrachloride-induced injury, only slight increases were found in S-7S and S-NC1 at 2 weeks, and even at 4–5 weeks the values were only about 2.5–4 times those in the controls [15]. In the present study, however, significant increases in both type IV collagen-related antigens were already found in serum 1 week after the injury, when no changes were seen in the liver total collagen content, and increases to about 8–9 times the control mean were present at 2 weeks, when the liver collagen content had increased to about 1.5-fold.

Recently data have been reported on changes in the hepatic concentrations of mRNAs for types I, III and IV collagen in rats with carbon tetrachloride-induced hepatic fibrosis [22]. The results obtained were quite different from those found in rats with DMN-induced fibrosis [16]. In the 'carbon tetrachloride' model, unlike in the 'DMN' model, increases in collagen mRNAs paralleled, but did not precede, increases in the hepatic collagen content, and the concentrations of type IV collagen mRNAs increased slightly later than those for type I and III collagens [22]. The present data support our previous suggestion that an enhanced production of basement-membrane collagen is an early event in the development of DMN-induced hepatic fibrosis [16]. The data also indicate that S-7S and S-NC1 are very sensitive indicators of changes in type IV collagen metabolism.

Gel-filtration experiments using the S-7S and S-NC1 antigens supported the suggestion that these serum antigens are mainly derived from type IV collagen synthesis. The type IV collagen molecules form in the tissues a network structure through association of the N-termini of four molecules via the 7S domains, and the C-termini of two molecules via the NC1 domains [13]. Tetrameric structures consisting of four molecules bound together at their 7S domains, but having free NC1 domains, have been described as intermediates in the assembly of the network [23,24]. In the present study, most of the S-NC1 and about half of the S-7S were found to be co-eluted from the Sephacryl S-400 column shortly after the void volume, both in control rats and in DMN-treated animals in which serum concentrations of these antigens were increased 10–11-fold. These high- M_r forms were converted into lower- M_r ones by incubation with bacterial collagenase, suggesting that they were linked

via a collagen triple helix. The high- M_r forms of the S-7S and S-NC1 antigens are thus likely to represent monomeric type IV collagen molecules that have escaped the type IV collagen network upon their synthesis. The other half of the S-7S antigen, which was eluted close to the tetrameric 7S domain standard, may represent degradation products of the tetrameric intermediates [23,24] in the assembly of the type IV collagen network. The low- M_r forms of the S-NC1 antigen may represent degradation products of either single molecules or the tetramer intermediates. However, the present data do not exclude the possibility that some of the tetramer-size 7S antigen and low- M_r NC1 antigen may represent degradation products of the existing basement membranes.

The gel-filtration data obtained here resemble those obtained in rats with carbon tetrachloride-induced injury [15], even though the time courses and magnitudes of increases in S-7S and S-NC1 were markedly different. A similar gel-filtration pattern of serum type IV antigens has also been found in rats with streptozotocin-induced diabetes [8]. In contrast, in patients with alcoholic hepatitis, most of the S-NC1 has been found in low- M_r forms, corresponding in size to NC1 domains of two type IV collagen polypeptide chains [6]. This form was suggested to represent degradation of the existing type IV collagen network [6], but the possibility cannot be excluded that it represents degradation products of newly synthesized single molecules or their oligomeric assemblies.

The M_r values obtained here for serum type III procollagen-related antigens are consistent with the suggestion that the serum S-Pro(III)-N-P measured with the radioimmunoassay used in the present study represents (a) intact procollagen molecules or molecules lacking their C-terminal propeptide, (b) intact N-terminal propeptides produced in the cleavage reaction catalysed by the procollagen N-terminal proteinase and (c) unidentified forms with M_r values higher than that of the intact propeptide. At least forms (a) and (b) are likely to represent synthesis of new type III procollagen, as is also evident on the basis of similarities in the increases of S-Pro(III)-N-P and the hepatic concentration of type III collagen mRNA found here. It may be noted that, because of the specificity of the present antibodies for the C-terminal part of the propeptide, this assay does not measure degradation products consisting of the N-terminal globular domain of the propeptide.

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