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Gene expression of type I, III and IV collagens in hepatic fibrosis induced by dimethylnitrosamine in the rat

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Dimethylnitrosamine (DMN)-induced hepatic fibrosis was used as an experimental model to study collagen-gene expression during liver fibrogenesis. Increase in the concentrations of the mRNAs for type I, III and IV collagens was found to be an early event in the development of hepatic fibrosis, as the mRNAs for all three collagen types showed a definite increasing tendency by day 7 of DMN treatment. Prolyl 4-hydroxylase (EC 1.14.11.2) and galactosylhydroxylysyl glucosyltransferase (EC 2.4.1.66) activities were also distinctly elevated at this stage, whereas no increase could be detected in the liver collagen content. The increase in the mRNAs for type I collagen was the smallest and that for type IV collagen the greatest at all the time points studied. The relative concentrations of the mRNAs for the three collagen types on day 21 of DMN treatment were 350% of the control mean for type I collagen, 490% for type III and 660% for type IV. The data further indicate that the proportions of the mRNAs for the three collagen types are 1.0:0.9:0.2 in normal rat liver, 1.0:1.4:0.8 on day 14 of DMN treatment, and 1.0:1.3:0.5 on day 21. The early marked increase in the mRNA for type IV collagen suggests that enhanced production of basement-membrane collagen may be an early event in the development of hepatic fibrosis.

INTRODUCTION

Hepatic fibrosis is a widespread alteration in the liver, which consists primarily of increased deposition of collagen in the tissue (for reviews, see [1–3]). Normal rat liver contains about 1 mg of collagen/g fresh wt. of tissue, type I and III collagens each forming about 40% of the total liver collagen, and types IV and V each about 5–10% [1]. Exact quantification of collagen types is nevertheless difficult, and hence values reported by various laboratories show some inconsistencies [1–3]. In cirrhosis the total liver collagen content increases up to 6–8-fold, and all collagen types are affected [1]. The ratio of type I to III collagen has been reported to remain within normal limits in the cirrhotic rat liver [1], whereas type IV and V collagens may proportionally increase even more than type I or III collagens [1–3].

The mechanisms leading to the increased hepatic collagen accumulation in liver fibrosis are not understood. Numerous studies have demonstrated that a fibrotic liver produces larger quantities of collagen than a normal liver (for review, see [1]). However, since the rate of collagen degradation also appears to be increased [4–7], the amount of collagen deposited must depend on the balance between the rates of synthesis and degradation. The enhanced collagen production has been shown to be accompanied by many changes in the biosynthetic pathway, such as increases in the pool of free proline [1], the concentration of functional prolyl-tRNA [1] and the activities of most of the post-translational enzymes involved in collagen biosynthesis [8–10]. Increases in the concentration of the mRNAs for type I collagen in three animal models for hepatic fibrosis have been reported [11–13], but the amounts of the mRNAs for the other collagen types have not been determined.

The present work was initiated to study changes in the concentrations of the mRNAs for type I, III and IV collagens during the development of dimethylnitrosamine (DMN)-induced hepatic fibrosis in the rat. Special emphasis was placed on the questions of (*a*) how possible increases in these mRNAs with time are related to those in the activities of the enzymes catalysing intracellular post-translational modifications of collagen and to changes in the amount of liver collagen, and (*b*) whether the time courses and the magnitude of the increases are the same for each collagen mRNA.

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 dimethylnitrosamine in doses of 1 μl (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. Treated animals were killed on days 7, 14 and 21 of the experiment. Some of the control animals were killed at the beginning of the experiment, and some together with the treated animals on days 7, 14 and 21. The final groups comprised 24 control rats, 16 rats with

Abbreviation used: DMN, dimethylnitrosamine.

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7 days' injury, 11 with 14 days' injury and 8 with 21 days' injury.

Rats were anaesthetized with diethyl ether and their livers 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.

Assays of enzyme activities and hydroxyproline

The livers were thawed, and homogenized in a Teflon/glass homogenizer at about 1500 rev./min for 60 s in a cold (0 °C) solution consisting of 0.2 M-NaCl, 0.1 M-glycine, 50 μM-dithiothreitol, 0.1% (w/v) Triton X-100, 0.01% (w/v) soya-bean trypsin inhibitor and 0.02 M-Tris/HCl buffer adjusted to pH 7.4 at 4 °C. The homogenization volume was 1 ml/30 mg of liver. After homogenization the samples remained in ice for 15 min. A portion of the homogenate was taken for protein measurements [14], and the rest was centrifuged at 15000 ***g*** for 30 min at 4 °C. The supernatant was used for the enzyme assays, and the pellet was kept for the hydroxyproline measurements.

Prolyl 4-hydroxylase was assayed in the supernatant with as substrate a [^{14}C]proline-labelled protocollagen made with chick-embryo tendon cells (see [15]). A prolyl 4-hydroxylase preparation, partially purified by $(NH_4)_2SO_4$ precipitation and stored in batches, was used as a standard for the different series of enzyme analyses [15]. Galactosylhydroxylysyl glucosyltransferase was assayed by determining the amount of radioactive glucosylgalactosylhydroxylysine formed from UDP-[^{14}C]glucose in a gelatinized calf skin collagen substrate [16]. A human serum sample containing a known amount of galactosylhydroxylysyl glucosyltransferase activity was used as a standard. Liver hydroxyproline content was measured after hydrolysis of the pellet in 6 M-HCl at 120 °C for 16 h, by the method of Kivirikko *et al.* [17]. Under these extraction conditions more than 93–98% of total liver hydroxyproline remains in the pellet [18].

RNA extraction

The total hepatic RNA was purified from 30–200 mg samples of liver by proteinase K digestion, followed by phenol/chloroform/3-methylbutan-1-ol extraction and subsequent high-salt fractionation. After ethanol precipitation, the RNA was further purified by using 6 M-guanidine hydrochloride [19]. The amount of RNA was determined by spectrophotometric absorption at 260 nm. The DNA and RNA contents of the tissue homogenates after proteinase K treatment were determined after trichloroacetic acid precipitation of the samples. The DNA solubilized by heating in 5% trichloroacetic acid was measured by the indole method [20], with calf thymus DNA as a standard. The RNA was dissolved in 0.5 M-NaOH and determined by the orcinol method [21], with yeast RNA as the standard.

Preparation of labelled procollagen-cDNA probes

Isolation and characterization of the human procollagen cDNA clones, α12 for proα1(I), Hf32 for proα1(I), E6 for proα1(III) and HT21 for proα1(IV) chains, have been described previously [22–24]. The sizes of the inserts contained in the pBR322 plasmid vectors range from 2.2 to 2.6 kb. The plasmid DNAs were isolated on CsCl gradients and nick-translated with [^{32}P]dCTP and [^{32}P]dGTP. The specific radioactivity was measured after each nick translation and ranged from 5×10^8 to 8×10^8 c.p.m./μg [25].

Assay of procollagen mRNA

Three serial dilutions of denatured total RNA (1–10 μg) were dotted on to nitrocellulose paper by using a vacuum manifold (Minifold II; Schleicher and Schuell). The filters were air-dried and heated at 78 °C for 2 h to bind the RNA to the filter. Prehybridization of the filters was carried out in a solution containing 0.75 M-NaCl, 0.075 M-sodium citrate, 0.5 M-sodium phosphate, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll, 250 μg of denatured salmon sperm DNA/ml and 50% (v/v) formamide for 12 h at 42 °C. The RNA–cDNA hybridization was carried out for 24 h at 42 °C in the same solution containing the labelled cDNA probe [26]. The filters were then washed four times for 5 min each at room temperature with 0.3 M-NaCl/0.03 M-sodium citrate (pH 6.8)/0.1% SDS, and twice for 30 min each at 58 °C with 0.015 M-NaCl/1.5 mM-sodium citrate/0.1% SDS. The blots were exposed to Kodak X-Omat films in cassettes with intensifying screens. The amounts of mRNA were quantified by using a Kontes K495000 densitometer connected to a Spectra-Physics SP4100 Computing Integrator.

Statistical analysis

Analysis of variance was used to compare changes in the parameters analysed between days 0, 7, 14 and 21 of liver injury [27]. Correlations were calculated by linear regression analysis.

RESULTS

General effects of DMN treatment

The body weight of the animals was not influenced by DMN administration during the first 14 days of the experiment, but was 87% of the control value on day 21 ($P < 0.01$). The livers were hard and granular on day 21, and about half of the animals had ascites in the peritoneal cavity. Mean liver weight was unchanged on day 7, being 104% of the control mean, but decreased thereafter to 88% on day 14 ($P < 0.05$) and 56% on day 21 ($P < 0.001$). The ratio of liver weight to body weight had significantly decreased by day 21 (Table 1). The concentration of total liver protein per g fresh wt. of tissue had significantly decreased by days 7, 14 and 21, whereas no significant changes were found in the concentration of liver DNA (Table 1).

Enzymes of collagen synthesis and collagen content

Significant increases in the activities of prolyl 4-hydroxylase and galactosylhydroxylysyl glucosyltransferase were found by day 7 of DMN administration (Table 2), with the increases in the former being much greater than those in the latter. Both enzyme activities reached their maximal values on day 14 (Table 2). The collagen content of the liver, expressed as μg of hydroxyproline/g of liver protein, was unchanged on day 7, but was about 160% of the control mean on day 14 and 400% on day 21 (Table 2).

Table 1. Effect of DMN on liver/body weights, hepatic protein and DNA content

The livers were homogenized and analysed for protein and DNA as described in the Materials and methods section. Results are means ± S.D. Value significantly lower than the control value: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Group	No. of animals	Liver wt. (mg/g body wt.)	Liver protein (mg/g of liver)	Liver DNA (mg/g of liver)
Controls	24	38.7±4.3	160.5±21.1	1.71±0.19
DMN, 7 days	16	41.5±3.8	136.3±16.3***	1.86±0.23
DMN, 14 days	11	35.6±4.6	140.1±16.6**	2.17±0.52
DMN, 21 days	8	22.3±4.4***	144.7±12.1*	1.68±0.45

Table 2. Effect of DMN on liver prolyl 4-hydroxylase (PH) and galactosylhydroxylysyl glucosyltransferase (GGT) activities and hydroxyproline content

The livers were homogenized and centrifuged at 15000 *g* for 30 min at 4 °C. PH and GGT were analysed in the supernatant and hydroxyproline in the pellet as described in the Materials and methods section. Results are means ± S.D. Value significantly higher than the control value: *$P < 0.01$, **$P < 0.001$.

Group	Activity (d.p.m./μg of protein)		Hydroxyproline (mg/g of protein)
	PH	GGT	
Controls	11.5±2.9	5.17±0.72	1.10±0.41
DMN, 7 days	26.5±11.1*	7.39±1.27**	1.18±0.31
DMN, 14 days	82.0±30.4**	9.11±1.58**	1.74±0.78*
DMN, 21 days	76.8±27.5**	8.28±1.15**	4.02±1.77**

Assay of collagen mRNA concentrations

The amount of total RNA per μg of DNA after proteinase K digestion of the liver tissue remained constant in the controls and in the rats treated with DMN for 7, 14 or 21 days (results not shown). About 30–40% of the RNA present in the original homogenate was consistently recovered after the final purification. The yield of purified total RNA was about 5 mg/g fresh wt. of liver tissue. The specific hybridization of the cDNA probes to mouse type I, III and IV procollagen mRNAs has been demonstrated previously in Northern-blotting analyses [28]. There is a remarkable conservation between mouse and human 3′ untranslated sequences, which are covered by the proα1(IV)-cDNA clone HT21 [28].

Changes in collagen mRNA concentrations during DMN treatment

The type I, III and IV collagen mRNAs were quantified by the slot-blot hybridization technique. A linear hybridization result was obtained with RNA amounts ranging from 1 to 15 μg with all the cDNA probes used, and three different amounts of total RNA (1.0, 5.0 and 10.0 μg) were chosen for the slot-blot analyses.

A typical slot-blot hybridization analysis of total liver RNA with the cDNA probe for the proα1(I) chain is shown in Fig. 1. Each slot blot represents 10 μg of total RNA from a separate animal, i.e. one control rat and one rat each after days 7, 14 and 21 of DMN treatment. The data obtained with the cDNA probe for the proα2(I) chain were essentially identical.

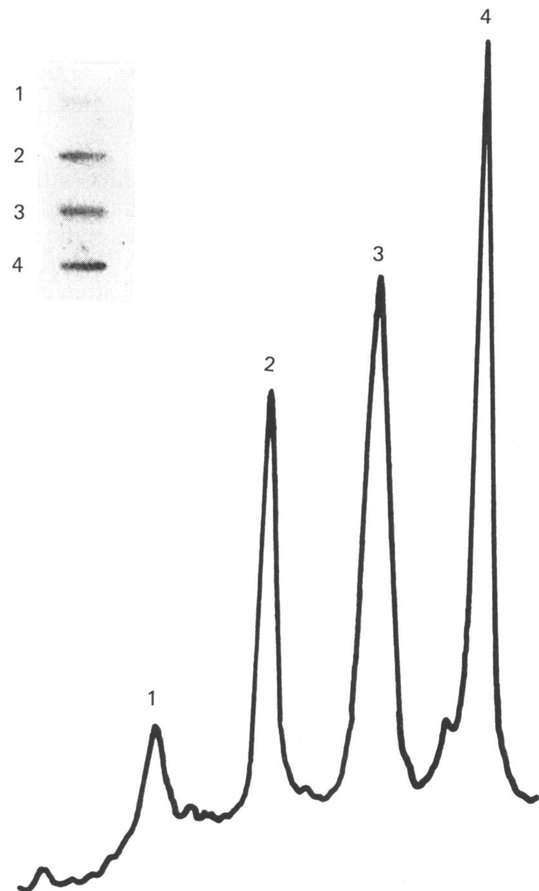

Fig. 1. Typical slot-blot hybridization analysis of procollagen type I mRNA in a control rat liver (band 1), and livers from animals treated with DMN for 7 days (band 2), 14 days (band 3) and 21 days (band 4)

Total RNA (10 μg) was dotted on to a nitrocellulose filter in each slot and hybridized with the ^{32}P-labelled cDNA probe for proα1(I) collagen. The RNA–[^{32}P]DNA hybrid bands were detected by autoradiography and quantified by densitometry.

The mRNAs for all three collagen types of three to nine different animals at each experimental time point

Table 3. Increases in hepatic proα1(I), proα1(III) and proα1(IV) mRNAs in the course of DMN-induced liver injury

Total hepatic RNA was extracted at each experimental time point and analysed by slot-blot hybridization by using specific cDNA probes, as described in the Materials and methods section. The results are expressed as percentages of the control values (means ± S.D.) after densitometric quantification of the autoradiography films. Significant differences compared with the control values: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Group	Procollagen mRNA (% of control value)		
	α1(I)	α1(III)	α1(IV)
Controls	100	100	100
DMN, 7 days	163±43	201±74	292±192*
DMN, 14 days	256±103**	348±96**	634±99***
DMN, 21 days	354±117***	486±227**	655±208***

were analysed after treatment of the rats with DMN (Table 3). A definite increasing trend was noted as early as on day 7, and the values on day 21 were about 350% of the control means for the mRNAs for the proα1(I) or proα2(I) chain (the latter value is not shown), about 490% for the proα1(III) chain and about 660% for the proα1(IV) chain (Table 3). The increase in the mRNAs for the proα1(I) and proα2(I) chains was the smallest, and that for the proα1(IV) chain was the greatest, at all time points studied.

Ratios of collagen mRNAs

The ratio of the proα1(I) mRNA to the proα2(I) mRNAs was about 2:1 in the normal rat liver and remained unchanged during DMN treatment. This value was obtained by correcting the hybridization data with respect to the length and the measured specific radioactivity of the cDNA probes. The ratios of the proα1(III) and proα1(IV) mRNAs to the proα1(I) mRNA were calculated by similar correction methods. These values were further converted into relative amounts of mRNAs for the three collagen types (Fig. 2): (*a*) by using a ratio of 2:1 for the mRNAs coding for the proα1(I) and proα2(I) chains, (*b*) by taking into account the fact that type III collagen consists of only one type of polypeptide chain, and (*c*) by assuming that the amount of proα1(IV) mRNA is twice that of proα2(IV) mRNA [29–31].

The ratio of mRNAs for type III collagen to those of type I collagen in normal rat liver was estimated to be 0.9:1 (Fig. 2), and this value increased to 1.4:1 in the DMN-treated rats. The ratio of type IV to type I collagen mRNAs was about 0.2:1 in normal rat liver; type IV collagen mRNAs thus represent nearly 10% of the sum of type I, III and IV mRNAs. The highest ratio (0.8:1) of type IV to type I collagen mRNAs was detected on day 14 of DMN treatment. Therefore, at this stage type IV collagen mRNAs represent 25% of the total of the mRNAs for the three collagen types, and on day 21 about 20%. It should be noted that even the absolute amounts of type IV collagen mRNAs on days 14 and 21 of DMN treatment were higher than those of either type I or III collagen mRNAs in the normal rat liver.

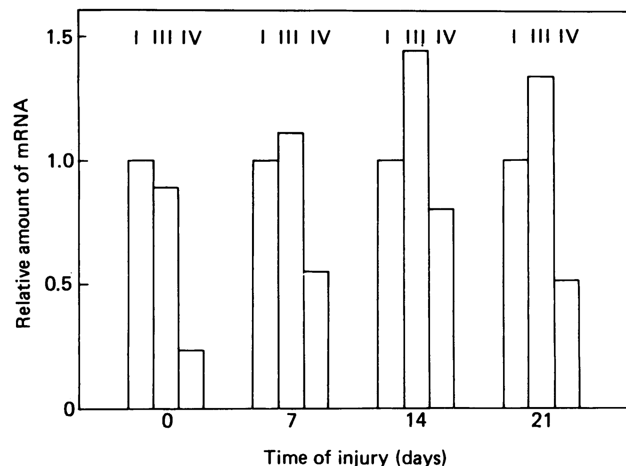

Fig. 2. Type I:III:IV procollagen mRNA proportions in the course of DMN-induced liver injury

Total hepatic RNA was extracted and analysed by slot-blot hybridization by using specific cDNA probes, as described in the Materials and methods section. Analyses of all three types were carried out simultaneously under identical conditions, and the values were corrected with reference to the lengths and specific radioactivities of the cDNA probes and for chain composition of different collagen types as described in the text. The amount of type I procollagen mRNA was taken to be 1.0 on each day of the experiment, and the amounts of type III and IV procollagen mRNAs were calculated in relation to this value.

DISCUSSION

DMN-induced liver fibrosis was used here as an experimental model to study the expression of the type I, III and IV collagen genes during hepatic fibrogenesis. This model, which has been characterized previously [32], differs from the other frequently used model of fibrosis induced by carbon tetrachloride in that there is no excessive fat accumulation in the hepatic tissue. The results concerning the time course of increase in the two enzyme activities of collagen synthesis and liver hydroxyproline content are in accordance with those reported previously [32].

Increases in the concentrations of the mRNAs for type I, III and IV collagens were found to be early events in the development of DMN-induced hepatic fibrosis. The mRNAs for all three collagen types showed a definite increasing tendency by day 7 of DMN treatment. The two enzyme activities of collagen synthesis measured here were also distinctly elevated at this stage, whereas no increase could be detected in the liver collagen content. The mRNAs for the proα1(IV) chain showed the largest early increase among the mRNAs studied, and also the largest increase at day 21.

Estimation of the proportions of the various mRNAs by hybridization techniques is based on several assumptions, in particular on the assumption that the affinities of the various cDNA probes for their respective mRNAs are comparable [33]. This is substantiated by our results showing that the ratio of the proα1(I) to proα2(I) mRNAs was 2:1, which is in excellent agreement with values previously determined using RNA from skin fibroblasts [33,34]. It should further be noted that the calculations shown in Fig. 2 were based on the

assumption that in liver the ratio of the concentration of the proα1(IV) mRNA to that of proα2(IV) mRNA is 2:1. In any event, the ratios determined for the proα1(I) and proα1(IV) chains are still valid, since in both cases the concentration of the proα2 mRNAs was considered to be half of that of the proα1 mRNAs.

The relative concentrations of the mRNAs for the three collagen types in normal rat liver, i.e. about 1.0:0.9:0.2 (Fig. 2), are consistent with the respective proportions of 1:1:0.15 reported for these three collagen types in normal rat liver [1]. This suggests that the steady-state concentrations of these three collagen types in normal rat liver may be regulated primarily at the level of transcription. The finding that the concentrations of the mRNAs for type III collagen increased more than those for type I collagen in hepatic fibrosis does not in itself agree with the tendency for the ratios of these two collagen types to remain within normal limits in cirrhotic rat liver [1]. However, these ratios have not been determined for very early stages of fibrosis, or fibrosis induced by DMN. It is therefore unclear as to whether type III collagen predominates at relatively early stages of DMN fibrosis, or whether other factors, such as differences in the translational efficiencies of the mRNAs or in their rates of degradation, may contribute to the ratios found in the fibrotic livers.

The early marked increase in the concentration of the mRNA for the proα1 chain of type IV collagen is especially significant, as it suggests that the enhanced production of basement-membrane collagen may be an early event in the development of hepatic fibrosis. In agreement with this suggestion, evidence for early deposition of type IV collagen in ethanol-induced liver disease has been reported, and the concentration of type IV collagen is thought to increase more than that of type I or type III collagen in rats with hepatic cirrhosis [2]. The deposition of basement membranes along the liver sinusoids transforms these into capillaries [2], and thus contributes to the pathogenesis of fibrosis by impairing the exchange of metabolites and the functioning of the liver. The present data suggest that this process may be initiated during the very early stages in the development of hepatic fibrosis.

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