Type IV collagen antigens in serum of diabetic rats: a marker for basement membrane collagen biosynthesis

D. G. Brocks[1], H. P. Neubauer[1] and H. Strecker[2]

[1]Pharmaceutical Research Division, Department of Biochemistry and [2]Radiochemical Laboratory, Hoechst A. G. Frankfurt/Main, FRG

Summary. The nature of type IV collagen antigens in the serum of streptozotocin diabetic rats was studied using radioimmunoassays for the N-terminal (7S-collagen) and C-terminal domain of type IV collagen. Type IV collagen antigen cross-reacting with antibodies to the C-terminal domain was elevated from 32.0 ± 5.36 ng/ml ($n=10$) in serum of normal rats to 94.9 ± 24.5 ng/ml ($n=10$, $P<0.0001$) in serum of streptozotocin diabetic rats and could be normalized to 40.1 ± 8.30 ng/ml ($n=18$) by insulin treatment. Molecular sieve chromatography of serum demonstrated a high molecular weight fraction containing the C-terminal and N-terminal domains and small-er material containing only the N-terminal domain. Degradation of the high molecular weight material by collagenase indicates that it consists of intact collagen type IV. Its relative proportion increased from 42% to 54% 4 weeks after diabetes induction. Together with unaltered clearance rates of 7S collagen in normal and diabetic rats, the data suggest that the increase of collagen type IV antigens in diabetic states reflects increased synthesis of collagen type IV.

Key words: Basement membrane, collagen type IV, serum analysis, diabetes.

Basement membrane thickening during the course of diabetes mellitus is a well established fact [1]. In vivo studies with radioactively labelled precursor amino acids have demonstrated increased glomerular basement membrane synthesis in diabetic rats [2–4]. Such experiments are, however, laborious and of limited usefulness for followup studies. Recently radioimmunochemical methods have become available to study basement membrane metabolism by measuring serum concentrations of basement membrane proteins like 7S-collagen, the N-terminal cross-linking domain of type IV collagen, and laminin or laminin fragments [5]. Earlier studies have shown increased serum levels of 7S-collagen in streptozotocin diabetic rats which are normalized by insulin treatment [6]. It remains unknown whether the increased serum levels are due to (a) a higher rate of synthesis of basement membrane collagen; (b) different clearance rates of 7S-collagen in diabetic and normal rats; or (c) an increased degradation of basement membrane collagen. In this study we report on experiments which favour the first interpretation.

Materials and methods

Animals

Normally-fed male Wistar rats of the strain HOE WISKF (Hoechst AG, Frankfurt/Main, FRG), initially weighing 200–250 g, were used throughout the study. Diabetes was induced by injection of freshly dissolved streptozotocin (70 mg/kg, Calbiochem, Frankfurt/Main, FRG) into the tail vein. Rats treated with insulin were subcutaneously injected with 9 IU Insulin (Long Insulin, Hoechst AG. Frankfurt/Main, FRG) twice daily (⅓ of the dose in the morning, ⅔ of the dose in the late afternoon).

Methods

The N-terminal and C-terminal domains of type IV collagen, 7S-collagen and non-collagenous domain 1 (NC1) were isolated from a transplantable murine tumour (EHS), which produced basement membrane material. The material was purified as described [7, 8].

Antisera were raised in rabbits by two injections of 0.5 mg antigen together with complete Freund's adjuvant at 4-week intervals. Antisera were collected 4–8 weeks after the booster injections. Serum levels of 7S-collagen and of type IV collagen NC1 were determined by radioimmunoassay following an established protocol [9]. Radiolabelling of the antigens with ^{125}I was performed by the chloramine T method [10] for 7S-collagen and the Bolton and Hunter [11] method for NC1. The radioimmunoassays were of the sequential saturation type: 0.1 ml of antiserum at a dilution capable of binding 50% of the tracer was incubated overnight at $+4\,^{\circ}$C with 0.2 ml of unlabelled standard antigen solution or an aliquot of the unknown sample. About 1 ng of ^{125}I-labelled antigen was then added (7S-collagen: about 20000 dpm in 0.1 ml, NC1 about 10000 dpm in 0.1 ml), and the incubation continued for 6–7 h. Separation of bound and unbound tracer was achieved by precipitation with a second antibody (goat antiserum to rabbit IgG – 0.5 U in 0.5 ml, Calbiochem, Frankfurt/Main, FRG).

Half-life studies

For the evaluation of the biological half-life of 7S-collagen, ^{125}I-labelled protein (15 mCi/mg) was injected into the tail vein of normal Wistar rats (HOE WISKF) (10 μCi/150 g body weight, mean weight
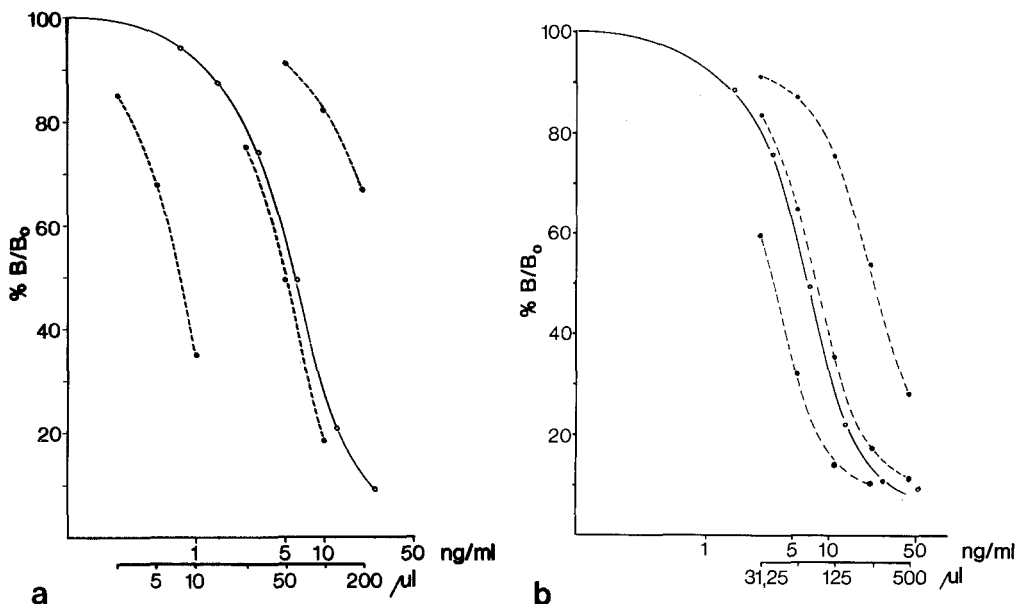
Fig. 1a and b. Linearity of 7S-collagen (a) and type IV collagen NC1 (b) radioimmunoassays. Standard curves (——) and serum inhibition curves (- - -) of different rats are shown
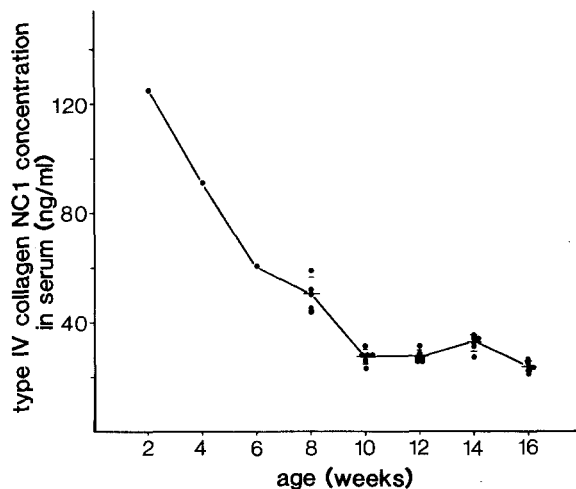

Fig. 2. Serum concentration of type IV collagen NC1 as a function of age. The data are mean values ± SD for six rats in each group, except for the 2-, 4- and 6-week old animals whose sera were pooled out of 6 individual sera

Table 1. Recovery of type IV collagen NC1 from rat sera

	Amount of NC1 (ng/ml)				
Experiment	Endogenous	Added	Calculated	Found	Yield
a	48.2	7.5	55.7	53.5	96%
b	48.2	30.9	79.1	77.5	98%

280 g) or streptozotocin diabetic rats (10 μCi/150 g body weight, mean weight 157 g) 4 weeks after induction of diabetes. At 2, 4, 8, 24 and 48 h intervals blood was taken from the retroorbital venous plexus, and the radioactivity in serum was determined in a γ-counter.

In a second experiment a solution of non-labelled 7S-collagen in PBS was injected into the tail vein of normal rats (9 μg/kg body weight, mean weight 300 g) and streptozotocin diabetic rats (40 μg/kg body weight, mean weight 132 g) 7 weeks after diabetes induction. At 1, 2, 4, 8 and 24 h intervals blood samples were taken and analyzed for

7S-collagen serum concentration by radioimmunoassay. Background levels of circulating 7S-collagen amounted to 28.4 and 92.1 ng/ml in normal and diabetic rats respectively and were increased 10-fold by the treatment.

Gel-filtration chromatography

For the determination of the size distribution of antigenic material present in serum, serum samples were analyzed by gel-filtration chromatography, using Biogel A5m (200–400 mesh, 1.6 × 140 cm column) equilibrated in phosphate-buffered saline, 0.04% Tween 20, 0.02% NaN_3. Fractions of 2.2 ml were collected. Each fraction was analyzed by radioimmunoassay for 7S-collagen and NC1. The gel-filtration column was calibrated using a standard mixture of globular proteins (BioRad, Munich, FRG).

Statistical analysis

Statistical analysis was performed using the one-tailed Student's t-test for unpaired data.

Results

Specificity of the radioimmunoassays

7S-Collagen. The sensitivity of the assay was 5.4 ± 0.3 ng/ml (mean ± SD, $n = 16$) at the 50% intercept of the standard curve. The intraassay coefficient of variation for serum samples was 2.5% ($n = 5$), and the interassay coefficient of variation was 6.0% ($n = 7$). Recovery studies of exogenous 7S-collagen added to serum revealed recovery rates between 84 and 112%. The linearity of the assay is shown in Figure 1 a.

Type IV collagen NC1

The sensitivity of the assay was 5.8 ± 0.7 ng/ml (mean ± SD, n = 16) at the 50% intercept of the standard curve. The intraassay coefficient of variation for serum
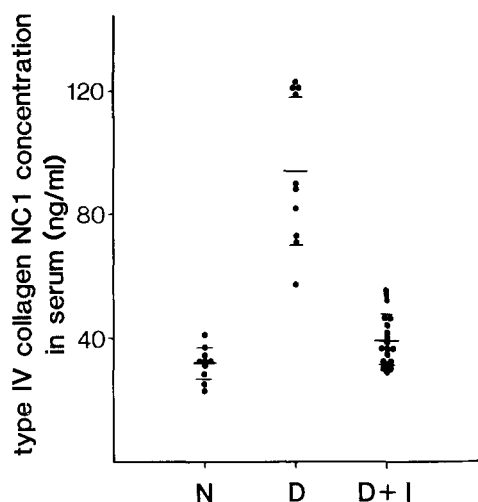
Fig. 3. Type IV collagen NC1 serum concentrations in normal and streptozotocin diabetic rats. Diabetes was induced by streptozotocin 8 weeks prior to the study. Blood glucose concentration was 5.77 ± 0.72 mmol/l, 22.20 ± 5.50 mmol/l and 10.32 ± 2.16 mmol/l in normal, diabetic and insulin treated diabetic rats respectively. Glycosuria was zero in control group (N), 6.6 ± 1.7 g/24 h in diabetic rats (D), and 3.1 ± 1.3 g/24 h in diabetic + insulin-treated rats (D + I). Mean body weight was 391 ± 34 g for controls and 158 ± 24 g and 321 ± 25 g for diabetic and diabetic + insulin rats respectively
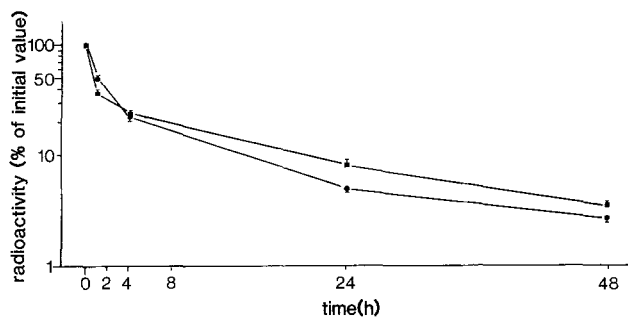

Fig. 4. Disappearance of injected ^{125}I-labelled 7S-collagen from rat serum as a function of time. ●——● = diabetic rats. ■——■ = normal rats. The mean value $\pm$ SD for 5 rats are given as a percentage of initial concentration measured 5 min after injection
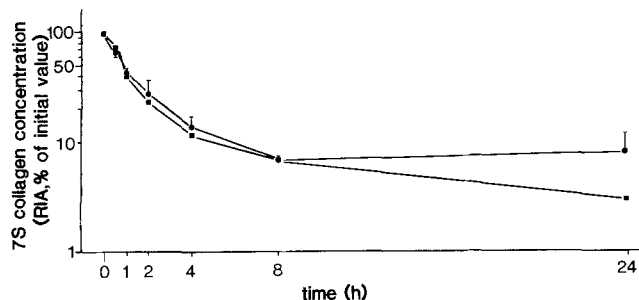

Fig. 5. Disappearance of injected 7S-collagen from rat serum as a function of time. ●——● = diabetic rats; ■——■ = normal rats. 7S-collagen concentration was measured by radioimmunoassay after a single injection of 7S-collagen and given as mean for 2 normal rats and as mean $\pm$ SD of 5 diabetic rats. Results are expressed as a percentage of initial concentration after injection

samples was 2.7% (n = 5) and the interassay coefficient of variation was 8.6% (n = 13). Table 1 shows the results of recovery experiments in serum. Satisfying recovery rates of 96 and 98% were found. The linearity of the assay is documented in Figure 1 b. The age dependence of the concentration of NC1 antigen in rat sera is shown on Figure 2. Highest values, which decreased gradually to reach stable values at an age of 10–12 weeks (295–335 g body weight), were found in infant rats.

Type IV collagen NC1 concentrations in serum of diabetic rats

Figure 3 shows that type IV collagen NC1 is markedly elevated in serum of diabetic rats. This increase could be normalized by treatment with insulin. Similar results have previously been reported when analyses were carried out with a 7S-collagen assay [6].

Half-life studies

The increase in basement membrane antigens could be due to a different clearance rate of these antigens in normal and diabetic animals. We therefore determined the biological half-life of radioactively labelled 7S-collagen and of non-labelled 7S-collagen in both groups of rats.

Figure 4 shows the decline of radioactivity in the serum of normal and streptozotocin diabetic rats after injection of ^{125}I-labelled 7S-collagen. No difference in the biological half-life was found. In order to eliminate the possibility of different levels of deiodases being present in serum of normal and diabetic rats, a similar experiment was performed with non-labelled 7S-collagen. The disappearence of antigen from serum was followed by radioimmunoassay (Fig. 5); it failed to show any significant difference in the half-life between normal and diabetic rats.

Nature of antigenic material in serum

The nature of the material present in serum which showed crossreaction in the radioimmunoassays for 7S-collagen and type IV collagen NC1 has not yet been determined. We analyzed the size distribution of antigenic material of serum samples from normal and diabetic rats by gel-filtration chromatography and radioimmunoassays. Material possessing 7S-collagen epitopes emerged in two peaks from the column (Fig. 6 a), corresponding in apparent molecular weight to 7S-collagen standard and one with a considerably higher molecular weight. The relative amount of 7S-collagen in the latter peak was increased in sera from diabetic rats (Fig. 6 b). The relative amount of antigenic material present in these two peaks changed during the development of diabetes over a period of 4 weeks (Fig. 7).

Analysis of the size distribution of antigenic material containing epitopes of NC1 demonstrated one peak
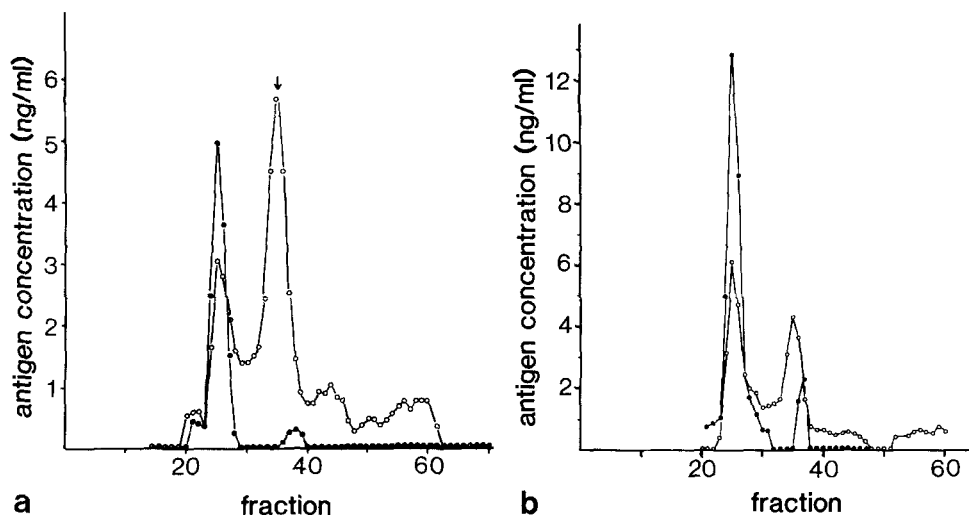
a

b

Fig. 6a and b. Size distribution of type IV collagen antigens in rat sera after gel-filtration. A 1.5 ml serum sample from a normal (a) or diabetic rat (b) was analyzed by 7S-collagen (O——O) and type IV collagen NC1 (●——●) radioimmunoassay after gel-filtration chromatography (Biogel A 5 m, 1.6 × 140 cm, phosphate buffered saline pH 7.2 0.04% Tween). Arrow: Elution Position of standard 7S-collagen
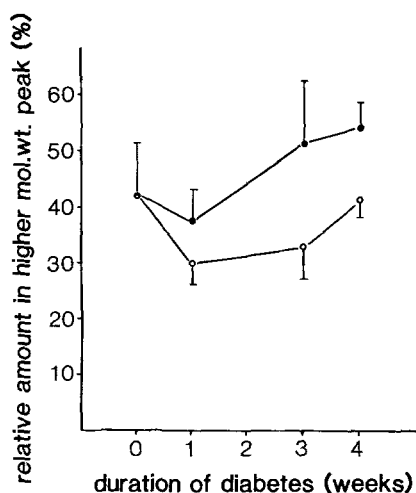

Fig. 7. Effect of diabetes on the relative amount of type IV collagen antigen present in the high molecular weight fraction after gel filtration (see Fig. 6). Diabetes was induced in 4 rats by intravenous injection of 70 mg/kg streptozotocin (●——●); 4 control rats were treated with 0.9% NaCl (O——O). Blood was withdrawn at weekly intervals and analyzed after gel-filtration chromatography for 7S-collagen. The relative amount of 7S-collagen is given in percent (mean ± SD). Values at 1 and 3 weeks ($p < 0.05$) and at 4 weeks ($p < 0.005$) differed significantly.
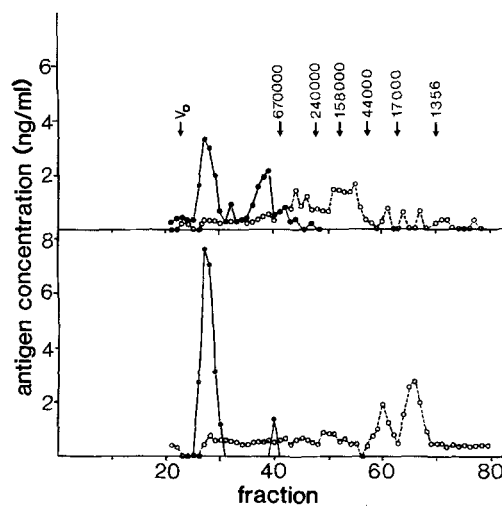
Fig. 8. Effect of collagenase treatment on the size distribution of antigenic material related to type IV collagen in serum. 1 ml of serum from a streptozotocin diabetic rat (NC1 content 110 ng/ml) was analyzed by gel-filtration (●——●). Another aliquot (1.5 ml) was incubated with 0.75 mg collagenase (CLSPA Worthington) for 24 h at room temperature. 5 mmol/l EDTA were added to stop enzymatic activity and 1.2 ml of the sample was analyzed by gel-filtration (O————O). The upper half represents 7S-collagen radioimmunoassay; the lower half NC1 radioimmunoassay

(Fig. 6). This peak coincided exactly with the higher molecular weight peak detected by the radioimmunoassay for 7S-collagen.

The 7S and NC1 antigens were also analyzed in the serum from a streptozotocin diabetic rat by gel-filtration chromatography before and after treatment with bacterial collagenase (Fig. 8). The high molecular weight peak containing NC1 material disappeared completely after treatment and was converted to smaller material with approximate molecular weights of 40 kD and 20 kD. A heterogenous pattern of low molecular weight components was also detected in the treated serum by the radioimmunoassy for 7S-collagen. When an

albumin solution was treated with collagenase under similar conditions no degradation was seen (data not shown).

Discussion

The antigens studied in this paper – 7S-collagen and type IV collagen NC1 – form the N-terminal and C-terminal domains of type IV collagen [12], the major structural component of basement membranes [13–15]. Earlier studies have shown that 7S-collagen can be detected in serum by radioimmunological methods [5]. The se-

rum concentration of this antigen is elevated in diabetic animals and can be normalized by insulin treatment [6]. The precise nature and metabolic fate of the antigen measured in serum remains to be established. The comparison of clearance rates of 7S-collagen in normal and diabetic animals (Figs. 4, 5) showed a similar biphasic profile. In the initial phase about 80% of the antigen disappeared within 2–4 h, presumably due to tissue uptake of 7S-collagen. The slower phase we attribute to the degradation and excretion of the circulating antigen. As no difference could be detected between normal and diabetic rats, these experiments indicate that differences in the excretion rate cannot explain the elevated serum concentrations in diabetic animals.

Recently a method became available for the isolation of the C-terminal non collagenous domain NC1 of type IV collagen [8]. Using a radioimmunoassay specific for this domain, elevated serum levels were detected in streptozotocin diabetic rats. These could again be normalized by insulin treatment (Fig. 3).

By using a combination of radioimmunoassays specific for the 7S and NC1 domains of type IV collagen, we could discover some features of a high molecular-weight form of serum antigens. Both assays detected a high molecular-weight antigen which was probably intact collagen type IV or small oligomeric variants. As expected, this material could be degraded by bacterial collagenase, producing NC1 antigens comparable in size to its monomeric and dimeric subunits [8] and a heterogenous population of small 7S collagen peptides. The instability of the 7S epitopes indicates that the collagen IV serum antigen is below the size of tetramers which cause a stabilization against collagenase [7, 12]. The analysis of non-treated serum revealed a second antigenic peak which contained 7S but not NC1 epitopes (Fig. 6). This material was similar in size to authentic, tetrameric 7S collagen [7] and presumably originates from tissue forms of cross-linked collagen type IV.

The type IV collagen antigen in the high molecular weight peak increases during the development of streptozotocin diabetes. This indicates increased biosynthesis of type IV collagen, and is in agreement with findings of Hasslacher et al. [16], who showed a positive correlation between the rate of glomerular basement membrane synthesis and 7S-collagen in serum. The data also show that 7S-collagen material in the second peak of rat serum has a molecular weight comparable to 7S-collagen isolated from intact tissues. This material could be due to degradation of basement membranes; thus the 7S-collagen may measure both neosynthesis and catabolism of type IV collagen. The NC1 radioimmunoassay, however, detects primarily intact type IV collagen and may be a suitable method for the exclusive analysis of increased basement membrane production.

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Dr. D. Brocks
Hoechst AG
Pharma Forschung
Biochemie H 825
D-6230 Frankfurt/Main 80
FRG