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Aortic collagen alterations in human diabetes mellitus. Changes in basement membrane collagen content and in the susceptibility of total collagen to cyanogen bromide solubilisation

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Summary. Structural alterations in arterial extracellular matrix components have been suggested to play a role in the development of arterial disease among patients with diabetes mellitus. This study examines the quantity and quality of collagenous components in aortas from diabetic patients. In order to obtain data about the arterial tissue concentration of type IV and V collagen in diabetic and non-diabetic patients, aortas from 21 patients with diabetes (9 with Type 1 (insulin-dependent) diabetes and 12 with Type 2 (non-insulin-dependent) diabetes), were collected at autopsy together with aortas from groups of sex- and age-matched patients. Intima and media samples from normal and fibrous plaque areas from the individual vessels were evaluated. Pulverized, dried and defatted tissue samples were subjected to chemical solubilization with cyanogen bromide and subsequent immunochemical quantitation of the dissolved type IV and V collagen in an ELISA. It was found that the concentration of type IV collagen was increased in the tunica media both in plaque and non-plaque areas in the samples from the diabetic patient groups as compared to the non-diabetic groups. No consistent differences in type IV collagen concentrations were found between diabetic and non-diabetic patients in tunica intima. The type V collagen concentrations and the total collagen content were not altered in the diabetic samples. The fraction of the total collagen that was solubilized during cyanogen bromide treatment was determined, and it was found that this fraction was decreased in most tissue areas in the diabetic patient groups. These findings suggest that patients with diabetes develop alterations in arterial collagenous components that may play a role in the development of arterial disease in diabetes.

Key words: Atherosclerosis, diabetic complications, extracellular matrix, elastin, non-enzymatic glycation.

The pathogenesis behind the increased mortality and morbidity of cardiovascular disease among diabetic patients is unknown [1]. The extent of macroscopic visible atherosclerotic lesions in coronary arteries and aortas from diabetic subjects is larger than in vessels from non-diabetic subjects [2–4], but epidemiology studies have shown that well-known atherosclerotic risk factors, i.e. hypertension and hypercholesterolaemia, can explain only a minor part of the excess incidence of heart- and large vessel disease among diabetic patients [5–7] Elements of the diabetic condition per se seem to play a role in the development of arterial disease, and it is therefore interesting that several studies indicate that structural changes different from classic atherosclerotic lesions occur in arteries from diabetic patients. Increased amounts of a periodic acid-Schiff-(PAS) positive substance in coronary arteries from patients with diabetes have been described in a few investigations [8–10] and it seems that a particular pattern of linear arterial calcification is frequently seen in arterial tunica media among diabetic subjects [11–14]. Biomechanical studies have, in addition, shown increased stiffness of non-atherosclerotic aortic tissue from diabetic patients, indicating that alterations occur in the connective tissue [15].

One important feature of the diabetic microangiopathy is thickening of basement membranes. A possible explanation for the association between heart disease and manifestations of the diabetic microangiopathy [9, 16–18] may be that common pathogenetic pathways exist for diabetic micro- and macrovascular disease. Arterial structural alterations in diabetes have been suggested to also involve the basement membranes, which surround vascular smooth muscle cells both in vivo and in vitro [4, 19–22]. In the present study we have quantitated basement membrane collagen and also other collagenous components in arteries from patients with diabetes, using a method, whereby aortic collagenous components are solubilized by chemical degradation followed by immunochemical quantitation of solubilized collagen epitopes [23].

Table 1. Clinical data in diabetic and control groups

Patient groups	Type 1 diabetes	Control group 1	Type 2 diabetes	Control group 2
Total number *(n)*	9	18	12	24
Sex (female/male)	3/6	6/12	4/8	8/16
Age (years)	57 ± 10	58 ± 10	74 ± 6	74 ± 6
Blood pressure *(n)*				
Hypertension	5	5	3	7
Normotension	4	11	8	15
Unknown	0	2	1	2
Duration of diabetes (years)	33 ± 11		12 ± 10	
Late diabetic manifestations *(n)*				
Nephropathy	5		3	
No signs	4		8	
Unknown	0		1	
Retinopathy	7		2	
No signs	2		8	
Unknown	0		2	
Causes of death *(n)*				
Cardiovascular[a]	6	8	8	12
Neoplasia	1	6	3	5
Other[b]	2	4	1	7

Values are presented as mean ± SD. *(n)* indicates number of subjects.
[a] Acute myocardial infarction, cardiac insufficiency, and stroke.
[b] Infections, accidents, etc.

Subjects and methods

The present report is based on investigations of mid-thoracic aortic samples obtained from vessels which had been examined in earlier studies [4, 15]. The descending part of the thoracic aorta was collected at autopsy from 9 patients with Type 1 (insulin-dependent) diabetes and 12 patients with Type 2 (non-insulin-dependent) diabetes as well as from two groups of sex- and age-matched non-diabetic subjects (control group 1 and control group 2). Patients were excluded if more than 40 h had elapsed from time of death to sampling of tissue. Also patients suffering from connective tissue diseases or having received glucocorticoid treatment, chemotherapeutic agents or radiotherapy to the thoracic region within the previous 6 months were excluded. The tissue from two Type 2 diabetic patients were lost during processing of the samples and these patients and their sex- and age-matched non-diabetic control subjects from the initial groups [4] were therefore disregarded. The clinical data from the various groups of patients are presented in Table 1. Patients treated for hypertension or subjects with a patient file with more than one blood pressure measurement above 140/100 mm Hg were considered hypertensive.

Aortas. Approximately 15 cm^2 of the middle part of the descending thoracic aorta was used for examination in the present study. The vessel was cut longitudinally and the tunica adventitia was removed. Two parts of the artery were studied separately: areas with normal intima and areas with fibrous plaques without complications. This subdivision was based on the macroscopic appearence of the arteries: areas of the vessels with faint yellow intima without elevations or discolouring were considered as non-atherosclerotic, whereas regions with elevated white areas without ulcerations, aneurysms or thrombosis were considered as uncomplicated fibrous plaques. Areas which could not be defined as belonging to one of these parts were not considered. The surface area of the two investigated parts of the aortas was determined by point-counting. After separation of the aorta into areas with or without plaque, the intima was carefully dissected from the tunica media and the resulting four samples of the individual aortas were then weighed (wet weight – ww). The separation of tunica intima from tunica media was followed by microscopic observations of several separated specimens. It was confirmed that the cleavage occurred in approximately the same layer as the lamina elastica interna, however, especially in plaque aeras some of the upper layer of medial elastic membranes was part of the tunica intima sample. After lyophilisation to constant weight the samples were weighed (dry weight – dw) and defatted by two 24-h extractions in 3 ml chloroform:ethanol 3:1. The samples were again lyophilized and weighed (dry, defatted weight – ddw) and finally pulverized in a Retsch Mill. The percent water content and the total extractable lipid content was expressed as ((ww – dw)/ww) * 100 (%) and ((dw-ddw)/ww) * 100 (%) respectively. It was not possible in three aortas to obtain normal tissue and in seven aortas no uncomplicated atherosclerotic tissue could be defined. The number and wet weight (mean ± SD) of available tissue areas in the various patient groups are shown in Table 2.

Cyanogen bromide treatment. Pulverized tissue samples or collagen standards (5–8 mg) were dissolved in 2000 µl 70% formic acid and CNBr-crystals (approximately 15–25 mg) (Sigma, St. Louis, Mo., USA) was added. The solution was incubated in a nitrogen atmosphere during constant magnetic stirring at 30 °C for 3 h. The reaction was stopped by adding 3 ml H_2O and subsequently lyophilizing the solution three times. After the last lyophilisation, the cleaved tissue was solubilized in 1 ml PBS (10 mmol/l phosphate, pH 7.2, 0.15 mol/l NaCl) and centrifuged at 7,000 *g* for 20 min at 4 °C. The pellet was washed once in 0.5 ml PBS and used for hydroxyproline quantitation. The combined supernatants were used for quantitation of collagen type IV and V and hydroxyprolin. The CNBr-solubilisation protocol was undertaken after optimization experiments. It was found that reduction of the tissue with dithiotreitol or mercaptoethanol did not improve the efficiency, neither did recyanogen bromide treatment of the pellet. In 11 samples it was not possible to obtain sufficient amounts of material for CNBr-treatment: three samples of normal intima in the control group 1, three samples of normal intima, two of plaque intima, and two samples of plaque media in the Type 2 diabetes group and two samples of plaque media in the control group 2.

Collagens and antibodies. Type I, III, IV and V collagens, used for standards and antigens were prepared by differential salt precipitations and cation chromatography (carboxy-methylated cellulose)

Table 2. Amount of analysed tissue, water content, total lipid content, dry weight/surface area and DNA content

	Type 1 diabetes	Control group 1	Type 2 diabetes	Control group 2
Available tissue areas (n)				
normal areas	9	18	11	22
plaque areas	7	14	12	23
Wet weight of starting material (mg)				
normal intima	320 ± 162	214 ± 202	160 ± 120	210 ± 157
plaque intima	577 ± 399	896 ± 601	527 ± 347	581 ± 378
normal media	806 ± 603	569 ± 446	354 ± 228	451 ± 299
plaque media	437 ± 238	459 ± 277	382 ± 209	337 ± 240
Water content (% of wet weight)				
normal intima	78 ± 3	79 ± 4	76 ± 6	77 ± 3
plaque intima	74 ± 8	76 ± 4	73 ± 4	70 ± 9
normal media	75 ± 2	76 ± 2	76 ± 2	75 ± 4
plaque media	75 ± 1	76 ± 2	75 ± 2	76 ± 3
Total lipid content (% of wet weight)				
normal intima	2.3 ± 2.0	2.5 ± 1.7	4.6 ± 4.3	3.5 ± 3.3
plaque intima	5.6 ± 2.7	7.5 ± 1.7	8.8 ± 3.7	10.4 ± 5.6
normal media	0.4 ± 0.4	0.4 ± 0.4	0.7 ± 0.6	0.7 ± 0.7
plaque media	1.1 ± 0.5	0.7 ± 0.6	1.5 ± 1.4	1.3 ± 1.1
Dry defatted weight/surface area (mg/cm^2)				
normal intima	10.4 ± 4.2	8.7 ± 4.2	8.8 ± 5.2	10.3 ± 3.4
plaque intima	26.1 ± 13.7	24.3 ± 7.1	19.4 ± 6.9	28.0 ± 14.3
normal media	26.7 ± 6.0	28.8 ± 5.8	24.3 ± 6.5	28.1 ± 7.3
plaque media	22.5 ± 4.1	18.3 ± 3.3	18.8 ± 4.4	17.9 ± 4.7
DNA/dry defatted weight (µg/mg)				
normal intima	2.4 ± 1.1	2.3 ± 0.5	1.7 ± 2.2	1.4 ± 0.4
plaque intima	1.5 ± 0.6	2.0 ± 1.2	1.1 ± 0.9	0.9 ± 0.5
normal media	2.2 ± 1.1	3.1 ± 1.2	1.5 ± 0.6	1.5 ± 0.8
plaque media	3.6 ± 0.8	2.9 ± 0.8	1.7 ± 0.9	2.1 ± 1.4

Values are expressed as mean ± SD

from a pepsin digest of human placentae as described elsewhere [24–26]. The purity of each collagen was tested by SDS-polyacrylamide electrophoresis both native and after cyanogen bromide under both reducing and non-reducing conditions (Fig. 1). Polyclonal antibodies were prepared by immunizing rabbits with pepsin digested and CNBr-treated type IV and V collagen. An intradermal multi-injection method was used. The antiserum was purified by immunosorption on individual CNBr-activated sepharose 4B columns using CNBr-treated type I, III, IV and V collagens as ligands, prepared according to the manufacturer's recommendations (Pharmacia, Up-

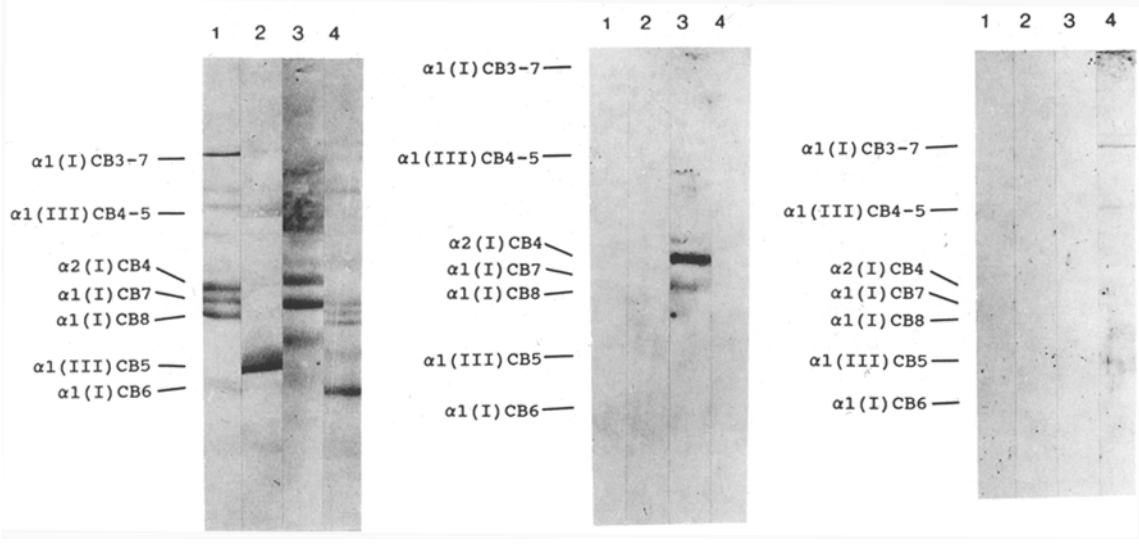

Fig. 1. Immunoblots using anti-CNBr-type IV collagen antibodies or anti-CNBr-type V collagen antibodies on SDS-separated CNBr-treated collagen standards. Twenty-five microgrammes of CNBr-treated collagen type I (1), III (2), IV (3) or V (4) was electrophoretically separated in 12.5% SDS-PAGE gel, transferred to nitrocellulose and either: Left panel: Stained with amido black. Middle panel: Probed with anti-CNBr-type IV collagen antibodies. Right panel: Probed with anti-CNBr-type V collagen antibodies

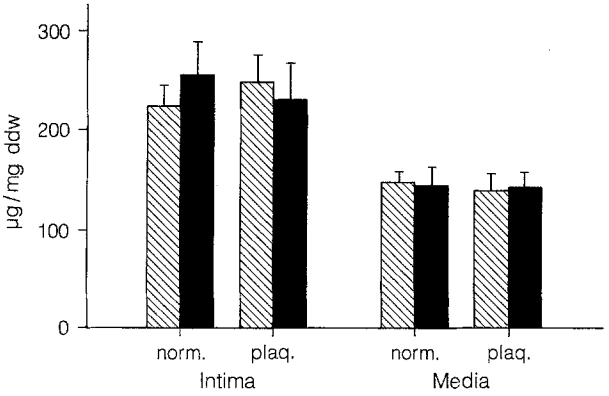

Fig. 2. Total collagen concentration in the different aortic tissue compartments (norm. are results from normal macroscopical appearing areas of the arterial tissue, plaq. show results from tissue areas with fibrous plaques). Mean ± SEM in the Type 1 diabetic (■) and the respective control group (▨) is shown. ddw, Dry defatted weight

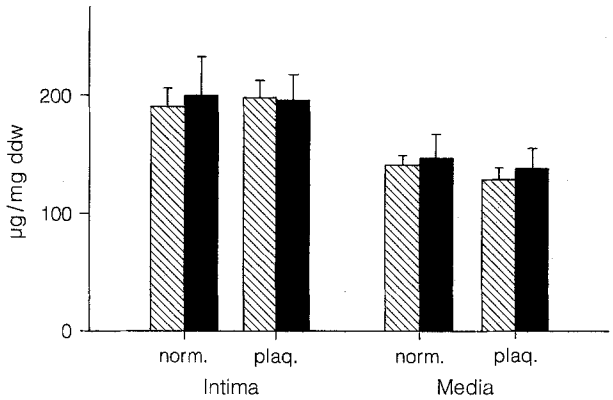

Fig. 3. Total collagen concentration in the different aortic tissue compartments (norm. are results from normal macroscopical appearing areas of the arterial tissue, plaq. show results from tissue areas with fibrous plaques). Mean ± SEM in the Type 2 diabetic (■) and the respective sex- and age-matched control group (▨) is shown. ddw, Dry defatted weight

psala, Sweden). The purified polyclonal antibodies against CNBr-treated pepsinated type IV and V collagen was specificity tested by ELISA, immunoblotting and immunochemistry. In indirect ELISA's, performed as described later only minor cross-reaction against other collagens could be detected. Immunoblotting experiments were done with CNBr-treated collagens separated by SDS-PAGE using a 12.5% polyacrylamide separation gel with a 4.0% stacking gel. After electrophoretic separation the CNBr-peptides were transferred using electrophoresis onto 0.45 µm nitrocellulose membranes (BioRad, Richmond, Va., USA) using a semi-dry electroblotting in a buffer, pH 8.3 of Tris/glycine/methanol. Following transfer, strips of nitrocellulose membranes were cut out and used for either staining in 0.05% amido black, 25% 2-propranol, 10% acetic acid or immunovisualisation with the two antisera. For immunovisualisation, nitrocellulose membranes was blocked by incubation in PBS, 0.05% Tween 20, 0.1% bovine serum albumin (BSA). After five washes in PBS, 0.05% Tween 20, the nitrocellulose membranes were incubated overnight at 4°C with diluted antiserum (1/100) in PBS, 0.05% Tween 20, 0.1% BSA. After five washes in PBS, 0.05% Tween 20 the membranes were incubated with peroxidase conjugated swine anti-rabbit immunoglobulins (Dakopatts P 217, Glostrup, Denmark) diluted 1:200 in PBS, 0.05% Tween 20, 0.1% BSA. The nitrocellulose membranes were washed as above and developed using 0.04% (w/v) 3-amino-9-ethylcarbazole with 0.01% hydrogen peroxide as substrate in a 50 mmol/l sodiumacetate, pH 6.0. In these experiments the immunoreactivity in the anti-CNBr-IV antibodies was found to be localized to two peptides derived from CNBr-treated type IV collagen, which migrated in the same range as the α1-CB-I 7 and 8 peptides of type I collagen in 12.5% SDS-PAGE (Fig. 1). No cross-reaction was observed against CNBr-peptides from type I, III and V collagen. Immunoblotting with anti-CNBr-V antibodies gave positive reactivity against 4–5 peptides derived from CNBr-treated type V collagen. No cross-reactivity against CNBr-peptides from type I, III and IV collagens was detected (Fig. 1).

Type IV and V collagen measurements. Antibody capture ELISA's were used for collagen quantitations. Wells of 96 multi-well microtitreplates (NUNC, Roskilde, Denmark) were coated with CNBr-treated type IV or type V collagen standards in different concentrations between 1 and 100 ng/ml or CNBr-treated aortic tissue diluted 1/300 in PBS (150 µl/well). The plates were incubated overnight at 4°C and after washing in PBS containing 0.05% Tween they were incubated for 2 h with anti-CNBr-IV collagen or anti-CNBr-V collagen diluted 1/500 in PBS containing 0.05% Tween and 0.1% BSA. After another wash the wells were incubated with swine-anti-rabbit Ig coupled to horseradish peroxidase (Dakopatts P 217) diluted 1/1200 for 2 h. After a final wash, wells were coloured using ortophenylendiamine as substrate for the peroxidase. Absorbance was read at 492 nm in an automatic ELISA reader. Standard curves were constructed based on values from dilutions of pepsin digested and CNBr-treated type IV and V collagen isolated from human placentae. The amount of standard collagens was calculated from hydroxyproline measurements and previous knowledge of the aminoacid composition in the two collagen types [27, 28]. The concentration of type IV and V collagens was expressed as µg/mg dry defatted weight. Dilution curves of CNBr-solubilized tissue from five individual aortic samples were parallel to the standard curves obtained with the two collagen types, when CNBr-supernatant dilutions between 1/800 and 1/100 were used in the ELISA's. Standards (3–6 µg) of type IV and V collagen added to a pool of pulverized aortic samples before CNBr-treatment could be recovered in the type IV and V ELISA quantitations at levels between 60 and 80%.

Hydroxyproline measurements. After hydrolysis of the pellets or fractions of the supernatants for 16 h at 116°C in 6 mol/l HCl, the hydroxyproline content was determined by a slight modification of the method of Stegeman and Stalder [29]. The use of reagent amounts in

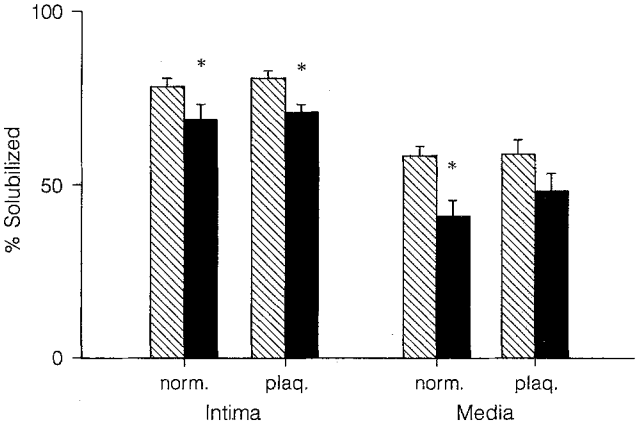

Fig. 4. The percentage of hydroxyproline-containing molecules solubilized by CNBr treatment in the different aortic tissue areas (norm. are results from normal macroscopical appearing areas of the arterial tissue, plaq. show results from tissue areas with fibrous plaques). Mean ± SEM in the Type 1 diabetic (■) and the respective sex- and age-matched control group (▨) is shown. * $p < 0.05$ compared to the control group

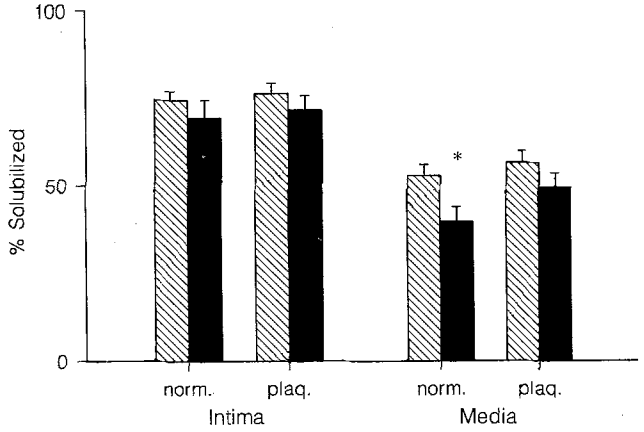

Fig. 5. The percentage of hydroxyproline-containing molecules solubilized by CNBr-treatment in the different aortic tissue areas (norm. are results from normal macroscopical appearing areas of the arterial tissue, plaq. show results from tissue areas with fibrous plaques). Mean ± SEM in the Type 2 diabetic (■) and the respective sex- and age-matched control group (▨) is shown. * $p < 0.05$ compared to the control group

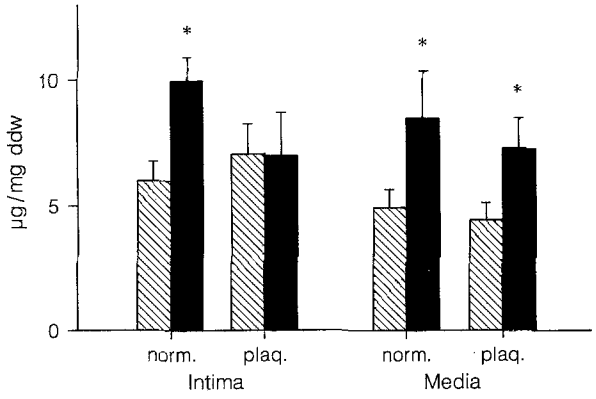

Fig. 6. Type IV collagen concentrations in the different aortic tissue compartments (norm. are results from normal macroscopical appearing areas of the arterial tissue, plaq. show results from tissue areas with fibrous plaques). Mean ± SEM in the Type 1 diabetic (■) and the respective sex- and age-matched control group (▨) is presented. * $p < 0.05$ compared to the control group. ddw, Dry defatted weight

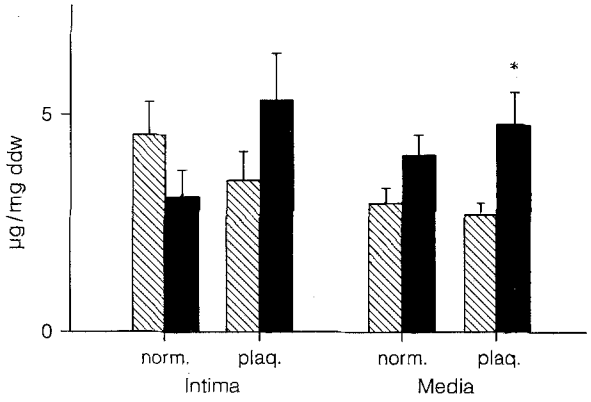

Fig. 7. Type IV collagen concentrations in the different aortic tissue compartments (norm. are results from normal macroscopical appearing areas of the arterial tissue, plaq. show results from tissue areas with fibrous plaques). Mean ± SEM in the Type 2 diabetic (■) and the respective sex- and age-matched control group (▨) is presented. * $p < 0.05$ compared to the control group. ddw, Dry defatted weight

the assay was scaled down so that it could be performed in micronic tubes (Flow Laboratories, The Netherlands) (final volume was 875 µl). Two hundred µl from each tube was transferred to microtitre wells and read automatically at 540 nm in an ELISA reader. From these measurements the fraction of dissolved hydroxyproline containing molecules was determined. The total collagen concentration (the sum of dissolved and non-dissolved hydroxyproline containing molecules) was estimated as described by Neuman and Logan [30].

DNA measurement. Samples of 5–8 mg dry, defatted and pulverized tissue was treated with 3 × 20 s sonication in 1 ml 0.15 mol/l NaCl, 0.015 mol/l Na_3citrate, 2 mmol/l EDTA, pH 7.0 and subsequently extracted in the same buffer overnight during constant magnetic stirring. After centrifugation with 8000 *g* for 15 min in 4 °C, DNA content was measured in the supernatant by a spectrofluorometric determination [31, 32]. A $^1/_{25}$ dilution of the samples and standards (calf thymus DNA, Sigma) from 0–1 µg/ml were mixed with bisbenzimid H 33258 (Fluka, Buchs, Switzerland) in a final concentration of 1.5×10^{-6}, and exitated at 360 nm, while emission was read at 450 nm.

Statistical analysis

All numerical parameters are expressed as mean ± SD or mean ± SEM as indicated. Student's *t*-test for unpaired observations and simple correlation analysis is used in the statistical evaluation. $P < 0.05$ in a two-tailed test is considered statistically significant.

Results

Basal values of aortic water content, extractable lipid content, dry weight/surface area and DNA content are presented in Table 2. There were no differences between the diabetic groups and the non-diabetic groups regarding these basal parameters.

The total concentrations of collagen in any of the tissue areas considered were the same in samples from the diabetic and non-diabetic subjects (Figs. 2 and 3). However, the collagen concentrations were higher in the tunica intima than the tunica media, both in plaque and non-plaque areas ($p < 0.05$ for all comparisons between intima and media) (Figs. 2 and 3).

The percentage of hydroxyproline-containing molecules dissolved by cyanogen bromide treatment is shown in Figures 4 and 5. It could be demonstrated that this fraction was decreased in tissue areas from diabetic patients compared to the non-diabetic patients, however, only in four tissue areas was the decrease statistically significant ($p < 0.05$ in normal intima, plaque intima and normal media in Type 1 diabetes and normal media in Type 2 diabetes). Moreover, as indicated in Figures 4 and 5, the fraction of solubilized hydroxyproline-containing molecules was lower in the tunica media than in tunica intima, both in plaque and normal areas in both diabetic groups and the control groups ($p < 0.05$ for all comparisons). No correlations were seen between the fractions of solubilized hydroxyproline-containing molecules and the duration of diabetes in both diabetic groups, this was the case for all tissue areas. The tissue concentrations of type IV collagen are presented in Figures 6 and 7. The results for the tunica media samples showed that the content of type IV collagen was approximately 60% higher among the diabetic pa-

Table 3. Aortic content of type IV and V collagen expressed per μg DNA

	Type 1 diabetes	Control group 1	Type 2 diabetes	Control group 2
Type IV collagen/DNA content (μg/μg)				
normal intima	4.2 ± 0.6	3.4 ± 0.7	4.2 ± 1.7	3.5 ± 0.8
plaque intima	6.4 ± 2.4	5.2 ± 1.4	7.6 ± 1.7	5.3 ± 1.9
normal media	5.5 ± 1.8[a]	1.8 ± 0.3	3.4 ± 0.6[b]	2.1 ± 0.4
plaque media	2.2 ± 0.4	1.8 ± 0.5	3.6 ± 0.9[a]	1.6 ± 0.2
Type V collagen/DNA content (μg/μg)				
normal intima	6.4 ± 1.8	6.4 ± 1.2	10.5 ± 7.0	6.8 ± 1.5
plaque intima	9.3 ± 3.1	12.2 ± 3.8	18.5 ± 6.9	15.2 ± 6.8
normal media	5.1 ± 2.5	2.9 ± 0.4	3.7 ± 0.9	3.8 ± 0.5
plaque media	1.9 ± 0.4	3.0 ± 0.5	4.3 ± 1.0	2.8 ± 0.5

[a] $p < 0.05$,
[b] $p = 0.12$ vs non-diabetic group.
Values are expressed as mean ± SEM

tients compared to non-diabetic subjects both in normal and plaque areas ($p < 0.05$, except in normal media in the Type 2 diabetic group where a p-value of 0.09 was calculated). The data from the type IV collagen measurements

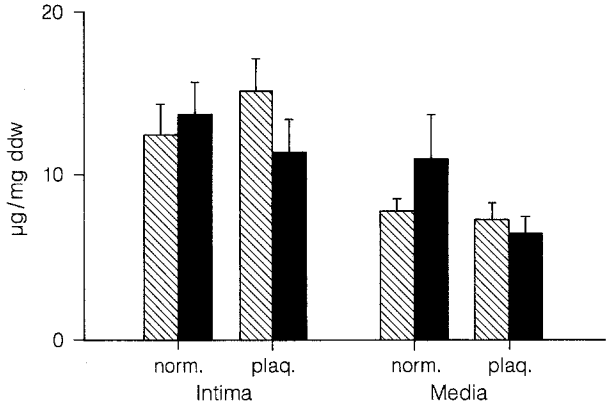

Fig. 8. Type V collagen concentrations in the different aortic tissue compartments (norm. are results from normal macroscopical appearing areas of the arterial tissue, plaq. show results from tissue areas with fibrous plaques). Mean ± SEM in the Type 1 diabetic (■) and the respective sex- and age-matched control group (▨) is presented. ddw, Dry defatted weight

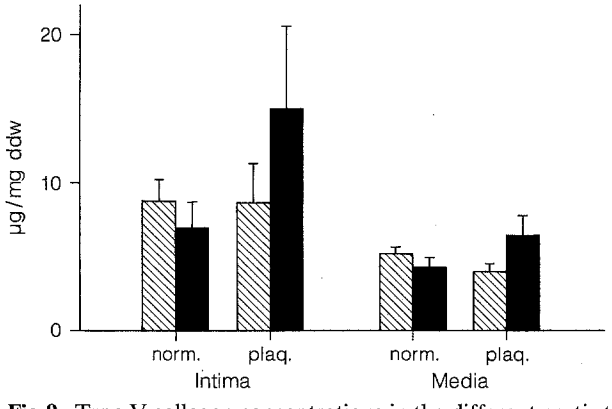

Fig. 9. Type V collagen concentrations in the different aortic tissue compartments (norm. are results from normal macroscopical appearing areas of the arterial tissue, plaq. show results from tissue areas with fibrous plaques). Mean ± SEM in the Type 2 diabetic (■) and the respective sex- and age-matched control group (▨) is presented. ddw, Dry defatted weight

in tunica intima were heterogenous as indicated in Figures 6 and 7, however higher concentrations in the normal intima areas in the Type 1 diabetic group were found compared to the non-diabetic group ($p < 0.05$). The type IV collagen concentration expressed per DNA content is shown in Table 3. Increased type IV collagen per DNA (μg/μg) was seen in the diabetic tunica media samples compared to the non-diabetic ($p < 0.05$ in normal media in Type 1 diabetes and in plaque media in Type 2 diabetes). No statistically significant differences were observed when the concentrations of type IV collagen in the tunica intima (both plaque and normal areas) were compared to values in the tunica media in all patient groups. When values of arterial type IV collagen concentrations from patients with hypertension were compared to those without or when values from males were compared to values obtained in females no differences were found. This was tested in all tissue areas in all groups. No correlations were obtained between the type IV collagen concentrations and diabetes duration. A tendency towards negative correlations between type IV concentrations expressed per tissue dry weight and age were observed in the various tissue areas in all patient groups. In contrast, no correlation was seen between age and the type IV collagen concentration expressed in relation to the DNA content.

The results of the measurements of type V collagen are shown in Figures 8 and 9. When the diabetic patient groups were compared to the non-diabetic groups no differences were found. However, the type V collagen levels were observed to be higher in the tunica intima samples compared to values from tunica media. The calculated p-values were less than 0.05 both in plaque and normal areas, except in normal areas in both Type 1 and Type 2 diabetes where the differences were not statistically significant. As for type IV collagen no relationship could be demonstrated between the content of type V collagen and gender or blood pressure.

Discussion

The main finding in the present study is the elevated tissue concentration of type IV collagen in aortic tunica media and the decreased solubility of total collagen in aortas

from patients with both Type 1 and Type 2 diabetes. The data calculated in the present study are based on examinations of tissue obtained at autopsy. This approach may give rise to selection bias, since not all relevant aspects of selection of patients and control persons can be controlled and since the prevalence of cardiovascular disease in the autopsy population may not reflect the prevalence in the general population. However, this is the only way that large amounts of human arterial tissue can be obtained. It is therefore not possible to rule out that factors, difficult to control retrospectively and known to play a role in development of arterial disease, such as dyslipoproteinaemia, smoking habbits and hypertension, could play an unknown role in some of the results. In order to obtain comparable tissue samples for the present investigation, we chose to select areas in the thoracic aorta with and without atherosclerosis in each individual case and investigate these separately. This selection was based on macroscopic observations of the vessel wall, which is an efficient approach but which may give rise to variation. It is, however, noteworthy that our biochemical data, especially in the results from tunica media, revealed the same set of differences between diabetic and non-diabetic samples both in vessel areas without atherosclerosis as well as in areas with atherosclerosis. This indicates that the tunica media alterations observed in the diabetic patients seems to be generalized and not influenced by atherosclerosis.

It is difficult to achieve information about the tissue concentration of collagens due to their large degree of insolubility. The method used to evaluate the concentration of type IV and V collagen in the present study combine, however, a high efficiency of solubilizing collagen molecules with a low degradation of type IV and V collagen epitopes during the solubilisation procedure as estimated by recovery experiments [23]. The method is based on chemical solubilisation of the aortic tissue with CNBr followed by immunochemical measurement of non-degraded type IV and V collagen epitopes in the dissolved tissue. The ability of CNBr-treatment to bring tissue proteins in solution is based on the ability of the chemical compound to specifically cleave peptides at methionine sites, and on the assumption that the derived smaller peptides are soluble. The dissolving-efficiency of the CNBr-treatment in this study was judged by measuring the percentage of total hydroxyproline brought into solution. Hydroxyproline is found in large amounts in collagen, but also to some extent in elastin [33, 34]. Since elastin only contains a very limited amount of methionine [33, 34] it is likely that this molecule is not dissolved during CNBr-treatment and a part of the non-solubilized hydroxyproline-containing molecules may therefore be elastin. The higher solubilization efficiency of tissue from tunica intima compared to tunica media, which contains large amounts of elastic fibres may therefore reflect differences in elastin content. The decreased solubility of hydroxyproline-containing molecules in samples from the diabetic patients may be due to differences in the amount of elastin, however several facts seem to favour another explanation, that collagen molecules in diabetes are less likely to dissolve due to non-enzymatic glycation, resulting in increased cross-linking of collagens [35, 36]. This notion is supported by the finding that increased amounts of non-enzymatically epsilon-amino-lysine-bound glucose can be measured in aortas from diabetic individuals [35]. Moreover, it is also in agreement with data from studies of rats with experimental diabetes, where a decreased solubilisation of the tissue collagen with CNBr have been ascribed to increased cross-linking of aortic collagen [36].

A tendency towards negative correlations between age and the aortic type IV collagen concentration expressed per dry weight was observed in both diabetic and non-diabetic patient groups. The difference in concentration of type IV collagen content between the two non-diabetic groups in tunica media may reflect decreased concentration with age, since the mean age was almost 20 years higher in the age- and sex matched control group for the Type 2 diabetic patients compared to the control group for the Type 1 diabetic patients. It has been shown that ultrastructural defined basement membranes in different locations thickens with age among diabetic patients as well as non-diabetic subjects [37, 38]. One could therefore expect increased tissue concentrations of basement membrane components with age and superficially our data seem to be contradictory to the ultrastructural investigations. A simple explanation could be that other components of the vessel wall accumulate to a higher degree with age, making it difficult to compare changes in the relative amount of type IV collagen with absolute basement membrane thickness. This notion agrees with our finding that expressed per DNA content, the type IV collagen concentration does not seem to change with age.

Only a few studies about the tissue concentrations of type IV and V collagen in relation to atherosclerosis are available. In 1981 Ooshima [39] reported higher concentrations of type V collagen in atherosclerotic intima compared to media. Later Morton and Barnes [40] and Murata et al. [41, 42] found higher amounts of type V collagen in proteolytic dissolved intimal tissue, both in areas with and without plaques in relation to media samples. In the reports by Murata et al. [41, 42] the proteolytic dissolvable type IV collagen was also considered and found to be increased in pools of intima samples with a high degree of atherosclerotic alterations. In the present study, we could confirm the earlier findings concerning higher type V collagen concentrations in intimal tissue, however no alterations in the tissue concentration of type IV collagen between intima and media were seen in our study. The higher concentration of type V collagen in intima has earlier been ascribed to hypercellularity in the atherosclerotic plaque [40], however this does not seem to be the explanation in our study, since the amount of type V collagen per DNA amount was higher in intima than in media.

An unsolved, but important problem is the question of whether the presence of a diabetic arterial disease is part of a generalized angiopathy. Clinical and patho-anatomical studies have demonstrated a correlation between manifestations of the diabetic microangiopathy and macrovascular diseases among diabetic patients [9, 16–18]. Furthermore, it has been previously demonstrated that one prominent feature of diabetic microangiopathy is thickening of basement membranes as recently reviewed

by Williamson et al. [43]. Therefore, the larger concentrations of basement membrane collagen in tunica media of diabetic aortas may prove to be of considerable interest. Attempts to quantitate the collagen content in glomerular basement membrane from diabetic patients have been reported [44–47]. The fractional collagen content of isolated glomerular basement membranes has been determined by amino-acid analysis and some data indicate increased amounts of collagen-specific aminoacids in diabetes, but this is still controversial. We have measured the total basement membrane collagen concentration in a mixture of tissue, making comparison to these earlier data on isolated glomerular basement membranes difficult. However, Karttunen et al. [48] used proteolytic digestion of the tissue in combination with immunochemical determination of the 7S-part of type IV collagen to determine the type IV collagen concentration in kidneys. No alterations in the total tissue concentration of type IV collagen in diabetes was found, however only four diabetic patients were investigated. It has been shown, using both semiquantitative and quantitative techniques, that PAS-positive material accumulates in coronary arteries from Type 1 diabetic patients [9, 10]. The precise nature of the accumulated substance is not known, although some of it seems to be the basement membrane associated glycoprotein fibronectin, as suggested by immunohistochemical and biochemical investigations on diabetic aortas [4]. The results obtained in the present study suggest that some of the previously described PAS-positive material in the tunica media may represent accumulation of basement membrane collagen. Few studies have focused on arterial changes in experimental diabetes, nevertheless our results fit well with the work of Reinilä [49], who has described, semiquantitively, thickening of basement membranes in aortas from a small number of rats with experimental diabetes. Also, our data are compatible with results from earlier experimental studies, where it was shown that both fibronectin and basement membrane-like material accumulates in cultures of smooth muscle cells from rabbit aortas, when the cells are exposed to serum from diabetic patients [50, 51]. In conclusion, the data obtained in the present investigation support the idea that a macroangiopathy, different from atherosclerosis, exists in diabetic patients.

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