Biochemical and biophysical alterations of the 7S and NC1 domain of collagen IV from human diabetic kidneys

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Summary Glycation of basement membrane collagen IV has been implicated as a major pathogenetic process leading to diabetic microvascular complications. To evaluate the relevance of carbohydrate-induced modifications on collagen IV in diabetic nephropathy, we isolated the cross-linking domains 7S and NC1 from the glomerular basement membrane (GBM) of patients with diabetes mellitus. Modifications characteristic for glycated proteins were identified when the domains from diabetic kidney were compared with the same domains from human placenta as an unmodified control. In both domains a marked formation of inter- and intramolecular cross links could be demonstrated by SDS-PAGE. Further-

more circular dichroism studies showed a decrease in helicity of the 7S domain from human diabetic kidneys of 13%, indicating denaturation already at room temperature. Thermal transition profiles, showing a shift of the denaturation temperature towards a lower temperature, with loss of a distinct second melting point, confirmed this observation. Our data provide further evidence for a possible role of protein-modification by glycoxidative reactions in the onset of diabetic nephropathy in vivo. [Diabetologia (1998) 41: 1073–1079]

Keywords Circular dichroism, collagen IV, diabetes, glycation, nephropathy.

Glycation and glycoxidative reactions have been recognised to be a major pathogenetic principle for biochemical and biophysical alterations of proteins in diabetes mellitus [1–3]. Both processes have been shown to increase in humans with age and even more in patients with diabetes mellitus with the duration of the disease [4–8].

Glycoxidative reactions, e.g. the Maillard reaction, are initiated by the condensation of a reducing sugar with an ϵ -amino group of lysyl- or hydroxylysyl-residues of proteins [9]. The resulting Schiff-base is stabilised subsequently by Amadori rearrangement and processed further in very diverse and not yet fully

elucidated chemical reactions resulting in a variety of cross-linked compounds. Only a small number of those, including pentosidine, have been characterised in structure and chemical composition [2, 10–16]. Glycation of proteins is associated with the formation of high-molecular aggregates that are stabilised by non-reducible cross-linking components [13, 17–18] with characteristic fluorescence spectra [6, 19, 20]. Circular dichroism (CD) spectroscopy studies of glycated proteins exhibited a damaging effect of the modifications on protein structure and stability [21, 22].

In diabetic nephropathy the disordered morphology and functional deficiency of the glomerular basement membrane (GBM) has been explained by glycation of the collagen IV network providing the mechanical scaffold of the membrane texture. Collagen IV monomers consist of a collagenous major triple-helical domain with two specific cross-linking domains, the NC1 domain at the C-terminal end and the N-terminus the 7S domain [23–26], facilitating

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Abbreviations: GBM, Glomerular basement membrane; CD, circular dichroism

the aggregation of collagen IV–monomers to a large, chicken-wired network by a highly specific interaction [27–29].

An influence of glycation of collagen IV and other integral proteins of GBM on their functional properties, e.g. collagen-network formation [30], basement membrane architecture [31] and binding of physiological and non-physiological ligands [32–35] has been shown by several *in vitro* and *in vivo* studies.

The effects of carbohydrate-induced reactions *in vitro* on two distinct domains of collagen IV (7S domain and NC1 domain) have been reported recently [22]. Our study was aimed to describe biochemical and biophysical properties of the collagen IV cross-linking domains 7S and NC1 from human kidneys obtained from patients with diabetes mellitus Type II. By comparison with native domains isolated from human placental basement membrane it became evident that glycoxidative modifications in diabetic nephropathy may have a decisive role in both the onset and progression of the disease.

Materials and methods

Extraction of the collagen IV cross-linking domains 7S and NC1 from human placenta and from human kidneys from patients with long-standing diabetes mellitus. Tissue from kidneys of three patients with diabetes mellitus Type II who had had the disease longer than 10 years was obtained from autopsies at the department of pathology after consent of the patient had been obtained in advance. The glomeruli were isolated from homogenized kidneys by sequential sieving. The GBM was then prepared by ultrasonic degradation of the glomeruli [36]. Normal human placental tissue was received from the department of Obstetrics and Gynaecology at the University of Lübeck and served as native control tissue. The 7S domain and the NC1 domain were prepared according to Weber et al. [26]. Briefly, 7S and NC1 were isolated by two consecutive collagenase digests (collagenase A, Boehringer Mannheim, Mannheim, Germany) of Guanidinium-hydrochloride (Sigma, Deisenhofen, Germany) extracted human placental membranes and human GBM, followed by molecular sieve chromatography on a superose 12 resin (Pharmacia, Freiburg, Germany). The identity of both the 7 S domain and the NC1 domain was shown by SDS-PAGE and immunoblotting using suitable antibodies.

Electrophoresis. SDS-PAGE was performed on slab gels [37] using 4% stacking and 10% separation gels. After electrophoretic separation gels were stained with Coomassie blue.

Immunoblotting. Electrophoretically separated proteins were transferred from SDS-PAGE-gels to nitrocellulose sheets (Schleicher and Schüll, Germany) using a protocol for semidry blotting [38]. The 7S and the NC1 domain were marked with specific antibodies from our own laboratory and stained by a colour reaction initiated by a second horseradish peroxidase-coupled antibody (DAKO, Germany).

Fluorimetric analyses. Fluorescence spectra were measured at an excitation wavelength of 335 nm and 370 nm. Fluorescence was determined for emission spectra from 370/400 nm to 500

Fig. 1. 6% SDS-PAGE of 7S from human placenta (lane 1 and 3) and human diabetic kidney (lane 2 and 4) marker, non-collagenous protein (lane 5) lane 1–2: reducing conditions (addition of 20% mercaptoethanol to sample buffer) lane 3–5: non-reducing conditions

nm. Readings were obtained with a Perkin Elmer Luminescence Spectrometer LS 50 B. The fluorescence was normalised to the protein content of the sample as determined by dry weight measurement.

Circular dichroism and transition profiles of the 7S domain. CD spectra were recorded on a Jasco J-500A spectropolarimeter equipped with a temperature-controlled quartz cell of 1 cm length (Gilford, Oberlin, USA). For transition curves conversion to random coil corresponds to the ellipticity at 95 °C, typical for totally denatured collagen IV. Thermal transition curves were recorded at a fixed wavelength of 221 nm while the temperature of the sample was linearly raised at a rate of 25 °C/h using a temperature programmer (Gilford). Helicity was expressed as the per cent of the value at room temperature. It was defined to be 100% at room temperature and 0% at 95 °C. The sample concentration was 100 µg/ml in 0.05% acetic acid. A change of thermal stability due to radiation damage could not be detected. The statistical error at 221 nm for one measurement was calculated to be 5% of the ellipticity.

Results

Extraction of 7S and NC1 from human kidney and placenta and demonstration of intramolecular and intermolecular cross-links. SDS-PAGE of the 7 S domain from human placenta and kidneys from patients with long-standing diabetes mellitus was performed under reducing and non-reducing conditions. In our preparation from both tissues the 7S domain existed in both the long (360 kD) and the short form (250 kD). The domain from diabetic kidneys showed non-reducible cross-links (Fig. 1), while most of the native 7S domain from human placenta was separated into

Fig. 2. Western Blotting of a 6 % SDS-PAGE of 7S from human placenta (lane 1) and human diabetic kidney (lane 2) under reducing conditions (addition of 20 % mercaptoethanol to sample buffer)

Fig. 3. 10 % SDS-PAGE of the NC1 domain from human placenta (lane 2) and human diabetic kidney (lane 3). Molecular weight-standard (collagen I) with bands at 300 kD, 200 kD and 100 kD (lane 1)

oligomers ranging in size from 180-kD under reducing conditions [25]. In contrast, hardly any oligomers could be seen in the fraction from diabetic kidney, similar to what has been described earlier for the *in vitro* glycated 7S domain [21]. Western blotting with anti-7S-antibody confirmed the identity of the 7S domain (Fig. 2).

The typical gel electrophoretic pattern with a double band at 60 kD was found for the NC1 domain. SDS-PAGE and Western blotting of the NC1 domain from diabetic kidney showed the formation of high-molecular weight material (Fig. 3 and 4) with additional bands at molecular weights of 70 kD, 80 kD and 100 kD besides the normal double band at 60 kD. These high molecular weight bands showed a positive staining with anti-NC1 antibodies and represent NC1 trimers and tetrameres.

Detection of cross-links by fluorescence. Cross-link formation in both domains was also shown by increased fluorescence at two specific excitation wave-

Fig. 4. Western Blotting of a 10 % SDS-PAGE of the NC1 domain from human placenta (lane 1) and human diabetic kidney (lane 2)

lengths. Excitation at 335 nm showed a marked increase in the pentosidine-like fluorescence [13] at 385 nm for both domains from diabetic kidney. Emission at 440-nm upon excitation at 370 nm ("collagen-linked fluorescence" [34]) is increased 12-fold (7S) and 78-fold (NC1), respectively, in protein from the diabetic organ.

Conformation and thermal stability of the 7S domain. To assess the effect of glycation *in vivo* on triple-helical conformation the collagenous 7S domain was examined by CD-spectroscopy. For the native 7S domain the spectrum was in good agreement with that reported previously [25]. In contrast, the 7S domain from diabetic kidney showed a decrease in helicity of 13 % in relation to control measured by the difference between the signals at 221 nm for the domain at room temperature and at 95°C. Unlike the spectrum for 7S domain from human placenta the signal at 221 nm for the protein from human diabetic kidney after thermal degradation at 95°C did not disappear completely (Fig. 5).

Thermal transition profiles were recorded to detect a possible influence of glycation on the stability of the 7 S domain. For the 7S domain from human placenta we showed a melting profile with two distinct melting points as described previously by Ristelli et al. [25]. Our recordings, however, showed the second melting point to be at a higher temperature (80°C vs 72°C as measured by Ristelli et al.). The melting profile of the 7S domain from diabetic kidneys followed the profile of the control until the first melting point was reached. Beyond that temperature the degree of helicity decreased steadily and did not level off in a plateau. At 65°C the degree of helicity

was 50 %, whereas it was markedly higher in the 7S domain from human placenta (Fig.6).

Discussion

The notion that hyperglycaemia is the major pathogenic feature for the long-term sequelae of diabetes mellitus is widely accepted [2, 39]. Diabetic nephropathy is the most important complication with a cardiovascular mortality 37 times higher than that of the general population [40].

In addition to the characteristic histological findings in diabetic nephropathy specific alterations have been found in basement membrane glycoproteins from diabetic patients [31, 41–43]. Functional impairment of the carboxyterminal domain of collagen IV in interacting with the major triple-helical domain has been shown earlier [30]. To find additional evidence for the impact of altered cross-linking domains of collagen IV for the pathogenesis of diabetic nephropathy, we investigated the effects of long-standing diabetes mellitus on the 7S domain and the NC1 domain of collagen IV isolated from the GBM.

In line with recent observations derived from *in vitro* glycation experiments our study shows that the domains from collagen IV of diabetic GBM may be the target of cross-link mechanisms in a diabetic metabolic state. These cross-links were not destroyed by heat nor were they susceptible to reducing agents (5 min at 95 °C in the presence of mercaptoethanol). For the NC1 domain we were able to indicate the presence of cross-linking by showing trimers (high-molecular weight material at 90 kD) and tetramers (high-molecular weight material at 110 kD). These aggregates cannot be found in native tissue [30, 44].

A second line of evidence for cross-linking of collagen IV domains from kidneys of patients with diabetes mellitus was obtained by fluorescence spectroscopy. Fluorophores typical for glycosylated proteins form on both the 7S and the NC1 domain. On the NC1 domain they can be found to a greater extent than on the 7S domain probably because of its higher content in arginyl- and lysyl residues [25, 26].

There was, however, no appreciable change either in the amino acid composition or in the content of the hydroxylysyl-bound glycosides when glycosylated

Fig. 5a,b. Circular dichroism spectra of the 7S domain from (a) kidneys of patients with diabetes mellitus and (b) human placenta (as native control) at room temperature [—] and after thermal denaturation (30 min at 95 °C) [---]. Arrows display the difference in ellipticity between normal and thermally degraded 7S domain. For 7S-domain from kidneys of patients with diabetes mellitus the difference is only 87 % of the value for the control

Fig. 6. Melting profiles of the 7S domain from kidneys of patients with diabetes mellitus [---] and human placenta (as native control) [—] recorded with circular dichroism at 221 nm

and non-glycated domains were analysed (data not shown). This effect which had been observed earlier by Trueb et al. [45] could in part reflect problems with the extraction of proteins from modified tissue. The sensitivity of amino acid analysis may be too low to detect the subtle changes expected in the amino acid composition of the modified domains.

Formation of cross-links, however, had an impact on collagen triple helix stability and this has been shown previously for collagen II from intervertebral discs of elderly patients [22]. Our experiments indicated a denaturation with loss of the triple-helicity of the 7S domain already at room temperature. Melting profiles confirmed this observation showing a lower thermal stability for the glycosylated 7S domain with a shift of the second part of the melting profile (reflecting denaturation of the central part of the domain) towards higher temperature. Thus it seems likely that the cross-linking modifications that altered the conformational stability of the protein are located in the central part of the molecule. In vitro glycosylated 7S domain, however, investigated in a recent study failed to show the detrimental effects at room temperature [21], indicating that glycation of proteins in vivo and in vitro may follow different pathways.

The ideal control for studies on in vivo modified proteins would be a native domain from the same species and the same tissue. In our study we chose a native domain from human placenta as a readily available tissue. The triple-helical collagen IV molecule is composed of three α -chains that are expressed in varying quantities in different tissues. So far six different α -chains have been identified and cloned [46–49]. In the GBM only 27% of the chains are not solely composed of $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$. Other trimers can be formed by $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ and in small amounts $\alpha 5(\text{IV})$ either in combination or together with $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ [50]. In diabetic nephropathy

it is possible that $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ are expressed in larger quantities than in healthy kidneys [48, 51]. All α -chains show a remarkable homology [52] with the most highly conserved parts in the end regions of the chains and the number of lysyl residues involved in glycation reactions does not vary appreciably. It has been reported that there are regions on the NC1 domain in which all chains differ in amino acid composition and that these regions may have a role in homo dimerisation of the α -chains [53]. In contrast, a striking similarity can be observed for the interspaced cross-linking cysteine-residues in the otherwise strictly triple-helically 7S-domain. Therefore, it seems that triple-helicity of collagen IV isolated from different human tissues is comparable. In contrast, a 13%-decrease in triple helicity as well as an appreciable difference in thermal transitions profiles, as seen in our experiments, indicates a severe disturbance of molecular integrity although the mechanism by which the thermal stability of collagen IV as well as other collagen types is reduced, can, as yet, not be explained.

The loss in triple-helical conformation of the 7S domain as well as the central triple-helical domain and the formation of non-physiological oligomers may be of pathophysiological importance for the molecular basis of diabetic complications as the intact triple-helical conformation of collagen is important for a variety of functions. Self-aggregation to the collagen IV-network may be a function of the terminal domains, whereas binding of ligands, e.g. laminin and heparansulphate-proteoglycan [54–56] and interaction with adjacent cells [57, 17] can be attributed to the central triple-helical domain which is also a target for glycoxidative reactions. The impairment of one or some of these functions of collagen IV might be responsible for the decrease in renal performance in incipient diabetes mellitus [58].

Our data provide the first evidence for non-enzymatic modification on the 7S domain and the NC1 domain of collagen IV in the GBM of patients with diabetes mellitus similar to those reported for these domains after glycation in vitro by Raabe et al. [21] and Tsilibary et al. [30]. The effects on protein structure and stability, however, seem to be more severe in vivo than in vitro and have an impact on their structural integrity. These findings underline the importance of glycation as a possible pathogenetic principle for the onset and progression of diabetic microangiopathy and in particular for diabetic nephropathy.

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