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The AGE product N^{ε}–(carboxymethyl)lysine serum albumin is a modulator of proteoglycan expression in polarized cultured kidney epithelial cells

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Abstract

Aims/hypothesis. Changes in kidney function in diabetes could be due to changes in the kidney basement membranes. Proteoglycans are important constituents of this kidney extracellular matrix. This study explored the possibility that advanced glycation end products affect proteoglycan synthesis in cultured kidney epithelial cells.
Methods. Madin Darby Canine Kidney (MDCK) epithelial cells were cultured with either low glucose (5 mmol/l), low glucose with 10 μg/ml of N^{ε}-(carboxymethyl)lysine bovine serum albumin (CML-BSA) or high glucose (25 mmol/l). From day 7–8 cells were labelled with either [^{35}S]sulphate or [^{3}H]glucosamine for 24 h. Labelled macromolecules were purified by gel and ion exchange chromatography, and isolated proteoglycans analysed by gel chromatography and electrophoresis.
Results. The CML- BSA treatment reduced the proteoglycan synthesis in MDCK cells. Neither the type of glycosaminoglycan chains made nor the molecular size of the chains was affected.
Conclusion/interpretation. At concentrations found in the plasma of diabetes patients CML-BSA, decreases proteoglycan expression in kidney epithelial cells. Advanced glycation end products could, accordingly, promote pathological changes in kidneys of diabetics. [Diabetologia (2001) 44: 488–494]

Keywords Advanced glycation end product, diabetes, proteoglycans, kidney, MDCK cells, CML-BSA.

In patients with diabetes or chronic renal failure there is a progressive formation of advanced glycation end products (AGEs). The formation of AGE is proportional to the blood glucose concentrations over a long period of time [1]. Both serum and extracellular matrix proteins could be glycated and contribute to renal and vascular complications in diabetes [2]. The basement membrane of the kidney glomeruli increases in size in patients with diabetes [3]. One important class of molecules in this matrix is the proteoglycans. Changes in the amounts and structures of heparan sulphate proteoglycans can be seen in the basement membrane of glomeruli in diabetics [4]. Proteoglycans are important for the filtration in glomeruli and the proper construction of the matrix, through interactions with collagen IV and laminin [5].

MDCK II and I are kidney epithelial cells with different characteristics. The MDCK II cells resemble those of the proximal tubules, while MDCK I have characteristics of distal tubules cells [6]. We have previously established [7,8] that both MDCK I and II cells cultured on filters express mostly heparan sulphate proteoglycans, including perlecan. A major portion of heparan sulphate proteoglycans is secreted to the basolateral medium, which contains many of the components found in the basement membrane in vivo.

Although the epithelial cells do not face blood circulation they will be exposed to high glucose concen-

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Abbreviations: CML-BSA, N^{ε}-(carboxymethyl)lysine bovine serum albumin; cABC, chondroitinase ABC; GAG, glycosaminoglycan; MDCK, Madin Darby Canine Kidney.

trations and AGEs in the interstitium in the diabetic state. One such AGE is N^{ε}-(carboxymethyl)lysine (CML) serum albumin, which has been detected in kidneys of patients with diabetic nephropathy [9] and been found to increase in serum from patients with diabetes [10]. We aimed to investigate the effects of AGEs on the expression and sorting of proteoglycans in MDCK cells.

Subjects and methods

Cell cultures. We cultured MDCK I and MDCK II cells on polycarbonate filters at a density of 10^6 cells for each filter in Dulbecco's modified Eagle's medium (DMEM) or alpha-Modified Eagles medium (alpha-MEM), with 5 % fetal calf serum, antibiotics and 2 mmol/l L-glutamine. The media contained 5 mmol/l glucose (alpha-MEM), 25 mmol/l glucose (DMEM) or 5 mmol/l glucose with 10 ug/ml CML-BSA (Bio-Whittaker, Verviers, Belgium, RPMI 1640 was from Gibco BRL, Paisley, Scotland). The cells were cultured in these media for 7 days before labelling with either [^{35}S]sodium sulphate or [^{3}H]glucosamine (Amersham, Buckinghamshire, UK). Established monolayers were verified by measuring the transepithelial resistance with Millicell-ERS equipment (Millipore Corp., Bedford, Mass., U.S.A.). Cells were labelled with [^{35}S]sodium sulphate as described [7]. Separately cells were labelled with [^{3}H]glucosamine (0.1 mCi/ml) using alpha-MEM or DMEM, containing either 5 or 25 mmol/l glucose, respectively. The CML-BSA treated cells were labelled in low glucose medium.

Preparation of CML-BSA. We prepared CML-BSA according to a method described previously [11]. The purity of the CML-BSA preparation was assessed by amino acid composition analysis. Lysine was the only amino acid residue modified by CML formation. The CML-BSA preparation contained 20 CML-modified lysine residues for each molecule albumin corresponding to 34 % of the available lysine residues [12]. The CML-BSA used in this study is more modified than human serum albumin. We used this CML-BSA as standard for measuring CML in human serum and found that a concentration of 10 μg/ml CML-BSA corresponds to human concentrations of CML, the dominant part of CML being bound to human serum albumin [10].

Preparative gel filtration and ion exchange chromatography. Apical and basolateral medium and cell fractions were solubilized in 4 mol/l guanidine/2 % Triton X-100 in 0.05 mol/l sodium acetate buffer, pH 6.0 and subjected to Sephadex G-50 fine gel filtration (Amersham Pharmacia Biotek AB, Uppsala, Sweden) in 0.05 mol/l Tris-HCl buffer pH 8.0 with 0.15 mol/l NaCl. To measure amounts of [^{3}H]-labelled proteoglycans secreted to apical and basolateral media, [^{3}H]-labelled macromolecules were subjected to DEAE Sephacel (Pharmacia) ion-exchange chromatography. We applied 1 ml samples to 0.6 ml columns and washed them with 0.2 mol/l NaCl. Proteoglycans were eluted with 2 mol/l NaCl in 0.5 ml fractions. The [^{35}S]-labelled material was isolated by gel filtration on Sephadex G50 columns in 0.05 mol/l Tris-HCl pH 8.0 with 0.15 mol/l NaCl. [^{35}S]-labelled samples were not purified on DEAE columns because proteoglycans represent the majority of sulphated macromolecules in MDCK cells [7].

Analytical ion exchange chromatography and gel filtration. Both [^{35}S]sulphate- and [^{3}H]glucosamine-labelled samples were analysed on MonoQ (Pharmacia) ion exchange columns. Generally, 20.000 cpm from each sample with 0.25 mg/ml of chondroitin sulphate internal standard was applied and eluted with a gradient of 0.15 to 2 mol/l NaCl in 0.05 mol/l sodium acetate buffer pH 6.0. Fractions were collected and subjected to scintillation counting. The elution profile of the standard was visualised by adding 200 ul dimethylene blue reagent (8 mg/500 ml) to 50 ul of each fraction in 96 well plates and measured at 590 nm on Titertek (EFLAB, Helsinki, Finland) spectrophotometer. To determine the type of GAG chains, samples were treated with chondroitinaseABC (cABC, Seikagaku Kogyo, Tokyo, Japan) to degrade chondroitin/dermatan sulphate, or nitrous acid (HNO_2) to degrade heparan sulphate [13] and subjected to MonoQ ion exchange chromatography. We have not detected any degradation of the core proteins of the major proteoglycans expressed by MDCK cells after HNO_2 treatment (Kolset, unpublished observation). The [^{35}S]-labelled samples were subjected to NaOH treatment (0.5 mol/l final concentration) overnight at room temperature to liberate intact GAG chains from the protein cores. Samples were subjected to Superose 6 (Pharmacia) gel filtration in 0.05 mol/l Tris-HCl pH 8.0 with 0.15 mol/l NaCl to detect possible differences in GAG molecular sizes.

Gel electrophoresis. The [^{35}S]-labelled macromolecules recovered after Sephadex G-50 gel filtration or immunoprecipitations [7], or [^{3}H]-labelled proteoglycans, were boiled in sample buffer and subjected to SDS-PAGE under reducing conditions, using 4–20 % polyacrylamide gels (Novex, Encinitas, Calif., U.S.A.) and [^{14}C]-labelled molecular mass standards (Amersham). Samples were treated with nitrous acid or cABC before SDS-PAGE. The gels were treated with Amplify (Amersham), dried, and subjected to autoradiography with Fuji Medical X-ray film (Fuji, Tokyo, Japan).

Results

The MDCK I and II cells were cultured on semipermeable filters for one week in 5 mmol/l glucose, 10 μg/ml CML-BSA with 5 mmol/l glucose, or 25 mmol/l glucose. Thereafter the cells were labelled with [^{35}S]sulphate or [^{3}H]glucosamine for 24 h and medium and cell fractions were harvested and analysed.

The incorporation of [^{35}S]sulphate into macromolecules was measured after gel chromatography. The amounts in both cell strains decreased after exposure to CML-BSA (Fig. 1). The total incorporation (cell, apical and basolateral medium) was 50 % of the control (low glucose) sample in MDCK I and 55 % of the control sample in MDCK II cells. After incubation in high glucose the incorporation of [^{35}S]sulphate into macromolecules decreased in MDCK I cells (50 % of control) but not in MDCK II cells (Fig. 1). The apical to basolateral secretion ratio of [^{35}S]sulphated macromolecules was not considerably affected in either MDCK I or II, by either CML-BSA or high glucose. The amount of [^{35}S]macromolecules in basolateral medium of MDCK II was higher in cells exposed to high glucose but was not evident for isolated [^{35}S]proteoglycans, suggesting a possible effect of non-proteoglycan material on sulphation.

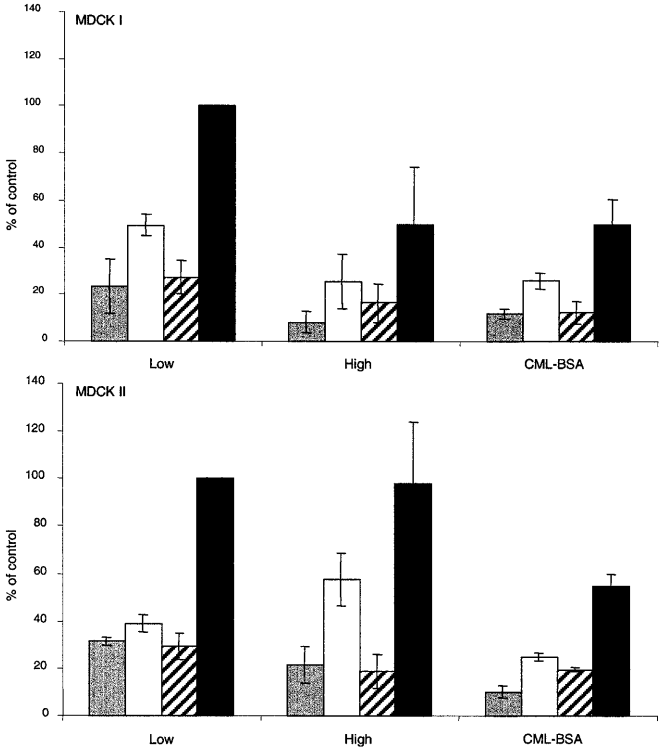

Fig. 1. Expression of [^{35}S]-labelled macromolecules in MDCK cells. The [^{35}S]-labelled macromolecules were recovered from the apical medium (▒), basolateral medium (□) and the cell fraction (▨). Total of fractions (■). Cells were cultured in high glucose, low glucose or in the presence of physiological concentrations of CML-BSA. Values are average of three separate experiments in both MDCK I and MDCK II cells, each experiment done in six parallels ± SD. The total in the control (low glucose) sample in each experiment is set to 100 %

The incorporation of [^{35}S]sulphate into macromolecules is a measure of both proteoglycans and other sulphated macromolecules, although proteoglycans are the major component in MDCK cells [7]. The [^{35}S]macromolecules were subjected to analytical MonoQ ion exchange chromatography. The amount of [^{35}S]proteoglycans was calculated on basis of the amount of material eluting at high ionic strength, related to the elution profile of chondroitin sulphate internal standard. We found CML-BSA decreased the amount of total [^{35}S]proteoglycans synthesised to 67 % and 64 % of controls in MDCK I and II, respectively (Fig. 2). Only minor decreases were seen in both cell types after high glucose treatment.

Incorporation of [^{3}H]glucosamine into macromolecules was almost completely inhibited in cells cultured in high glucose, probably reflecting low specific activity of labelled material due to the high glucose concentration. When MDCK I and II cells were, however, incubated with 10 μg/ml of CML-BSA the total amount of [^{3}H]macromolecules was 60 % of control samples, and 95 % of control samples, respectively (not shown). The [^{3}H]macromolecules were subjected to DEAE ion exchange chromatography to measure the amount of [^{3}H]proteoglycans, which decreased after exposure to high glucose (Fig. 3). After CML-BSA treatment, the amount of [^{3}H]proteoglycans was, however, 92 % of control in MDCK I and 81 % of control in MDCK II. These results show that CML-BSA reduced the expression of [^{35}S]-proteoglycans but had no effect on [^{3}H]labelled proteoglycans. The difference in effect between the two labelling techniques suggests a decrease in the sulphation of proteoglycans (see below).

To determine the type of GAGs expressed, [^{35}S]proteoglycans were subjected to cABC treatment and deaminative cleavage. The [^{35}S]proteoglycans were almost exclusively of the heparan sulphate type (Fig. 4), shown by susceptibility to deaminative cleavage and resistance to cABC treatment. It is also evident that the amount of heparan sulphate synthesised by MDCK I cells did not change when cells were treated with CML-BSA or high glucose concentrations. Similar results were obtained with material from MDCK II cells (not shown).

To investigate if the different treatments lead to the expression of different types of proteoglycans, both [^{3}H] and [^{35}S]labelled proteoglycans were subjected to SDS-PAGE. The pattern of proteoglycan expression is somewhat different in MDCK I and II cells after labelling with [^{35}S]sulphate. In both cell types, however, the molecular sizes and the amount of proteoglycans recovered did not change after the different treatments (Fig. 5 and 6). After labelling with [^{3}H]glucosamine, the proteoglycan pattern was somewhat different when compared with after the [^{35}S]sulphate labelling. The [^{3}H]proteoglycans expressed by both MDCK I and II cells were, however, almost identical, irrespective of pretreatment (not shown).

The [^{3}H]-labelled proteoglycan material was also subjected to SDS-PAGE after deaminative cleavage or chondroitinase ABC. There were no differences in the amount of heparan sulphate or chondroitin sulphate in MDCK II or I cells treated with high or low glucose or CML-BSA, as previously shown for [^{35}S]-labelled proteoglycans (Fig. 4).

The decreased expression of [^{35}S]proteoglycans found in cells treated with CML-BSA and the lack of effect on [^{3}H]proteoglycan synthesis suggests a decrease in sulphation of the proteoglycans or a decrease in the molecular size of the GAG chains. MonoQ ion exchange chromatography did not reveal any differences in elution profiles when compared with the internal standard, which suggests that the polyanionic properties of the GAG chains were not changed after CML-BSA treatment. The decrease in [^{35}S]sulphate incorporation, and the lack of effect on [^{3}H]glucosamine labelled material, suggests, however, minor changes in sulphation not detectable by ion exchange chromatography. Free GAG chains obtained by alkali treatment were also analysed by Superose 6 gel chromatography. No differences in elu-

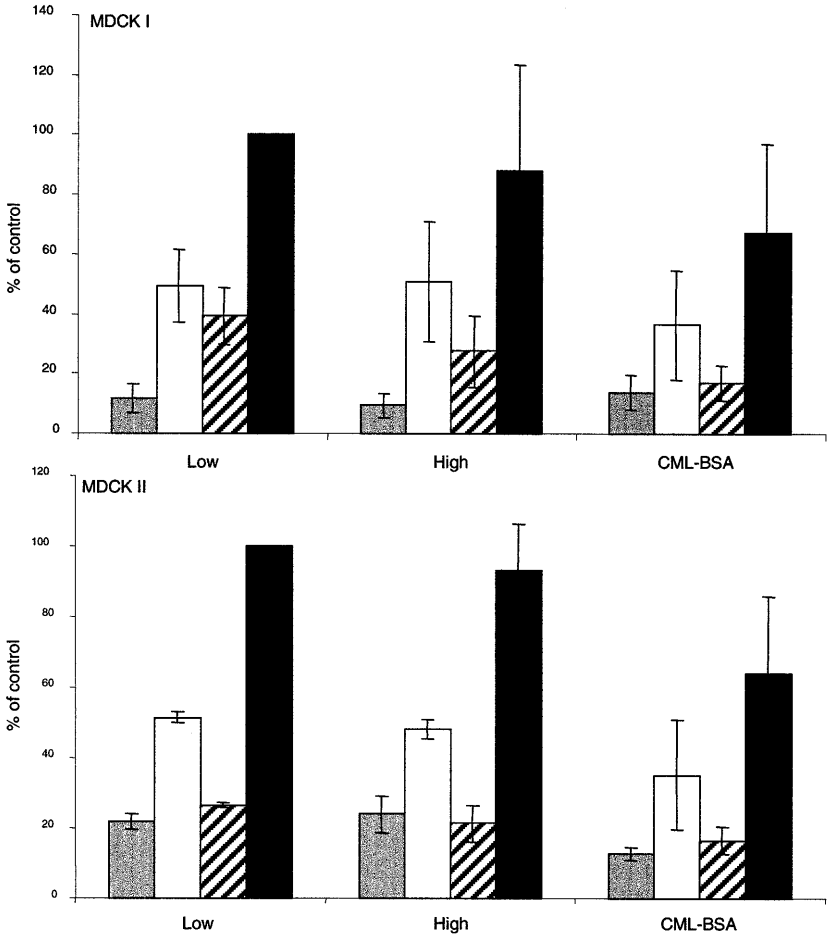

Fig. 2. Expression of [^{35}S]-labelled proteoglycans in MDCK cells. [^{35}S]- labelled macromolecules from MDCK I and II cells were recovered from the apical medium (▩), basolateral medium (□) and the cell fraction (▨). Total of fractions (■). All fractions were subjected to MonoQ ion exchange chromatography. The material eluting in the proteoglycan peak area was pooled and measured. The values shown are from two separate experiments, each done in triplicates ± SD. The total in the control (low glucose) sample in each experiment is 100 %

tion profiles of the GAG chains could be observed, showing no change in the size of these chains after CML-BSA exposure.

The effects of CML-BSA on proteoglycan expression could be due to the effect on one particular proteoglycan. Antibodies against syndecan and CD44 (proteoglycan form in MDCK cells, Kolset, unpublished), were used to precipitate [^{35}S]proteoglycans and analysed by SDS-PAGE. The amounts of [^{35}S]syndecan and [^{35}S]CD44 were lower in material from CML-BSA or high glucose treated cells (not shown), corresponding to the decreased amount of [^{35}S]macromolecules recovered. This suggests that the decrease in [^{35}S]proteoglycan expression with CML-BSA is not due to an effect on one particular proteoglycan.

Discussion

Clinical and experimental evidence increasingly suggests that AGEs are involved in different types of complications in patients with diabetes. The generation of the CML epitope has been associated with nephropathy [14, 15] and is present in the mesangial matrix and capillary walls in glomeruli of patients with diabetes [16]. The CML is present in atherosclerotic plaques [17], in the peripheral nerves and retinal vessels [18, 19], in blood circulation in patients with diabetes [10] and shown to be a major AGE epitope [20]. Clearly, the experimental use of CML-modified proteins is relevant to studies of events leading to vascular complications in patients with diabetes. Increased thickness of glomeruli basement membrane has been shown in diabetic patients with microalbuminuria [21]. Furthermore, changes in proteoglycan structure and expression pattern has been shown to be related to the diabetic state [4].

In our study we show that polarized MDCK I and II cells exposed to CML-BSA decrease proteoglycan expression when labelled with [^{35}S]sulphate but not after labelling with [^{3}H]glucosamine. It is intriguing that exposure of cultured kidney epithelial cells for only seven days to this AGE product affects proteoglycan expression. The effect is not due to changes

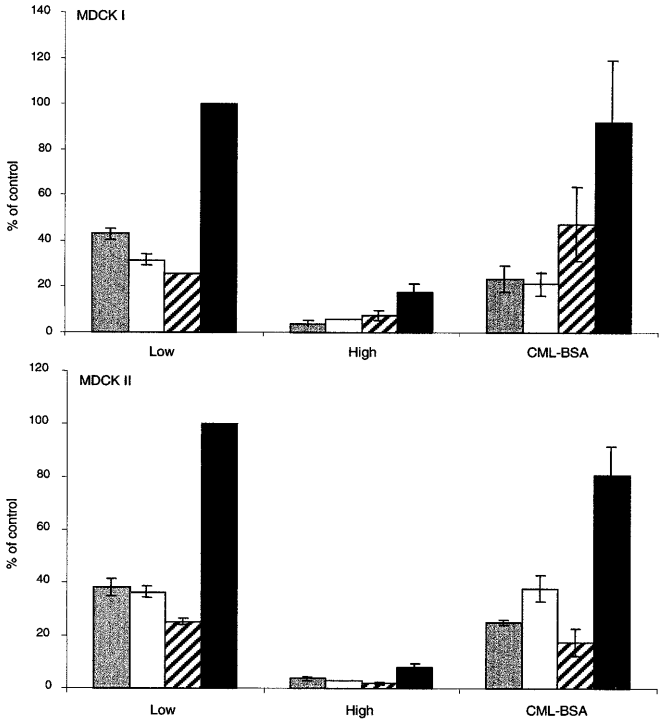

Fig. 3. The expression of [^{3}H]-labelled proteoglycans in MDCK cells. Material from MDCK I and II cells was subjected to preparative DEAE Sephacel ion-exchange chromatography. The figure presents the amount of [^{3}H]-labelled proteoglycans in apical medium (▩), basolateral medium (□) and cell fraction (▨). Total of all three fractions (■). The values presented are average from two separate experiments each done in triplicates ± SD. The total in the control sample in each experiment is 100 %

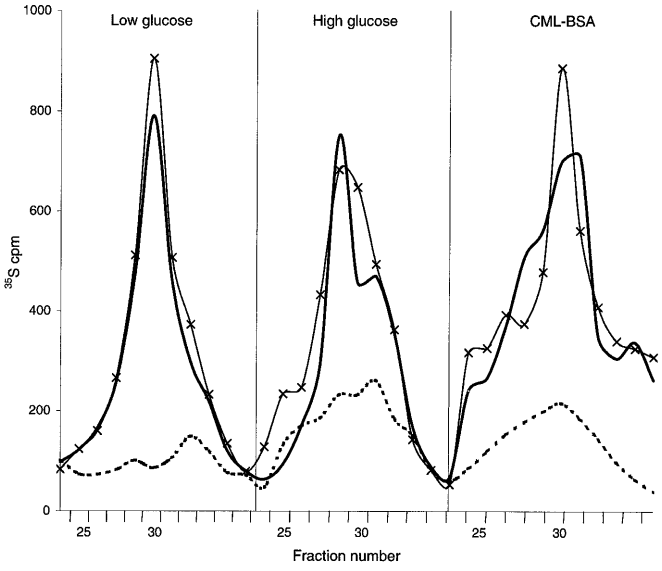

Fig. 4. The expression of [^{35}S]-labelled proteoglycans in MDCK cells. [^{35}S]- labelled macromolecules from the basolateral medium of MDCK I cells were subjected to MonoQ ion exchange chromatography after treatment with HNO_2 (. ..), cABC (-x-) and no treatment (—). Cells were cultured in high glucose, low glucose or in the presence of CML-BSA

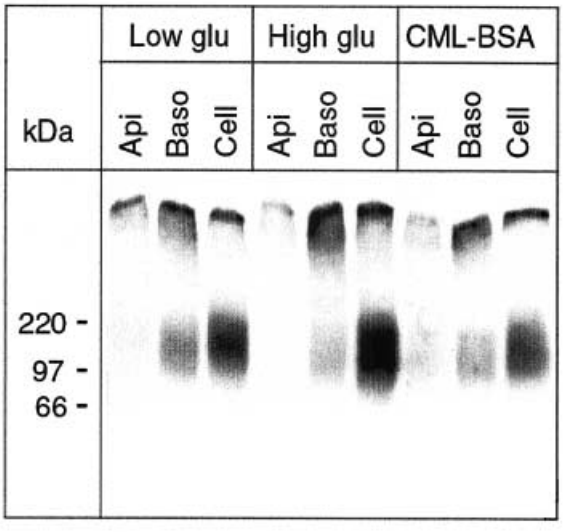

Fig. 5. SDS-PAGE of [^{35}S]-labelled proteoglycans from MDCK I cells. [^{35}S]-labelled proteoglycans were recovered from the apical and basolateral media and cell fractions of MDCK I cells and subjected to SDS-PAGE under reducing conditions

in the molecular size of the GAG or in the polyanionic properties of the GAG chains. The latter aspect, however, deserves more detailed structural analyses. Specific structural changes in heparan sulphate from human glomerular basement membrane of diabetics have recently been shown [22].

The concentration of CML-BSA used was similar to that observed in the circulation of patients with diabetes [10]. The effects observed could be mediated through interactions with cell surface receptors for AGEs but this was not the subject of our study. The binding of CML-BSA to cellular receptors for AGEs leads to cellular activation mediated through NF-$\varkappa$B signalling pathways [23]. A further understanding of changes induced by AGEs could provide a basis for the development of tools to interfere with processes leading to vascular and renal complications for example [24].

The effect of CML-BSA on proteoglycan expression in MDCK cells could also provide an insight into the effects of AGEs on the glomerular basement membrane filter. The MDCK cells express mostly proteoglycans of the heparan sulphate type [7]. By injecting antibodies to glomerular basement membrane heparan sulphate into rats, ensuing proteinuria could be shown [25]. Furthermore, in glomerulonephritis, a weak staining of heparan sulphate in the glomeruli has been correlated to albuminuria [26, 27]. Several reports have shown a reduced heparan sulphate synthesis in the diabetic kidney but this has not been confirmed in all studies (see: [4]). A reduced staining of heparan sulphate chains has, however, been observed in diabetic nephropathy [28], although the amount of

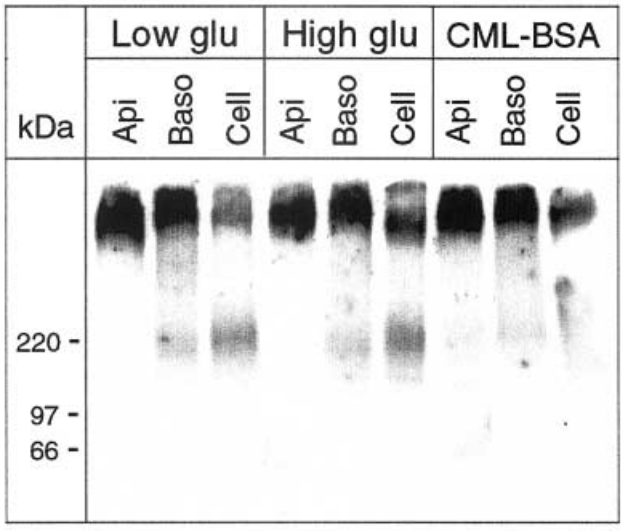

Fig. 6. SDS-PAGE of [^{35}S]-labelled proteoglycans from MDCK II cells. [^{35}S]-labelled proteoglycans were recovered from the apical and basolateral media and cell fractions of MDCK II cells and subjected to SDS-PAGE under reducing conditions

agrin core protein did not alter [26]. A lower degree of sulphation of heparan sulphate has also been reported [29]. The results presented here support the notion that heparan sulphate contents are lower in the diabetic kidney. A lower degree of sulphation of the heparan sulphate chains synthesised could not be detected. Incubation periods of 7 days with either CML-BSA or high glucose concentrations, however, could be too short to see such effects. Long-term cultivation of MDCK cells could be necessary to observe a decrease in sulphation. The data presented do, however, suggest that AGEs are involved in the modulation of proteoglycan expression in diabetes. Diabetic nephropathy is a serious complication and more information about the underlying mechanisms, including those involving AGEs, is needed to improve diagnostics and treatment.

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