Advanced glycation end-products induce cell cycle arrest and hypertrophy in podocytes

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Abstract

Background. Podocyte injury with loss of cells into the urine seems to be an early factor in diabetic nephropathy. Advanced glycation end-products (AGEs) are important mediators of structural and functional renal abnormalities in diabetic nephropathy. We and others have previously described that mice with a deletion in the gene for the cell cycle regulatory p27Kip1 are protected from some features of diabetic nephropathy.

Methods. The present study investigates a potential influence of AGE-modified bovine serum albumin (AGE-BSA) on podocyte growth and p27Kip1 expression in culture. The p27Kip1 expression was measured by western blots and real-time PCR. Cell cycle analysis, cell hypertrophy, proliferation and various markers of apoptosis and necrosis were assessed. The p27Kip1 expression was inhibited by siRNA or was overexpressed in podocytes with an inducible expression system.

Results. AGE-BSA was actively taken up into the cell as determined by immunohistochemistry, western blots and HPLC. Incubation with AGE-BSA induced in differentiated podocytes, but not in tubular cells, p27Kip1 mRNA and protein expression. This induction was associated with cell cycle arrest of podocytes, cell hypertrophy (as measured by increases in cell size and protein/cell number ratios) and an increase in necrotic, but not apoptotic cells. Inhibition of p27Kip1 expression with siRNA halted the AGE-BSA-mediated cell cycle arrest and hypertrophy, but did not interfere with AGE uptake into podocytes. In contrast, overexpression of p27Kip1 using an inducible expression system stimulated hypertrophy and cell cycle arrest of podocytes.

Conclusion. Our data demonstrate that AGE-BSA-induced hypertrophy and damage of cultured podocytes occurs by a mechanism involving p27Kip1. This effect can contribute to the loss of podocytes in diabetic nephropathy.

Keywords: advanced glycation end-products (AGEs); cell cycle regulation; diabetic nephropathy; podocytes

Introduction

Studies in the pathogenesis of diabetic nephropathy have traditionally focused on mesangial cells [1]. Increases in mesangial size and mesangial matrix expansion are hallmarks of nephropathy in diabetes mellitus [1]. However, the genesis of proteinuria in diabetes is not readily explained by mesangial expansion alone. Although loss of negatively charged proteoglycans of the glomerular basement membrane may partially account for proteinuria, this view has been recently challenged [2]. Attention has focused on podocytes, and a better understanding of podocyte biology has provided new insights into diabetic nephropathy [3–8]. A foot process widening of podocytes has been found in patients with type 1 and 2 diabetes [5,8]. In Pima Indians with type 2 diabetes, the decreased number of podocytes per glomerulus was the strongest predictor of progressive renal diseases, with fewer cells predicting more rapid progression [5]. In fact, podocytes can be detected in the urine of patients with diabetic nephropathy and in animal models [9,10]. Podocytopenia may exacerbate the development of proteinuria because a denuded GBM promotes synechiae formation, ultimately resulting in the development of glomerulosclerosis [11].

Irreversible changes caused by hyperglycaemia are closely related to the enhanced formation and accumulation of advanced glycation end-products (AGEs) through a reaction between sugars and the free amino groups of proteins, lipids and nucleic acids [12,13]. A critical role of AGEs in diabetic nephropathy has been demonstrated by elegant studies in db/db mice in which scavenging of AGEs with a soluble receptor attenuated diabetic nephropathy [14]. Interestingly, podocytes, but not mesangial cells, express the receptor for AGEs (RAGE) [14]. The influence of AGEs on podocyte growth and cell cycle regulation has been, however, incompletely studied. Our study demonstrates that AGEs induced cell cycle arrest in cultured podocytes with concomitant hypertrophy and that this effect depends on induction of the cell cycle regulatory protein p27Kip1.
Materials and methods

Podocyte culture

Conditionally immortalized mouse podocytes were cultured as previously described [15,16]. In brief, cells were first grown under permissive conditions at 33°C in RPMI 1640 media containing 8% fetal calf serum (FCS, Cambrex Bio Science, Verviers, Belgium), 50 U/ml interferon-γ (INF-γ) and 100 U/ml of penicillin/streptomycin in collagen-coated flasks. Subsequently, INF-γ was tapered down every third passage to 10 U/ml. Then, subcultivation under non-permissive conditions without INF-γ was started at 37°C with media changed on alternate days. Differentiation of podocytes grown for 10 days at 37°C was confirmed by the podocyte marker synaptopodin (polyclonal rabbit antibody SE-19, Sigma, Saint Louis, USA) and validated by immunocytochemical staining and western blot. Furthermore, the switch to a reduction in cell proliferation during differentiation was monitored by bromodeoxyuridine (BrdU) incorporation (details see below) through comparing cells at 33°C and INF-γ (10 U/ml) to cells that have been grown without INF-γ at 37°C for 10 days [16]. As an additional control, a mouse tubular cell line (MCT) was used [17].

Preparation of AGE-BSA and evaluation of podocyte uptake

BSA (Fraction V, Fatty Acid-Poor, Endotoxin-Free, Calbiochem, La Jolla, USA) was incubated under sterile conditions at 37°C for 50 days in PBS with and without the addition of glucose 90 mg/ml and filtrated (Millipore Lab-scale TFF System, Billerica, USA) and lyophilized. After glycation, AGE-BSA was characterized by a 58-fold higher N’-carboxy-methyl-lysine (CML) concentration than Co-BSA (11.6 nmol/mg protein versus 0.2 nmol/mg protein in Co-BSA) and 6-fold higher pentosidine (4.9 pmol/mg protein versus 0.8 pmol/mg protein in Co-BSA) concentrations. CML was measured by an ELISA (Roche Diagnostics, Mannheim, Germany) whereas pentosidine was determined by HPLC (Merck–Hitachi, Darmstadt, Germany) as previously described [18].

All experiments were conducted in RPMI media containing 0.1% FCS supplemented with 5 mg/ml AGE-BSA (corresponds to a solution of 75 μmol/l) or 5 mg/ml Co-BSA for the chosen incubation times. AGE uptake by podocytes was confirmed by immunocytochemical staining and western blot analysis for the detection of CML-modified albumin (polyclonal rabbit antibody, Roche Diagnostics, Mannheim, Germany). In addition, total pentosidine levels in podocyte cell lysates were determined by HPLC [18].

Immunocytological staining

Podocytes were cultured in chamber slides (Lab-Tek, Rochester, USA) for 24 h with AGE- or Co-BSA. Cells were fixed with 70% ethanol in a glycine buffer for 20 min at −20°C. Slides were incubated with 3% H2O2 for 10 min at room temperature to block endogenous peroxidase. The following primary antibodies were used: synaptopodin (polyclonal rabbit, SE-19, Sigma, Saint Louis, USA), p27Kip1 (polyclonal rabbit, Cell Signaling Technology, Inc., Danvers, USA) and CML (polyclonal rabbit, Roche Diagnostics). Staining was performed using the Vectastain® Elite ABC Kits (Vector Laboratories, Burlingham, USA) and aminothylcarbazole as a chromogen. Counterstaining was performed with Mayer’s haematoxylin. For negative controls, a primary antibody was replaced by rabbit or mouse immunoglobulin (Sigma) at the same concentration as the primary antibody. For imaging, documentation and analysis, a computer-assisted Axioplan microscope with an AxioCam HRC digital camera and AxioVision 4.1 software was used (Carl Zeiss, Jena, Germany).

Cell proliferation and viability tests

After 24 h of incubation in the corresponding medium, the podocytes were characterized by using proliferation tests. BrdU incorporation (Roche Diagnostics) was conducted as a parameter for DNA synthesis and MTT assay (Roche Diagnostics) to evaluate cell viability and metabolic activity. The MTT assay is based on the cleavage of the tetrazolium salt (MTT) in the presence of an NAD[P]H production through glycolysis and occurs in viable cells only. Podocytes were grown in a 96-microtiter plate with 5000 cells per well at 37°C for 24 h under their normal growth conditions or to the corresponding transfection protocol. The media were then changed into RPMI media supplemented with AGE- and Co-BSA and incubated for another 24 h. Subsequently, either BrdU or MTT labelling reagent was added for 4 h. Afterwards in BrdU test media were removed and the cells were fixed and DNA was denatured by the addition of FixDenat® for 30 min. After the removal of FixDenat® anti-BrdU monoclonal peroxidase-conjugated antibody was admitted for 90 min and incubated with Substrate solution® for 20 min. BrdU incorporation was measured at an absorbance of 450 nm in a photometer (TECAN, Crailsheim, Germany). For the MTT test, Solubilization Solution® was added and the microtiter plates was incubated overnight at 37°C. The absorbance of the formazan product was measured at 570 nm. Each measurement was performed with n = 8 per treatment group. The tests were repeated twice with a cumulative number of n = 16 per group.

Measurement of cellular hypertrophy

AGE-mediated effects on cell size and hypertrophy were determined by three independent parameters. By the use of a computer-assisted Axioplan microscope with an AxioCam HRC digital camera a quantitative assessment of cell size was performed. The podocyte surface of single stand, well-circumscribable cells taken from every region of the object slide was determined in μm2 by an investigator unaware of the origin of the groups. In addition, a ratio of protein content to cell number was determined as described [19]. After treatment either with Co- or AGE-BSE, cells were harvested and washed twice with PBS. A small aliquot of cells was counted in a Neubauer chamber after resuspension in PBS. The remaining cells were lysed in Complete Lysis M reagent (Roche Diagnostics) and total protein content was measured by...
a modified Lowry method. Total protein content was expressed as µg protein per 10^3 cells and normalized to the corresponding control. Each experiment was independently performed five times.

Finally, cell size was measured by FACS analysis (FACSCalibur, Becton Dickenson) as forward cell scatter (FSC). All experiments were performed at least in triplicates.

**Cell cycle analysis and evaluation of cell death**

For cell cycle analysis podocytes were harvested after 5, 18, 24, 29 and 48 h of incubation with Co-BSA, AGE-BSA or muristerone and stained with propidium iodide and analysed by a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lake, USA).

To differentiate between AGE-associated changes in early apoptosis and necrosis, the determination of phosphatidylserine on the outer leaflet of apoptotic cell membranes was performed by staining the podocytes with annexin-V-fluorescein and propidium iodide (Roche Diagnostics) simultaneously, as recommended by the manufacturer’s instructions. After detaching, the cells were washed with PBS, and centrifuged at 200 × g for 5 min. The cell pellet was resuspended in 100 µl of Annexin FLUOS labelling solution (containing of 20 µl annexin-V-fluos® labelling reagent, 20 µl propidium iodide solution and 1 ml Incubation buffer®) and incubated for 15 min at room temperature. Then, 0.5 ml Incubation buffer® was added per 10^6 cells. Analysis was performed using 488 nm excitation and a 515-nm band pass filter for fluorescein detection and a filter > 600 nm for propidium iodide detection.

For the detection of later stages of apoptosis, caspase activity of cells treated for 1, 4, 24 or 48 h was determined by staining with a CaspACER activity of cells treated for 1, 4, 24 or 48 h was determined by staining with a CaspACER

**Reverse transcriptase and real-time PCR**

Total cellular RNA was extracted from treated podocytes after direct lysis in culture flasks using an RNA isolation kit (RNeasy Mini kit; Qiagen, Valencia, USA) according to the manufacturer’s instructions. The standard protocol was supplemented by DNase digestion by using the corresponding RNase-Free DNase Set. Concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm. Complementary DNA (cDNA) was synthesized from total RNA with the reverse transcription system (Promega, Madison, USA) according to the manufacturer’s instructions (60 µl reaction mixture, 42°C and 60 min). Primers were designed according to mouse cDNA sequences in Gene Bank (NCBI). The sequences of the chosen oligonucleotide primers were as follows: for p27Kip1, 5′-ACAGATGATAAGCCCTGGAG-3′ (forward) and 5′-CTGACGAGTCGACCTTTG-3′ (reverse) with 224 base pairs, and for GAPDH, 5′-TGCAGCAATCATCTGCAG-3′ (forward) and 5′-GATGTACCATACCTTGCAAGGGT-3′ (reverse) with 337 base pairs.

To quantify the amounts of p27Kip1 mRNA, real-time PCR was performed with a LightCycler instrument (Eppendorf AG, Hamburg, Germany). The reaction mixture containing double-stranded DNA-specific SYBR Green I dye (Roche Diagnostics) and primers for GAPDH and p27Kip1 was added to cDNA dilutions. cDNA samples were amplified 40 cycles for p27Kip1 and 30 cycles for GAPDH expression. Each cycle consisted of 10 s denaturation at 95°C, 15 s of primer annealing at 59°C and an extension step at 72°C for 20 s. Expression of p27Kip1 was normalized against expression of GAPDH.

**Western blot analysis**

Podocytes or MCT cells harvested from flasks were lysed in a sodium dodecyl sulphate (SDS) sample buffer and heated at 100°C for 5 min. Aliquots of 10-µg protein/lane were electrophoresed in a 15% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a poly-vinyliden-fluoride (PVDF) membrane using a semidy transfer cell (Bio-Rad Laboratories Inc., Hercules, USA). Subsequently, the membrane was incubated in blocking buffer (1 × PBS, 0.1% Tween-20 and 5% non-fat milk) for 1 h at room temperature, and incubated overnight at 4°C in a 1:1000 dilution of a polyclonal rabbit anti-p27Kip1 antibody (Cell Signaling Technology, Inc., Danvers, USA) or by a 1:5000 dilution of a monoclonal mouse anti-β-actin antibody (Sigma) or by an anti-CML antibody at a 1:5000 dilution (polyclonal rabbit, Roche Diagnostics). The membrane was then washed four times for 10 min in 1 × PBS with 0.1% Tween-20, and incubated in blocking buffer with the corresponding horseradish peroxidase-linked secondary antibody (KPL, Inc., Gaithersburg, USA) at 1:5000 dilution. Detection of peroxidase was performed with a chemiluminescent agent (ECL) (Roth, Karlsruhe, Germany) and exposed to X-ray film. Quantification was done by measuring relative density and normalisation for β-actin as previously described [19].

**Transfection with inducible p27Kip1 expression construct and with p27Kip1-siRNA**

For transient transfections, cells were seeded either in six-well plates (2–8 × 10^5 cells/well) or in 96-well plates (2–8 × 10^3 cells/well). For inducible p27Kip1 expression a full-length mouse p27Kip1 cDNA was cloned into the pIND
Fig. 1. Immunocytochemical staining of podocytes incubated with AGE-BSA 5 mg/ml compared to Co-BSA 5 mg/ml (original magnification, 40×). Expression of the podocyte differentiation marker synaptopodin did not change after treatment with AGE-BSA. Intensive intracellular staining for CML in cells treated with AGE-BSA suggests active uptake and intracellular AGE metabolism. Treatment with AGE-BSA increased nuclear and cytoplasmic staining for p27Kip1.

Results

Immunocytochemistry

Experiments were performed in conditionally immortalized podocytes (provided by Dr Peter Mundel). These cells have been described in detail before and all studies were done on fully differentiated cells [15,16]. Differentiation of cells was confirmed by the expression of synaptopodin and morphological criteria as recently defined [16]. Proliferation of podocytes was low compared with other cells and differentiated podocytes had even a significantly lower BrdU incorporation than those growing at 33°C with IFN-γ (undifferentiated cells: 0.24 ± 0.01, differentiated cells: 0.21 ± 0.02 adsorption at 430 nm; n = 16, P < 0.0001). Podocytes were incubated for 24 h with 5 mg/ml glycated bovine serum albumin (AGE-BSA contains ε-carboxy-methyl-lysine (CML) 11.6 nmol/mg protein) or the same concentration control-BSA (Co-BSA). This concentration was selected to mimic the in vivo situation [18].

Incubation of podocytes with AGE-BSA does not influence expression of the podocyte marker synaptopodin indicating that AGE-BSA is not interfering with cell differentiation (Figure 1A). Differentiated podocytes grown in either Co-BSA or AGE-BSA show clear signs of arborisation and the presence of processes extending from cell bodies (Figure 1A). Immunohistochemical staining for CML, a representative member of the AGE family, demonstrates that AGE-BSA is actively taken up by podocytes into the cytoplasm (Figure 1A). Furthermore, p27Kip1 expression, a cell cycle inhibitor previously shown to play a pivotal role in diabetic hypertrophy (44), is increased after incubation with AGE-BSA (Figure 1A). Although nuclear p27Kip1 expression increased after AGE-treatment, there was also an extensive increase in cytoplasmic p27Kip1 expression (Figure 1A).

Statistical analysis

All data are reported as means ± standard deviation (SD). Statistical analysis was performed using the statistical package SPSS for Windows version 11.0 (SPSS, Inc., Chicago, IL, USA). Results were analysed with the Kruskal–Wallis test followed by the Mann–Whitney U-test. Differences were considered significant when P < 0.05.
Uptake of AGE-BSA into podocytes

Although staining for CML as shown in Figure 1A clearly suggested active uptake of AGE-BSA into podocytes, we further confirmed this observation with additional experiments. Figure 2A shows the concentration of pentosidine, a typical AGE, in cell lysates of podocytes. Cells grown in the presence of AGE-BSA exhibit a significant higher pentosidine concentrations in cell lysates compared with podocytes incubated with Co-BSA (Figure 2A). Moreover, western blots reveal a strong increase in intracellular CML concentrations in podocytes grown in the presence of AGE-BSA (Figure 2B).

p27Kip1 mRNA and protein expression

Since it has been previously shown that the cell cycle inhibitory protein p27Kip1 is associated with podocyte hypertrophy under diabetic conditions [37,40], we tested whether AGE-BSA modifies p27Kip1 expression. Western blot analysis revealed that p27Kip1 protein expression was significantly higher in AGE-BSA-treated podocytes compared to Co-BSA (Figure 3A). In addition, a significant increase in p27Kip1 mRNA was found with real-time PCR in podocytes after treatment with AGE-BSA suggesting that, at a minimum, part of the increased p27Kip1 protein expression may be due to transcription (Figure 3B). Figure 4 shows that tubular MCT cells challenged with AGE-BSA demonstrated a slight decrease, but not an increase in p27Kip1 protein expression.

Inhibition and overexpression of p27Kip1

To gain functional insight into the involvement of p27Kip1 in AGE-associated growth of podocytes, expression was either suppressed with p27Kip1-siRNA or transiently induced by
transfection with the ecdysone-inducible gene expression system pINDp27Kip1/pVgRXR. The efficiency of manipulating p27Kip1 expression was confirmed in western blots. As shown in Figure 5A, specific p27Kip1 siRNA, but not a control sequence, almost completely eliminated p27Kip1 expression in podocytes treated with Co-BSA as well as AGE-BSA. Treatment with p27Kip1-siRNA did not interfere with AGE uptake, but clearly reduced p27Kip1 expression in the cytoplasm and nuclei as detected by immunocytochemistry (Figure 5B). In Figure 5C muristerone-dependent induction of p27Kip1 is shown in podocytes treated with Co-BSA confirming that inducible induction occurred.

**Cell proliferation, cell viability, apoptosis and cell cycle analysis**

DNA synthesis was evaluated with BrdU incorporation, and cell viability was determined via metabolic activity using the MTT test. Figure 6A and B shows that DNA synthesis as well as metabolic activity was significantly reduced in AGE-BSA treated podocytes compared with Co-BSA. In contrast, transfection with p27Kip1-siRNA suppressed the AGE-mediated reduction in BrdU incorporation and metabolic activity, whereas transfection with a control sequence was without influence (Figure 6A and B). Induction of p27Kip1 expression by transfection of podocytes grown in Co-BSA with pINDp27Kip1/pVgRXR and application of the inducer muristerone mimicked AGE effects even in the presence of Co-BSA (Figure 6A and B).

For cell cycle analysis podocytes were harvested after 5, 18, 24, 29 and 48 h of incubation in either Co-BSA or AGE-BSA. After propidiumiodide staining flowcetric analysis was performed. AGE-BSA induced cell cycle arrest in 98.7% of the cells in the subG1/G1 phase after 48 h of treatment compared with Co-BSA (70.2% of podocytes subG1/G1 phase; Figure 7A). Transfection with p27Kip1-siRNA attenuated the subG1-phase cell cycle arrest and increased the total number of cells in the S and G2 phases suggesting that the AGE-BSA-mediated cell cycle arrest depends on p27Kip1 (Figure 7B). A Control primer showed no significant effect. However, podocytes incubated in Co-BSA with muristerone-induced p27Kip1 expression revealed a similar cell cycle analysis as found after AGE-BSA treatment (Figure 7B).

Cells were analysed after annexin-V-fluorescein staining by flowcetric analysis. A significant decrease of vital cells (annexin V and propidiumiodide negative) was accompanied by a significant increase of necrotic and late
Apoptotic cells after AGE-BSA incubation (annexin V and propidium iodide positive), but significant changes in early apoptosis (annexin V positive/propidium iodide negative) were not detected (Figure 8). Podocytes transfected with p27Kip1-siRNA did not show the AGE-mediated decrease of vital cells after AGE-BSA treatment (Figure 8). Transfection with the control sequence did not interact with the AGE effects. On the other hand, muristerone inducible p27Kip1 expression resulted in a comparable shift of podocytes with p27Kip1-siRNA did not show the AGE-mediated decrease of vital cells and an increase of necrotic cells in the presence of Co-BSA (Figure 8). Neither suppression nor induction of p27Kip1 led to significant changes in early apoptosis.

To further test a potential influence of AGE-BSA on apoptosis of podocytes, two additional assays were performed. As shown in Figure 9A, AGE-BSA did not induce apoptosis podocytes as demonstrated by the absence of small DNA fragments. Apoptotic U937 cells served as a positive control and a typical DNA fragmentation pattern is visible. In addition, pan caspase activity was measured by flow cytometry using the CaspACE® VAD-FMK in situ marker. Although total caspase activity increased in cells after incubation for 24–48 h, there was no significant difference between podocytes incubated in Co-BSA or AGE-BSA (Figure 9B).

Cellular hypertrophy

To evaluate effects on cell size as they are expressed in their adherent phenotype, the podocyte surface of single standing, well-circumscribable cells out of every region of the object slide was determined by quantitative imaging. The mean cell surface in AGE-BSA-treated podocytes significantly increased compared with cells incubated in Co-BSA (Figure 10A). Inhibition of p27Kip1 expression by siRNA eliminated the AGE-BSA-induced increase in cell size (Figure 10A). The ratio of protein content to cell number as a well-established hypertrophy index revealed a significant increase with AGE treatment (Figure 10B). This AGE-BSA-mediated hypertrophy was abolished through p27Kip1-siRNA. However, hypertrophy was induced in podocytes grown in Co-BSA after induction of p27Kip1 with muristerone (Figure 10B). These observations were confirmed by FACS analysis of cell size using FSC as a parameter. Figure 11 shows the FSC histograms with a significant right shift under AGE incubation which was antagonized by p27Kip1-siRNA, but not by a Control primer (Figure 11). An enlargement in cell size was also seen after induction of p27Kip1 in podocytes grown in Co-BSA (Figure 11).

Discussion

The molecular and cellular mechanisms of diabetic nephropathy are still incompletely understood. Beyond the paradigm of extracellular matrix expansion resulting in complex renal structural and functional changes, early podocyte injury has emerged to play a pivotal role in diabetic glomerular disease [7]. Early podocyte damage that antedates the development of glomerulosclerosis has been demonstrated in several experimentally induced models of diabetic nephropathy [4]. Podocyte depletion could be a major mechanism driving glomerulosclerosis and progressive deterioration of renal function [11,21]. Changes in the interaction of podocytes with the underlying glomerular basement membrane and apoptosis of podocytes contribute to this depletion. It has been previously shown that hyperglycaemia directly induces hypertrophy and later apoptosis in cultured podocytes [22–24]. Podocyte loss is closely correlated with the onset and magnitude of glomerular sclerosis [7] and a better understanding of podocyte pathophysiology...
Fig. 7. Cell cycle analysis. (A) Podocytes were either incubated with RPMI media (0.1% FCS) containing Co-BSA 5 mg/ml or AGE-BSA 5 mg/ml. Cells were harvested after 5, 18, 24, 29 and 48 h of incubation and FACS analysis was performed after propidiumiodide staining. AGE-BSA induced cell cycle arrest in the subG1/G1 phase after 48 h treatment in comparison to Co-BSA. Results of one typical experiment out of five independent experiments. (B) Inhibition of p27Kip1 expression with siRNA attenuated the subG1-phase cell cycle arrest and increased the total number of cells in the S and G2 phases indicating that the AGE-BSA-mediated cell cycle arrest depends on p27Kip1. A Control primer showed no significant effect ($n=4$, *$P<0.01$ versus Co-BSA, +$P<0.01$ versus not transfected podocytes). On the other hand, p27Kip1 expression induced by 5 µg/ml muristerone in podocytes grown in Co-BSA showed the pattern of cell cycle arrest as observed under AGE-BSA ($n=5$, *$P<0.05$ versus cells without muristerone).
Fig. 8. FACS analysis after annexin-V-fluorescein staining. AGE-BSA treatment induced a significant reduction in vital cells and a significant increase of necrotic and late apoptotic cells (n = 7, **P < 0.01, ***P < 0.001), which could be attenuated by p27Kip1-siRNA (n = 5, P = not significant). A Control primer was without effect. Overexpression of p27Kip1 in podocytes in the presence of Co-BSA induced an increase in necrosis (n = 5, *P < 0.01). However, early apoptosis was neither significantly influenced by AGE-BSA nor by p27Kip1 induction.

in diabetic nephropathy is necessary to develop innovative therapeutical strategies.

In the diabetic milieu hyperglycaemia and increased oxidative stress lead to enhanced formation and accumulation of AGEs that is further aggravated during renal insufficiency [12,13]. Glomerular AGE accumulation correlates with the severity of diabetic nephropathy [25]. In animal models, neutralisation or scavenging of AGEs prevents podocyte damage [14,26].

Studying molecular mechanisms of AGEs in vivo systems is difficult and we relied therefore on cell cultures of a podocyte cell line that has been widely used [16,27]. A recent review has stressed the merits of such an approach to use podocytes in culture for mechanistic studies [16]. Criteria for differentiated podocytes in vitro have been defined including arborized morphology, formation of cellular structures comparable with filtration slits, positivity for synaptopodin and absence of DNA synthesis [16]. We believe that our differentiated podocytes that were used for the AGE studies fulfilled all of these criteria.

The present study shows that AGE-BSA is actively incorporated into cultured podocytes. This was tested by immunohistochemical staining and western blots of cellular lysates for CML, as well as measurement of pentosidine in cellular lysates. This uptake of AGE leads to an increase in p27Kip1 expression, cell cycle arrest and hypertrophy. Podocyte hypertrophy was measured by three different methods clearly demonstrating the AGE-BSA-induced hypertrophy. The percentage of necrotic, but not apoptotic cells was significantly higher in podocytes incubated with AGE-BSA. Inhibition of p27Kip1 expression by siRNA attenuated cell cycle arrest. In addition, forced expression of p27Kip1 using an inducible vector-system-stimulated hypertrophy of podocytes grown in Co-BSA. These findings point to a crucial role of AGEs in podocyte hypertrophy by a cell cycle-dependent mechanism involving p27Kip1.

Podocytes are terminally differentiated cells and die rather than proliferate in diabetic nephropathy [28]. It has been previously shown that the switch from a proliferate and undifferentiated podocyte phenotype to a quiescent phenotype that does not proliferate is associated with an increase in p27Kip1 [29,30]. On the other hand, genetically engineered mice with p27Kip1 deletion exhibit a marked increase in podocyte proliferation after induction of nephritis [31].

Somewhat surprising was the observation that AGE-BSA failed to induce apoptosis and rather mediated necrosis because AGE-mediated apoptosis has been recently described in podocytes involving the FOXO4 transcription factor [32]. We used different assays for apoptosis. We found no induction of DNA fragments with AGE-BSA, no change in staining for annexin, and no difference in pan-caspase activity. We believe that these three independent assays clearly demonstrate that AGE-BSA does not induce apoptosis in podocytes compared with Co-BSA. However, we observed an increase in pan-caspase activity after prolonged incubation (>24 h) of podocytes in medium with 0.1% FCS and Co-BSA or AGE-BSA. Since we failed to detect DNA fragmentation or annexin V activation, we believe that this increase does not reflect apoptosis. Indeed, there is accumulating evidence that pan-caspases are activated by many other signalling events beyond death including cellular differentiation [33,34].

Animal models of diabetic nephropathy clearly showed that podocyte apoptosis occurs and that this effect is mediated by oxidative stress [24]. However, it has also been reported that viable podocytes can be found in the urine of diabetic rats suggesting that other mechanisms than apoptosis may contribute to the depletion of podocytes during diabetic nephropathy [10]. To our knowledge, no study has so far systematically studied whether necrosis or apoptosis is the leading event inducing depletion of podocytes in diabetic
nephropathy. One recent study looking at renal biopsies of patients with type 2 diabetes found an increase in glomerular and tubular apoptosis, but it remains unclear what glomerular cells were exactly involved [35]. Higher concentrations of AGE-BSA also induced necrosis of podocytes in a recent study [32]. At least in other models of podocyte injury in vitro such as puromycin aminonucleoside application, both necrosis and apoptosis contribute to podocyte death [36]. In addition, we do not think that the observed decrease in vital cells after AGE-BSA treatment represents an unspecific toxic effect because it was clearly associated with the concomitant induction of p27Kip1. Inhibition of p27Kip1 prevented AGE-BSA-induced necrosis and overexpression of p27Kip1 increased the percentage of necrotic podocytes grown in Co-BSA. This strongly argues against unspecific toxic effects of AGE-BSA. Certainly, further studies are necessary to define the exact role of podocyte necrosis in animal models of diabetic nephropathy.

We have previously shown that p27Kip1 expression is enhanced in glomeruli of diabetic db/db mice and BBdp rats [37,38]. Immunohistochemistry revealed that, besides mesangial cells, podocytes increase their p27Kip1 content in db/db mice during glomerular hypertrophy [37]. Xu and colleagues studied podocytes in culture and found an increase in p27Kip1 mRNA and protein expression in podocytes exposed to high glucose [22]. This increase in p27Kip1 expression was associated with podocyte hypertrophy. Interestingly, high glucose-induced p27Kip1 expression and hypertrophy of podocytes were attenuated by an angiotensin II receptor blocker indicating that some of the effects may be mediated by high glucose-induced angiotensin II [22]. This is in accordance with previous findings of our group showing that angiotensin II itself can induce p27Kip1 [39].

AGE-BSA-stimulated p27Kip1 protein expression has been associated with a significant increase in mRNA expression suggesting that stimulated transcription contributes, at least partially, to the stimulated p27Kip1 expression. This is in contrast to previous findings in cultured mesangial cells in which high glucose-induced p27Kip1 expression was post-transcriptional caused by enhanced phosphorylation with a decrease in p27Kip1 degradation...
Fig. 11. FACS analysis of cell size. The FSC histograms (left panels) show a right shift of the cells under AGE treatment as well as under induction of p27Kip1 in podocytes incubated in Co-BSA. These changes were highly significant (*P < 0.001, right panels). Treatment with p27Kip1-siRNA, but not a Control primer, not only antagonized the observed right shift, but even resulted in a significant left shift (P < 0.001). The shown data are an example of n = 3 independent experiments.
Although the majority of studies have described post-transcriptional regulation of p27Kip1 [29], an increase in p27Kip1 abundance due to stimulated gene transcription has been well documented [42,43]. For example, transcription of p27Kip1 is stimulated by E2F1, a transcription factor activated in the diabetic milieu [43,44]. Our siRNA experiments, which almost completely blunted p27Kip1 protein expression, also point to the importance of an increase in p27Kip1 mRNA. Thus, we suggest that AGE-BSA-mediated gene transcription is the main mechanism of the increase in p27Kip1 expression in podocytes.

In addition to an increase in nuclear accumulation of p27Kip1 in podocytes after challenge with AGE-BSA, an increase was also found in the cytoplasm. Several studies have established that cyclin-dependent kinase (CDK) inhibitors such as p27Kip1 might have an additional role in the cytoplasm because only nuclear localization of p27Kip1 would impair the catalytic function of CDKs [45]. CDK in the cytoplasm may act as a molecular bridge to other cell cycle proteins where it can bind and sequester cyclin/CDK complexes [46]. In addition, an interaction of p27Kip1 with other proteins, including the nuclear pore-associated protein mNPAP69, has been detected indicating that cytoplasmic p27Kip1 may regulate nuclear transport [46]. Moreover, it has been postulated that cytoplasmic localisation of CDKs inhibits apoptosis [47]. For example, inhibition of p27Kip1 induces neuronal cell death [47]. Thus, the AGE-BSA-induced p27Kip1 expression may prevent apoptosis and shifts the injury pattern to necrotic cell death. On the other hand, a potential influence of p27Kip1 on cell migration has been described [48]. In these experiments, a forced expression of p27Kip1 inhibits endothelial cell migration as well as the migration of vascular smooth muscle cells [48]. Since cell migration requires cytoskeleton reorganization and proteins associated with the podocyte slit membrane are associated with the cytoskeleton [6], it is tempting to speculate that an increase in p27Kip1 expression in the cytoplasm may alter the interaction between podocyte protein and microtubules resulting in an increase in proteinuria. Certainly, further studies are necessary to test this interesting hypothesis.

We believe that the AGE-BSA-induced cell cycle arrest and hypertrophy are detrimental for podocyte structure and function. Indeed, significantly more cells exposed to AGE-BSA were necrotic compared with Co-BSA, but there were no significant differences in apoptosis. Thus, one may speculate that the inability of podocytes to proliferate in the presence of AGE-BSA because of p27Kip1 induction results in hypertrophy as an attempt to cover ‘nude’ areas of the glomerular basement membrane. However, although podocytes remain differentiated in the presence of p27Kip1 hypertrophy is maladaptive for podocyte metabolism and the cells become necrotic leading to further podocyte depletion, resulting in a vicious circle. Since necrosis in contrast to apoptosis is associated with an inflammatory response, the necrosis of hypertrophic podocytes with the release of potential proinflammatory material may contribute to glomerular inflammation found early in diabetic nephropathy.

We and others have described that diabetic mice missing the p27Kip1 gene are partially protected from development of diabetic nephropathy [30]. We found that diabetic p27Kip1−/− mice developed significantly less albuminuria compared to diabetic wild-type animals after 6 weeks [30]. The increase in mean podocyte volume, as a crude parameter of hypertrophy, in diabetic animals was attenuated in p27Kip1−/− mice [30]. Although we have not measured AGE concentrations in these experiments, it has been described that AGEs are implicated in renal pathophysiology of streptozotocin-induced type 1 diabetes in mice [13]. It is therefore possible that AGE-induced cell cycle arrest with hypertrophy caused by upregulation of p27Kip1 also occurs in vivo. Certainly, additional factors (e.g. high glucose, angiotensin II and TGF-β) may contribute to induction of p27Kip1 in the in vivo situation.

In summary, AGE-BSA-induced p27Kip1 expression leads to hypertrophy, cell cycle arrest and subsequent necrosis, but not apoptosis, of cultured differentiated podocytes. This mechanism may contribute to the podocyte depletion observed in diabetic nephropathy.

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Conflict of interest statement. None declared.

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