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Original Article

Advanced glycation end products (AGEs)-induced expression of TGF-β1 is suppressed by a protease in the tubule cell line LLC-PK1

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Abstract

Background. Advanced glycation end products (AGEs) are assumed to play a key role in diabetic nephropathy (DN). Since little is known about their action in tubule cells, we investigated in LLC-PK1 cells: (i) whether AGE-bovine serum albumin (AGE-BSA) affects cell proliferation and expression of transforming growth factor-β (TGF-β1); and (ii) whether the AGE-induced effects can be modulated by trypsin due to interference with its binding proteins at the cell surface.

Methods. Arrested cells were exposed to vehicle (control), AGE-BSA (19–76 µM) and BSA (38 µM) in the presence or absence of trypsin (0.625–5.0 µg/ml) (2.5 µg/ml) for 24 h. We evaluated cell proliferation by cell count and by [^{3}H]thymidine incorporation, TGF-β1 expression by reverse transcription-polymerase chain reaction (RT-PCR), and TGF-β1 protein by ELISA. In addition, cell accumulation of AGEs was studied by immunohistochemical staining of the AGE imidazolone.

Results. AGE-BSA inhibited [^{3}H]thymidine incorporation, lowered cell number and increased cell protein content as well as TGF-β1 mRNA and protein as compared with control and BSA. Immunohistochemical staining revealed a marked intracellular accumulation of the AGE imidazolone. Co-incubation of AGE-BSA with trypsin ameliorated the impaired thymidine incorporation, the decreased cell count and the enhanced cell protein content. TGF-β1 overexpression was normalized, while TGF-β1 protein declined insignificantly. Intracellular imidazolone accumulation was strikingly suppressed.

Conclusions. In the tubule cell line LLC-PK1, AGE-BSA exerts an antiproliferative effect, most probably due to TGF-β1 overproduction. The co-administration of trypsin abrogated this alteration, very likely as a result of an interaction with AGE-binding protein(s), which is supported by the decreased intracellular AGE accumulation. These findings may be the starting point for the development of specific proteolytic enzymes to interfere with the interaction between AGEs and their receptors/binding proteins.

Keywords: AGEs; cell proliferation; imidazolone; TGF-β1 mRNA; trypsin and tubule cells

Introduction

Diabetic nephropathy (DN) is characterized by renal hypertrophy, thickening of the basement membranes and accumulation of extracellular matrix (ECM) in the mesangium and tubulointerstitium [1]. The pathogenesis is multifactorial and still not completely understood. Various haemodynamic, hormonal and biochemical factors appear to be involved [2]. Several lines of evidence suggest the central role of advanced glycation end products (AGEs) [3], which accumulate in experimental and human DN in the glomeruli and tubulointerstitium, in relation to severity of the renal disease [4–6]. Their pathogenetic relevance was verified in *in vivo* experiments in euglycaemic rodents by parenteral administration of AGE-albumin, leading in the glomeruli to TGF-β1 overproduction, enhanced gene expression of ECM proteins and morphological lesions similar to those of DN [7]. In *in vitro* studies in endothelial and mesangial cells, AGEs caused enhanced formation of oxygen radicals, synthesis and release of inflammatory cytokines/growth factors and expression of the adhesion molecule VCAM1 [8]. These responses were mediated via one or more of

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the various specific receptors of AGEs (in particular RAGE), expressed on numerous cell types, such as mononuclear phagocytes, endothelial, mesangial and tubule cells [9–12]. In addition, many AGEs can promote a receptor-independent cross-linking of matrix proteins, which decreases susceptibility to enzymatic degradation and results in tissue remodelling [3].

To counteract the toxic intra-/extracellular effects of AGEs, numerous drugs were developed and studied in *in vitro* and *in vivo* experiments. These include blockers of AGE formation (aminoguanidine [12] and the thiazolidine derivative OPB-9195 [13]), the protein cross-link breaker *N*-phenacylthiazolium bromide [14], anti-oxidants (taurine, vitamin E, alpha-lipoic acid and pyridoxamine [15]), as well as the administration of the soluble receptor of AGEs [16]. Another approach could be the blocking of AGE binding to their cell surface receptors by proteolytic enzymes. Thus, in cultured endothelial and lung cells, the AGE–RAGE interaction is suppressed by the serine protease trypsin, associated with decreased oxidative stress [9,17]. It is conceivable that the protease-induced lowering of AGE-binding also protects renal cells from maladaptive responses, such as the enhanced cytokine/growth factor formation and their consequences. Therefore, in the current study, we investigated the potential modulatory action of the serine protease trypsin on various AGE effects. Our interest focused on TGF-β1 formation, which is of fundamental importance in the development of cell hypertrophy and accumulation of ECM [18,19]. Since tubular cells are the main target of renal hypertrophy in diabetes, we examined the porcine tubule cell line LLC-PK1. Surprisingly, nothing is known about AGE effects on TGF-β1 expression in the tubule cells. The involvement of this growth factor seems to be probable, since exposure to AGE-BSA was followed by alterations characteristic of TGF-β1 effects, such as cell hypertrophy, impaired protein degradation and lowered cathepsin activity [20]. Additionally, we studied the intracellular accumulation of the AGE imidazolone in the presence and absence of trypsin.

The results obtained show that the exposure of LLC-PK1 cells to AGE-BSA enhanced cell protein content and increased the expression of TGF-β1 in a dose-dependent manner. Cell proliferation was reduced. Co-administration of trypsin abrogated these alterations. Immunohistochemical staining showed a marked intracellular AGE accumulation, which was lowered noticeably by trypsin, indicating a blockade of cellular AGE uptake.

Materials and methods

Generation and characterization of AGE-modified albumin

Bovine serum albumin (1 mM; BSA fraction V Sigma, Germany) was glycated by incubation with 1 M glucose in 50 mM potassium phosphate, pH 7.3, 1 mM EDTA under sterile conditions at 50°C for 40 days according to a modified protocol of Schmidt *et al.* [9]. Formation of AGEs was followed by measuring the time-dependent increase of fluorescence at 440 nm when excited at 370 nm [21]. The AGE-BSA solution was then lyophilized and redissolved in 50 ml bi-distilled water. Glucose and other compounds were removed by extensive dialysis against 5×5 l bidistilled water for 48 h. Residual glucose was <0.1 mM after this procedure. The dialysed AGE-BSA was lyophilized again, resulting in a brown powder. Shortly before use, AGE-BSA was redissolved in phosphate-buffered saline (PBS). Control BSA was incubated under the same conditions but in the absence of glucose.

AGE-modified BSA was characterized by immunological and spectroscopical methods. The preparation was recognized by two different AGE-specific antibodies using both the ELISA (imidazolone and carboxymethyllysine antibodies) and western blot techniques (data not shown). The AGE-BSA showed fluorescence spectra typical for AGEs ($\lambda_{max\ emission} = 440$ nm at an excitation wave-length of 370 nm). After purification by chromatography on a polymyxin column (Pierce, Germany) no endotoxin could be detected by the Limulus Amebocyte Lysate (LAL) test (E-TOXATE; Sigma).

Cell culture and experimental treatment

LLC-PK1 cells, a porcine cell line that exhibits the properties of proximal tubules, were grown at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) with 100 mg/dl glucose supplemented with 10% foetal calf serum (FCS; Gibco-BRL), 25 mM Hepes, 100 U/ml penicillin and 100 ng/ml streptomycin. Cells were plated separately on 100 mm dishes for isolation of RNA or six-well plates for the cell proliferation assay.

After reaching 70–80% confluence, the subconfluent cells were synchronized by serum-free medium (SFM) for 24 h, followed by treatment with vehicle (control), BSA or AGE-BSA (19–76 μM) related to BSA content with or without trypsin (0.625–5 μg/ml) and high glucose (450 mg/dl).

Examination of a potential AGE-BSA degradation by trypsin

Two AGE-BSA batches and unmodified BSA (25 μM in PBS) were incubated with a high dose of trypsin (50 μg/ml; MUCOS GmbH, Geretsried, Germany) for 24 h at 37°C. In neither case was any degradation observed after sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Prolonged incubation for >72 h under the same conditions caused significant degradation, but some undegraded AGE-BSA still was present after this time (data not shown).

Cytotoxicity was evaluated by both Trypan Blue exclusion and release of lactate dehydrogenase.

Cell number was analysed using the Casy-1 System (Schaerfe, Reutlingen, Germany), based on the Coulter Counter principle.

Cellular proliferation assay

Cellular proliferation was determined using [^{3}H]thymidine incorporation. The subconfluent monolayer was quiescented by being grown in SFM for 24 h, then incubated for 12, 16, 20 and 24 h, respectively, in SFM in the absence

(control) or presence of AGE-BSA or non-glycated BSA with or without trypsin at various concentrations, as well as an anti-TGF-β1 antibody. During the last 4 h, cells were labelled with 0.5 μCi per well of [^{3}H]thymidine (50 μCi/mmol; Amersham, Germany). The cells were rapidly washed three times with ice-cold PBS and solubilized in 2% SDS, followed by precipitation with 1 ml of 20% trichloroacetic acid (TCA). The precipitates were collected with a cell harvester onto a glass microfibre filter (Schleicher Schuel, Dassel, Germany) and washed sequentially with 10 and 5% TCA, and finally with ethanol. Incorporated [^{3}H]thymidine was measured in a liquid scintillation counter.

Cell protein content

Total protein concentration in cell lysate was measured using bicinochoninic acid (BSA protein assay; Pierce, Bonn, Germany [22]).

Measurement of TGF-β1 expression by reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from subcultured LLC-PK1 cells treated with vehicle, BSA or AGE-BSA in the presence or absence of trypsin by single step method [23]. The purity and concentration was determined by measuring the optical densities at 260 and 280 nm. The A_{260}/A_{280} ratio ranged from 1.70 to 1.95. The integrity of RNA was confirmed by electrophoresis on 1% agarose gels, and the RT-PCR reaction was performed as described previously [24].

Briefly, cDNA was synthesized by reverse transcription from 1 μg of total RNA in a volume of 25 μl containing 1 × First-Strand synthesis buffer (10 mM Tris-HCI pH 9.0, 50 mM KCI, 1.5 mM MgCI), 1 mM of dNTPs, 0.5 μl of ribonuclease inhibitor (40 U/μl), 0.5 μl of Maloney murine leukemia virus (MMLV) reverse transcriptase (50 U/μl, Stratagene, La Jolla, CA), and 0.4 ng/μl of random primers for 1 h at 37°C.

PCR amplification was performed by the standard procedure using primers (sense 5′-CTGAGGCTCAAGTTAAAAG-3′ and anti-sense 5′-GAACCCGTTAATTTCCAC-3′) deduced from the TGF-β1 sequence of pigs, giving a PCR product size of 246 bp [25]. GAPDH cDNA was co-amplified as an internal control using the following primer sequences (5′→3′); the sense 5′CGGAGTCAACGGATTTGGTCG-3′ and anti-sense 5′AGCCTTCTCCATGGTGGTGA AGAC-3′ with a final product size of 306 bp [23]. Both TGF-β1 and GAPDH were amplified for 35 cycles using the following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 1 min. The PCR products were analysed by 2% agarose gel electrophoresis and quantified by densitometry. A sample of RNA not subjected to RT-PCR was used as a negative control in each experiment.

TGF-β1 protein and bioactivity assay

After incubation with AGE-BSA (38 μM) for 24 h, total TGF-β1 protein was determined in the supernatant of cultured cells by an ELISA assay according to commercial instruction (Promega, Heidelberg, Germany). Its concentration was related to cell number.

For the evaluation of the bioactivity of TGF-β1 induced by AGE-BSA, the cell protein concentration as well as the antiproliferative effect was investigated after co-incubation with the polyclonal rabbit antibody of TGF-β1 (Promega) according to Wolf *et al.* [26]. The LLC-PK1 cells were made quiescent for 24 h in serum-free DMEM and were then incubated for an additional period of 24 h with AGE-BSA either alone or in the presence of 20 μg/ml of the neutralizing anti-TGF-β1 antibody. Incorporation of [^{3}H]thymidine into LLC-PK1 cells was used for measurement of cell proliferation as described before.

Immunohistochemical detection of imidazolone

To evaluate the cellular accumulation of AGEs, immunostaining of imidazolone (a common epitope of AGE-modified proteins [27]) was performed using the peroxidase–antiperoxidase method with the monoclonal anti-imidazolone (AG-1) antibody as follows. Cell culture medium was removed and cells washed with PBS. Cultured cells were mixed with 1.5 ml fibrin glue (Tissuecol Duo S; Immuno GmbH, Heidelberg, Germany), scraped off, transferred into reactive tubes and centrifuged at 1000 **g** for 10 s. The pellet was coagulated with a drop of a thrombin solution (Immuno GmbH). A spoon of cells in a cup were fixed with 0.1 M PBS containing 4% paraformaldehyde for 1 h at 4°C, washed three times for 30 min in PBS, further fixed in 40%, 70%, 96% and absolute ethanol as well as xylol for 20–30 min, embedded in paraplast, cut (5 μm sections) and mounted on silane-coated slides. The sections were dewaxed, dried overnight and irridiated with microwaves in 0.01 M sodium citrate buffer (pH 6.0), twice for 5 min at 850 W. After being washed in PBS (pH 7.4) the sections were treated with 0.3% hydrogen peroxide for 30 min, incubated with respective normal sera and then incubated for 1 h at 37°C with primary antibodies. After removing the normal rabbit sera, the sections were incubated with monoclonal anti-imidazolone antibody at 4°C overnight. Thereafter, they were washed with PBS and incubated with biotinylated secondary antibodies followed by a streptavidin/biotin-peroxidase complex (Veceastain Elste, Vector Burlingame, CA, USA) at 37°C for 30 min. They were then washed twice with PBS and the sections were completed by the addition of diaminobenzidine-H_2O_2 solution for 5–30 min. After being washed with PBS, the slides were counterstained with haematoxylin.

Analysis of the sections was carried out in an unbiased manner by means of a C.A.S.T. Grid system (Olympus, Albertslund, Denmark) (for more details see [28]). All cells were evaluated on the television screen monitor at a final magnification of ×3453 (objective used ×100, oil, Numerical Aperture (NA) = 1.35) that came into focus with unbiased counting frames systematically, randomly spaced throughout the sections (area of the counting frames 1477 μm^2). The distance between counting frames in mutually orthogonal directions x and y was 100 μm. Accordingly, on average, ~350 cells were evaluated per section, which were found in ~64 unbiased counting frames per section. Degree of imidazolone staining was divided into four rates: negative (−), weak positive (+), intermediate positive (+ +) and strong positive (+ + +). Afterwards, negative and weak positive data were rated as negative, and the others were counted as positive.

Statistical analysis

The mean for the replicates of each experiment was determined. Results are presented as mean ± SD, with n

indicating the number of experiments. A comparison between the two groups was made using paired and unpaired t-test. Group differences (more than three) were evaluated employing analysis of variance (ANOVA). Statistical significance was defined as a $P<0.05$.

Results

Effect of AGE-BSA on cell proliferation

With AGE-BSA (38 μM) treatment of LLC-PK1 cells, cell number decreased within 24 h from 3.95 ± 0.12 (control) to $3.34 \pm 0.22 \times 10^6$ (AGE-BSA; $P<0.05$, $n=9$), while that of BSA-treated cells remained unchanged ($4.01 \pm 0.3 \times 10^6$; $n=9$). The time course of [^{3}H]thymidine incorporation showed the maximal decline after 20 and 24 h, corresponding to 68% of control (serum-free). Co-administration of trypsin enhanced the thymidine incorporation to 80.1% of control (Figure 1). The AGE-BSA-induced decline of thymidine incorporation was not due to cell death, since both exclusion of Trypan Blue and release of LDH were not significantly changed in the different groups (data not shown).

Cell protein content and cell volume

Cell protein content rose from 0.730 ± 0.07 ng/cell (control) to 0.894 ± 0.06 ng/cell after AGE-BSA ($P<0.05$), and BSA incubation resulted in an insignificant rise to 0.806 ± 0.01 ng/cell. After co-incubation of AGE-BSA with trypsin, the cell protein content was nearly normalized (0.772 ± 0.05 ng/cell). Cell volume showed, after AGE-BSA, a significant rise from 2.828 ± 0.10 to 3.092 ± 0.102 fl ($P<0.05$), and was nearly normalized by co-incubation with trypsin (2.633 ± 0.098 fl). After BSA incubation, cell volume did not change (2.612 ± 0.097 fl).

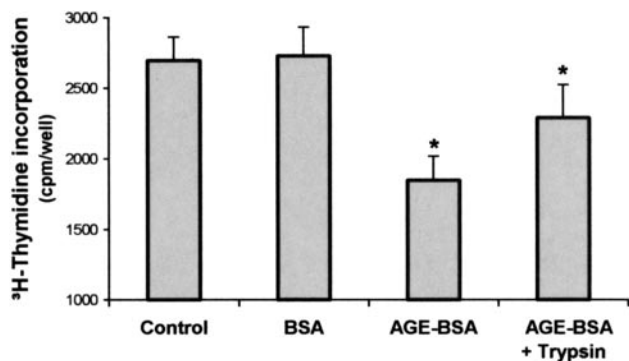

Fig. 1. Effect of AGE-BSA on [^{3}H]thymidine incorporation on subcultured LLC-PK1 cells. AGE-BSA (38 μM)-induced inhibition of [^{3}H]thymidine incorporation and its reversal by trypsin (2.5 μg/ml). Results from representative experiments are shown. Four samples were analysed in each experiment ($n=2$), values represent the mean ± SD, and comparisons were made by ANOVA. Significance was accepted at $^*P<0.05$. The AGE-BSA-treated group differed from controls as well as the BSA and AGE + trypsin-treated groups.

Expression of TGF-β1 mRNA in LLC-PK1 cells

We hypothesized that the AGE-BSA-induced cell effects could be mediated, at least in part, by a rise in endogenous TGF-β1 production. Therefore, TGF-β1 gene expression and TGF-β1 protein in the supernatant of cell culture were investigated. The results revealed induction of TGF-β1 mRNA by AGE-BSA (38 μM) as well as by high glucose medium (30 mmol/l glucose). AGE-BSA increased TGF-β1 mRNA levels standardized to GAPDH mRNA levels by 5.3 ± 1.2 (serum-free; $n=6$) and high glucose by 4.1 ± 0.9 of control ($n=6$; Figure 2). Elevated expression of TGF-β1 mRNA transcripts was not significantly changed after prolongation of the incubation period to 72 h (data not shown). AGE-BSA (19, 38 and 76 μM) increased the expression of TGF-β1 mRNA after 24 h in a concentration-dependent manner (Figure 3).

To elucidate whether trypsin modulates the expression of TGF-β1 mRNA, a co-incubation of AGE-BSA

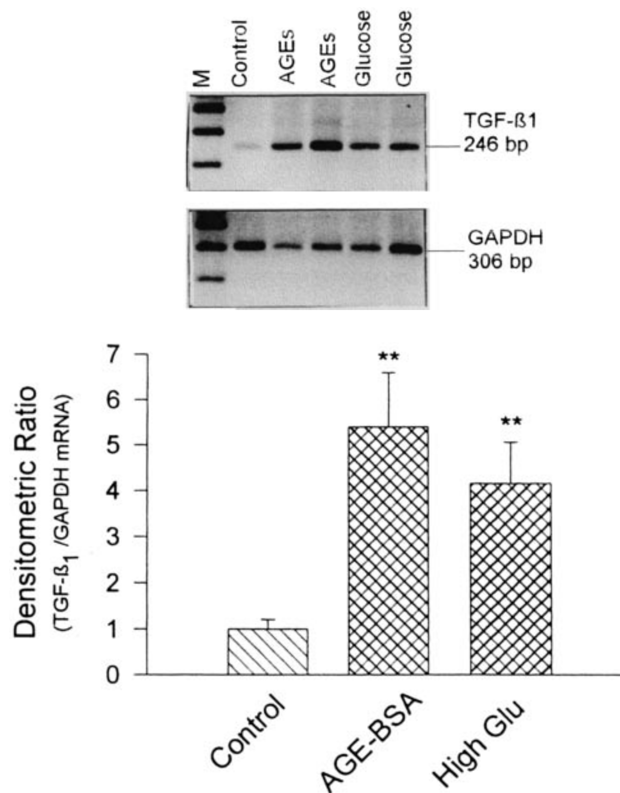

Fig. 2. Growth-arrested cells were cultured in vehicle (serum-free medium), AGE-BSA (38 μM) and high glucose (450 mg/dl) for 24 h. Products of reverse transcription were separated by flat bed electrophoresis in 2% agarose gels. The results, expressed as the densitometric ratio of TGF-β mRNA to GAPDH, were normalized for the control. The increase in TGF-β1 mRNA levels, induced by AGE-BSA, was similar to that of high glucose ambient culture. Lanes 2, 3, and 5 and 6 represent the amplification product of two independent repeat experiments with the same treatment, reverse transcription and amplification. Note that a relative density of 1.0 was assumed for lane 1 (control) at 24 h of cell culture. Densitometric results are shown as the means ± SD of two independent experiments ($n=6$; $P<0.01$ *vs* control).

(38 μM) with various concentrations of trypsin (0.625, 1.25, 2.5 and 5.0 μg/ml final) was performed for 24 h. This treatment significantly reduced the AGE-induced TGF-β1 mRNA expression, although it did not abolish the basal TGF-β1 expression. Incubation with unglycated BSA yielded no significant effects (Figure 3).

Effect of AGE-BSA on TGF-β1 protein

As shown in Figure 4, the incubation of LLC-PK1 cells with AGE-BSA (38 μM) resulted in a significant rise of total TGF-β1 protein in the supernatant in relation to cell number after 24 h. Co-incubation with trypsin (2.5 μg/ml) weakened this effect.

Neutralizing TGF-β1 antibody against the AGE-BSA-induced antiproliferative effect

To assess whether the AGE-BSA-induced cellular responses are mediated by TGF-β1, a neutralizing anti-TGF-β1 antibody was used. Addition of 20 μg/ml to AGE-BSA (38 μM)-containing media increased thymidine incorporation to levels that approximated the basal thymidine incorporation in cells grown in control medium; the antibody itself did not influence cell proliferation (Figure 5) and prevented the increase of cell protein content. Thus, the AGE-BSA-induced rise of cell protein from 0.40 ± 0.04 to 0.49 ± 0.02 ng/cell ($P < 0.05$) was reduced to 0.42 ± 0.01 ng/cell ($P < 0.01$) after co-incubation with the neutralizing anti-TGF-β1 antibody. These responses suggest that the growth inhibitory action of AGE-BSA was predominantly attributable to endogenous TGF-β1 bioactivity.

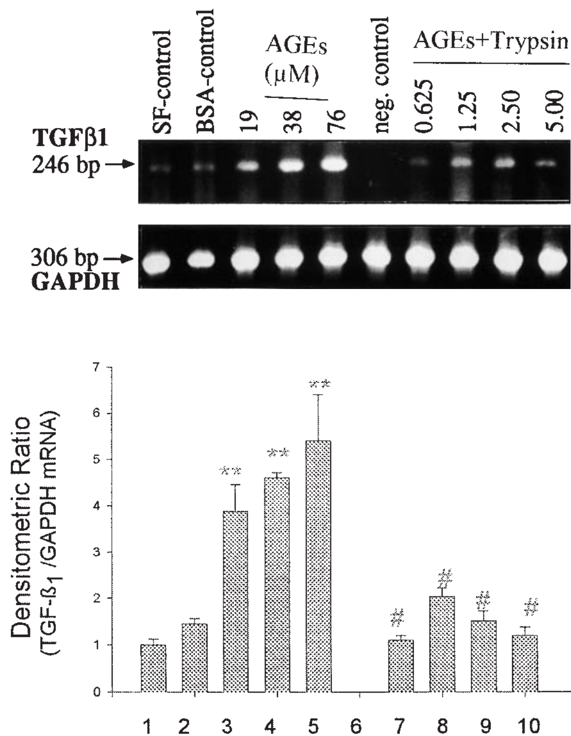

Fig. 3. Dose response of AGE-BSA and the effect of co-administered trypsin on TGF-β1 expression after 24 h. Amplification product of two independent experiments with separate treatments, reversed transcription and amplification at 24 h of cell culture. A relative density of 1.0 was assumed for lane 1, representing vehicle-treated cells; lane 2: BSA (38 μM)-treated cells; lanes 3–5: AGE-BSA-treated cells (19, 38 and 76 μM, respectively); lane 6: negative control; lanes 7–10: AGE-BSA (38 μM) + trypsin (0.625, 1.25, 2.5 and 5.0 μg/ml, respectively)-treated cells. It is evident that AGE-BSA increased TGF-β1 expression in a dose-dependent manner. After combined administration of AGE-BSA with trypsin, the increased TGF-β1 expression is reduced. Densitometric ratios of TGF-β1 to GAPDH mRNA represent means ± SD. $^{**}P < 0.01$ *vs* control and BSA-treated cells; $^{\#}P < 0.05$ *vs* AGE-BSA-treated cells. Unglycated BSA did not alter TGF-β1 expression significantly.

Immunohistochemical evaluation of AGE accumulation in the cells

Potential AGE accumulation in the cells was evaluated by an immunohistochemical detection method for the AGE imidazolone [31], which demonstrated in the AGE-BSA-treated group a mean positive staining of 65.5% of the cells, which averaged only to 34.4% after co-incubation of AGE-BSA with trypsin (Figure 6).

Discussion

This study was designed to investigate in tubule cells the effects of AGEs on TGF-β1 expression as well as on cell proliferation, and to examine the potential modulatory action of a co-administered protease. It could be shown that AGE-BSA and not BSA inhibited the proliferation of LLC-PK1 cells, as demonstrated by a decrease in cell number and in [^{3}H]thymidine incorporation. These findings are consistent with studies in mesangial [29,30], smooth muscle and endothelial cells, as well as retinal capillary pericytes [31].

In accordance with our recent studies [20], a rise in cell protein content and cell volume was found after

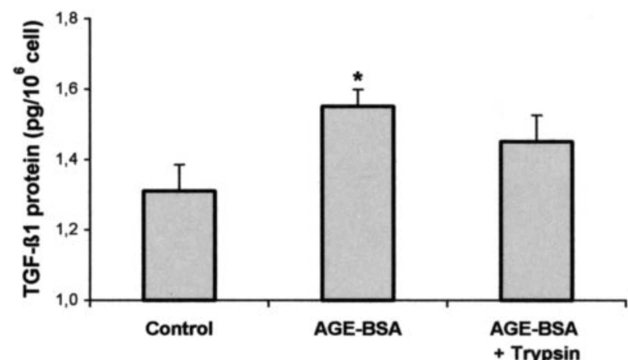

Fig. 4. Effect of AGE-BSA on TGF-β1 protein in cell supernatant. In the supernatant of the LLC-PK1 cell culture, incubation with AGE-BSA (38 μM) resulted in a significant rise of total TGF-β1 protein ($P < 0.05$) in relation to cell number after 24 h. Co-incubation with trypsin (2.5 μg/ml) lessened this response without significance.

incubation with AGEs. Incubation with BSA, on the other hand, did not alter these parameters significantly.

As a next step, we could show that the incubation of LLC-PK1 cells with AGE-BSA increased expression of TGF-β1 mRNA in a dose-dependent manner. In addition, a significant rise in TGF-β1 protein could be demonstrated in the supernatant 24 h after AGE incubation, which indicates that the expression of TGF-β1 was translated into protein. The physiological relevance of increased TGF-β1 synthesis was evaluated by co-incubation with a neutralizing anti-TGF-β1-antibody, which led to a reversal of both the AGE-enhanced cell protein content and the antiproliferative action.

Our data on increased TGF-β1 formation is consistent with similar *in vitro* studies in rodent mesangial cells after incubation with AGE-albumin [32], as well as *in vivo* investigations in the glomeruli of normal rodents after parenteral administration of AGE-albumin [33]. The AGE-BSA-induced TGF-β1 formation is of particular interest with regard to renal hypertrophy in the early stages of DN and the later development of renal fibrosis [2] and corresponds to our earlier finding of an impaired protease activity (cathepsin B + L activity) after incubation of LLC-PK1 cells with AGE-BSA [20]. Currently, activation of transcription factor by AGEs has been reported for the major inducible transcription factor NF-κB via the RAGE receptor [11]. In our own investigations in LLC-PK1 cells, we could demonstrate an activation of p42^{MAP}-kinase (Erk) as well as its downstream target, the AP1-complex, after incubation with AGE-BSA but not with BSA alone [34]. This indicates that AGEs appear to induce specific signal transduction pathways in LLC-PK1 cells.

Co-incubation of AGE-BSA with trypsin ameliorated the anti-proliferative action of AGE-BSA as well as the enhanced cell protein content. Moreover, TGF-β1 expression was markedly reduced. The elevated TGF-β1 protein levels showed only a tendency to lower values. This surprising observation can be attributed to various factors. One possible explanation is that the observation period might have been insufficient to detect a significant decline of TGF-β1 protein.

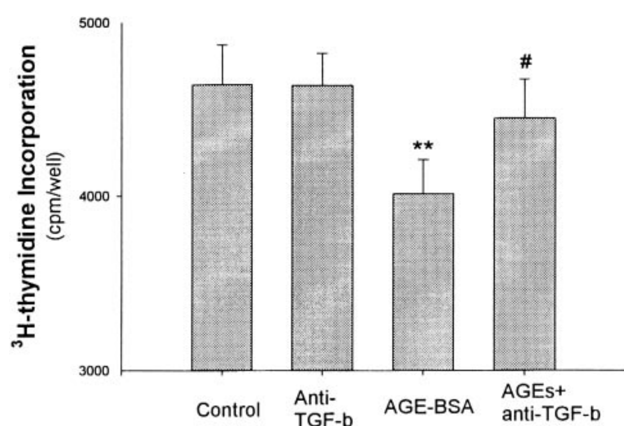

Fig. 5. Bioactivity of TGF-β1 in cells grown in AGE-BSA media. Bioactivity of TGF-β1, induced by AGE-BSA, was evaluated by co-administration of a neutralizing anti-TGF-β1 antibody. The quiescent LLC-PK1 cells were cultured for 24 h in serum-free media, in media containing AGE-BSA (38 μM) and after co-incubation with the neutralizing anti-TGF-β1 antibody (20 μg/ml). AGE-BSA decreased [^{3}H]thymidine incorporation ($P < 0.05$). The anti-TGF-β1 antibody restored the antiproliferative effect of AGE-BSA ($P < 0.05$). One typical experiment ($n = 4$) is shown.

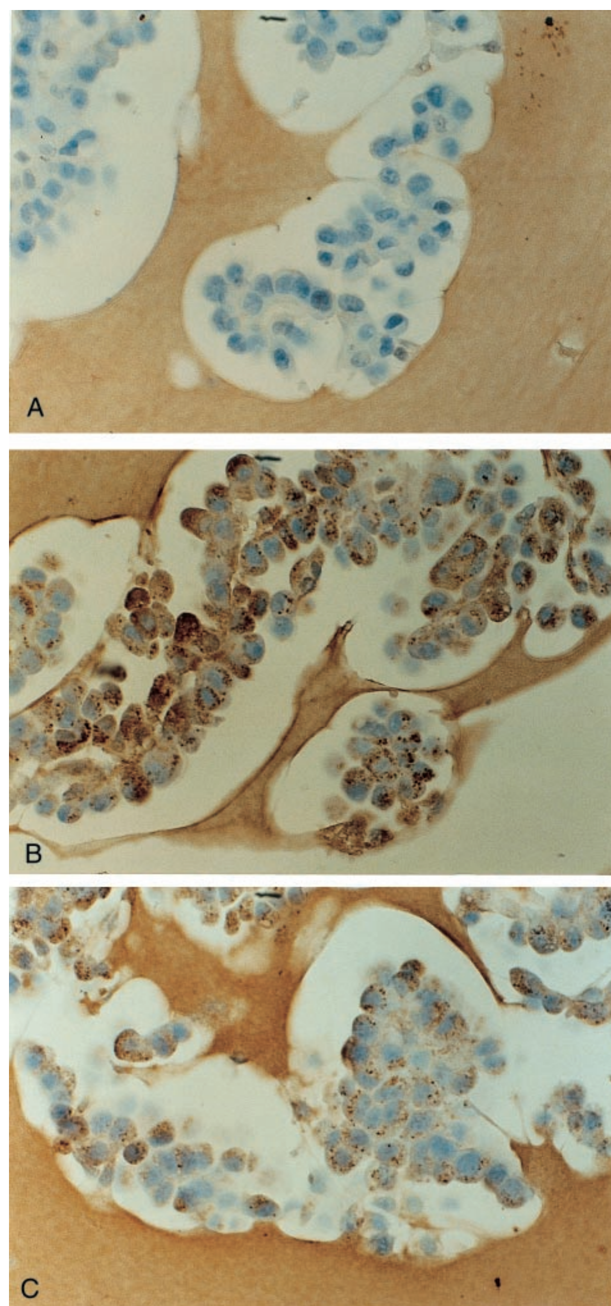

Fig. 6. Intracellular AGE accumulation, as evaluated by immunohistochemical staining of imidazolone in LLC-PK1 cells (original magnification ×400). The figure shows, in the untreated cells, that the nucleus stained blue and the cytoplasm stained a lighter colour (**A**). After AGE-BSA incubation (38 μM), most cell cytoplasms strongly stained brown (**B**). Co-incubation with trypsin (2.5 μg/ml) markedly reduced the intensity of brown staining of the cytoplasm (**C**).

Concerning the modulatory effect of trypsin on tubule cell function, a partial degradation of AGE-BSA into two smaller fragments, with a consequent decline of cellular uptake, could be involved. This possibility could be excluded by incubation of two AGE-BSA batches with a high concentration of trypsin. Within an observation period of 24 h, no degradation could be detected.

Therefore, we assume that the trypsin-mediated effects result from an impaired interaction between AGEs and their binding proteins/receptors at the tubule cell surface, as formerly demonstrated for endothelial cells by binding studies [9,17]. Our findings of the immunostaining of the AGE imidazolone in the tubule cells are in line with this data. By use of an imidazolone-specific antibody, a marked staining of the tubule cells could be demonstrated after AGE-BSA incubation, which was reduced by $>50\%$ in the presence of trypsin. Hence, it can be deduced that the amelioration of the AGE-BSA effects observed in our study resulted in particular from a decreased cellular AGE content. Trypsin is a widely used protease for enzymatic digestion of fixed tissues to enhance immunostaining. In our study, there was a strong fixation and paraffin-embedding step between the biological experiment (e.g. trypsin treatment of living cells) and the immunohistochemical detection of AGEs. Therefore, later interference of trypsin with the immunodetection of AGEs can be excluded.

Currently, we cannot offer data concerning the specificity and selectivity of the trypsin action. It is likely that other proteases, such as certain metalloproteases (MMPs), produce similar effects or even higher specificity and affinity to the AGE-binding proteins. Thus MMPs have been implicated in the cleavage of TNF-α and other receptors [35]. Therefore the findings presented here may be the starting point for the development of new specific proteolytic enzymes.

In conclusion, our data shows that AGE-BSA exerts marked effects on LLC-PK1 cells, most likely due to enhanced formation of TGF-β1. These alterations are substantially reversed by co-administration of trypsin, which lowers the intracellular accumulation of the AGE imidazolone. Thus, an interaction of this serine protease with AGE-binding proteins on the surface of LLC-PK1 cells is assumed.

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