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Toxic action of advanced glycation end products on cultured retinal capillary pericytes and endothelial cells: relevance to diabetic retinopathy

R. Chibber, P. A. Molinatti, N. Rosatto, B. Lambourne, E. M. Kohner

Division of Medicine, Department of Endocrinology, Diabetes and Metabolic Medicine, St. Thomas' Hospital, London, UK

Summary The toxic effects of advanced glycation end products (AGEs) on bovine retinal capillary pericytes (BRP) and endothelial cells (BREC) were studied. AGE-modified bovine serum albumin (AGE-BSA) was toxic to BRP. At a concentration of 500 μg/ml it reduced the BRP number to 48 ± 3 % ($p < 0.05$) of untreated controls, as determined by cell counting with haemocytometer. AGE-BSA was also toxic to bovine aortic endothelial cells (BAEC) reducing cell number to 84 ± 3.1 % of untreated controls. Under similar conditions, low concentrations (62.5 μg/ml) of AGE-BSA were mitogenic to BREC increasing the cell proliferation to 156 ± 11 % ($p < 0.05$) above that of untreated controls. At a higher dose of 500 μg/ml AGE-BSA decreased the proliferation of BREC to 85 ± 6 % of untreated controls. Immunoblot analysis demonstrated that BRP and BREC express the p60 AGE-receptor. Retinal capillary bed from the human also stained positively for the p60 AGE-receptor. Addition of 0.25 μg/ml of p60 AGE-receptor antibody was able to block the effects of AGE-BSA on BRP and BREC. The level of binding of [^{125}I]-labelled AGE-BSA to the cell surface was small but significant among the three cell types. There was also an increase in the internalized pool of radioligand in BRP and BREC but this was very much lower than in BAEC. In all the cell types the internalized pool of [^{125}I]-labelled AGE-BSA was much larger than the amount associated with the cell surface. Degradation products were not detected in the media over the 24-h incubation of the cells with [^{125}I]AGE-BSA. The binding of [^{125}I]-labelled AGE-BSA to the cell surface was prevented by the addition of p60 AGE-receptor. These results suggest that the interaction of AGE-modified proteins with the membrane-bound AGE-receptor may play an important role in the pathogenesis of diabetic retinopathy. [Diabetologia (1997) 40: 156–164]

Keywords Advanced glycation end products, diabetic retinopathy, pericyte, endothelial cell, AGE-receptor.

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Corresponding author: Professor E. M. Kohner, Division of Medicine, Department of Endocrinology, Diabetes and Metabolic Medicine, UMDS, 4th floor, North Wing, St. Thomas' Hospital, Lambeth Palace Road, London SE1 7EH, UK

Abbreviations: AGE, Advanced glycation end products; BRP, bovine retinal capillary pericytes; BREC, bovine retinal capillary endothelial cells; BAEC, bovine aortic endothelial cells; PBS, phosphate buffered saline; FCS, fetal calf serum; MEM, Minimal Essential Medium; SDS, sodium dodecyl sulphate; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; HBGF, heparin binding growth factor; LDH, lactate dehydrogenase; EDTA, ethylenediaminetetraacetic acid; ECL, enhanced chemiluminescence; FITC, fluorescein isothiocyanate; RAGE, receptor for AGE; PMSF, phenylmethylsulfonyl fluoride

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Glucose reacts spontaneously with amino groups of a wide range of proteins to form Schiff bases, which through multiple rearrangements, are transformed into irreversibly bound, chemically reactive adducts, called advanced glycation end products (AGE) [1]. AGE have been detected in plasma and on vessel walls [2] and their levels increase as a function of age and blood glucose level [3, 4]. Recent studies are beginning to establish a causal relationship between the accumulation of AGE in tissues and the development of diabetic complications [5].

Studies in vitro have shown that AGE can mediate monocytic chemotactic activity [6], and block the cytostatic effect of nitric oxide-dependent vasodilatation [7]. AGE modification of mesangial cell matrix has been reported to alter the mesangial proliferative response [8]. The addition of AGE-modified proteins to cultures of mouse mesangial cells results in the increased synthesis and production of extracellular matrix components, such as fibronectin and collagen [9]. AGE-modified proteins bind specifically to AGE-receptors on the surface of endothelial cells, resulting in receptor-mediated endocytosis, changes in cellular proliferation, endothelial cell permeability, coagulant functions [10] and altered expression of endothelial-derived relaxing activity [11]. Receptors that specifically recognize AGE adducts on proteins have also been identified on macrophages, mesangial cells and other cell types [12, 13].

In in vivo experiments the administration of AGE-modified albumin to normal rats raises tissue levels of AGE and significantly increases vascular permeability in the absence of hyperglycaemia [14]. There appears to be a good correlation between the degree to which AGEs accumulate in dermal collagen and the degree of diabetic retinopathy [15].

We hypothesized that the gradual accumulation of AGE-modified proteins in tissue and serum of diabetic patients may contribute to the dysfunction and/or degeneration of retinal vascular cells which occur during the early stages of diabetic retinopathy [16]. To address this hypothesis, we investigated possible in vitro toxicity and the interaction of AGE-modified bovine serum albumin (AGE-BSA) with cultured bovine retinal capillary pericytes and endothelial cells.

Materials and methods

Cell isolation and culture. Primary and subculture (up to the second passage) of retinal pericytes and endothelial cells were established from bovine retinas dissected from eyes of freshly slaughtered cattle. The retinas were homogenised in serum-free Minimal Essential Medium (MEM; Gibco, Paisley, UK) and filtered through an 85-μm nylon mesh.

Bovine retinal capillary pericytes (BRP). Pure cultures of pericytes were isolated and established as described previously [17]. The trapped microvessels were digested with collagenase-dispase (1 mg/ml) solution in serum-free MEM at 37 °C for 30 min. The digested microvessels were then filtered through a 53-μm nylon mesh and plated in tissue culture flasks. The culture medium was MEM supplemented with 10 % fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 mg/ml). Pericyte outgrowth from the microvessels was observed after 24 to 48 h and reached confluence within 7 days. BRP in culture were identified by morphological criteria and by immunocytochemical staining with pericyte specific marker, 3G5 monoclonal antibody (mAB) as described by Nayak et al. [18] and α-smooth muscle actin.

Bovine retinal capillary endothelial cells (BREC). The isolation and long-term culture of endothelial cells was carried out according to a modified procedure of Bowman et al. [19]. The trapped microvessels were digested with collagenase-dispase (1 mg/ml) for 90 min at 37 °C and filtered through a 53-μm nylon mesh. The digested microvessels were then plated in fibronectin-coated tissue culture flasks. The growth medium for BREC was MEM supplemented with 10 % pooled human serum. The cells were characterised using morphological criteria and by immunostaining with an antibody against factor VIII related antigen.

Bovine aortic endothelial cells (BAEC). Aortas from freshly slaughtered cattle were washed with phosphate buffered saline (PBS) and cleared of excess adventitia. The intercostal vessels and one end of the aorta were clamped and the vessel was filled with 0.2 % w/v collagenase-dispase in PBS and incubated for 15 min at 37 °C. The cells were removed by flushing with 30 ml of PBS and centrifuged at 2000 *g* for 10 min. The cells were resuspended in MEM supplemented with 10 % FCS and plated in tissue culture flasks. BAEC were characterised morphologically and by immunostaining with an antibody against factor VIII related antigen.

Preparation of AGE-modified bovine serum albumin (BSA). AGE-BSA was prepared according to the procedure of Makita et al. [20]. Briefly, 50 mg/ml of BSA (fraction V, low endotoxin; Sigma, Poole, Dorset, UK) was incubated in PBS, pH 7.4 containing 50 mmol/l glucose, 100 IU/ml penicillin, 100 mg/ml streptomycin, and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF) for 6 weeks at 37 °C. The amount of AGE formed on the albumin was assessed as described previously [20].

Toxicity of AGE-BSA. Ten thousand cells were plated in 3-cm tissue culture dishes which were coated with fibronectin for experiments with BREC. After 24 h to allow for cell attachment, fresh growth medium containing various concentrations of AGE-BSA (0, 7.9, 15.7, 31.3, 62.5, 125, 250, 500 μg/ml) were added. To the control dishes, equal concentrations of unmodified BSA were added. After 4 days, the cells were washed in PBS, and counted using trypan blue and a haemocytometer. The experiment was conducted using duplicate dishes per experiment and repeated on at least four separate occasions.

To examine whether the AGE-receptor antibody could block the effect of AGE-BSA on BRP, BREC and BAEC, approximately 1×10^4 cells were plated in 3-cm tissue culture dishes. After overnight incubation, fresh growth medium containing 125 μg/ml of AGE-BSA with or without approximately 0.25 μg/ml of p60 AGE-receptor antibody. The medium was replaced after 2 days and cells counted using trypan blue and a haemocytometer.

To study direct cellular cytotoxicity of AGE-BSA the release of lactate dehydrogenase (LDH) by the cells was

measured. Near confluent cultures of BRP and BAEC in 6-cm tissue culture dishes were exposed to various concentrations of modified or unmodified BSA for 2 days in 0.1 % FCS. A sample of the medium was centrifuged (3000 *g*, 4 °C, 5 min) and the LDH activity of the supernatant determined using Ektachem 950 IRC (Johnson and Johnson, UK). The results are expressed as IU LDH activity/L.

Radiolabelling of AGE-BSA. AGE-BSA was radiolabelled with [^{125}I] using chloramine-T as described [21]. Briefly, 4 μl of [^{125}I] (100 mCi/ml, Amersham, Aylesbury, UK) and 15 μl of chloramine-T (2 mg/ml) were added to 50 μg of AGE-BSA in phosphate buffer (pH 7.5). The reaction mixture was incubated for 90 s at room temperature. After further additions of chloramine-T, free iodine was removed by gel filtration using Sephadex G-25 column (PD-10, Pharmacia, Milton Keynes, UK). The specific activity after four iodinations was 1.2×10^4 cpm/ng of protein.

Binding and cellular processing of [^{125}I] AGE-BSA. Pre-confluent cultures of BRP, BREC and BAEC in 3-cm tissue culture dishes were washed with PBS and incubated with 1 ml of binding buffer (MEM containing 1 % FCS) containing 3 μg of [^{125}I] AGE-BSA alone or in the presence of 200-fold molar excess of unlabelled AGE-BSA. After incubating at 37 °C for various times (0.5, 1, 2, 4, 6, 8 and 24 h) the reaction was terminated by washing the dishes ten times with cold binding buffer. The material bound to the membrane was then eluted by incubating the cells at 37 °C for 5 min with 2 mg/ml heparin (Sigma) in elution buffer (in mmol/l: 5 EDTA, 10 HEPES, pH 7.5, 137 NaCl, 11 glucose, 4 KCL and 1 mg/ml BSA). The internalized pool of AGE-BSA was determined by solubilizing the cells in 0.3 mol/l NaOH and 1 % SDS for 30 min at room temperature. The amount of degraded radioligand in media overlying the cells was determined by adding 50 μl of 3 % BSA in MEM to 50 μl of the media, followed by 100 μl of ice-cold 20 % TCA. The samples were incubated for 30 min on ice and the protein was then precipitated by centrifugation at 14000 *g* for 1 h at 4 °C. The amount of degraded radioligand in the media was also confirmed by a second protocol in which the cells were incubated with 3 μg/ml of [^{125}I] AGE-BSA in 1 ml of binding buffer (MEM containing 0.1 % BSA) for 40 min at 37 °C. After washing, the cells were incubated with 1 ml of MEM containing 0.1 % BSA in the presence and absence of 50 mmol/l chloroquine. At various time points a 50-μl sample was removed and processed as above. Radioactivity was measured using a gamma counter (1261 Multigamma; LKB Wallac, Turku, Finland). The experiment was carried out for each cell type in duplicate and repeated on three separate occasions.

To examine if anti-p60 antibody can inhibit the binding of [^{125}I] AGE-BSA cells were grown to near confluency in 24-well plates. The cells were washed in PBS and 0.5 ml of binding buffer was added. Radiolabelled AGE-BSA (3 μg/ml) alone or in the presence of a 200-fold molar excess of unlabelled material was then added and the cells incubated at 4 °C for 4 h. Antibody to the p60 AGE-receptor was added to some of the cells at concentrations of 0.025, 0.05, and 0.25 μg/ml. Following incubation the wells were washed rapidly 10 times with ice-cold binding buffer, and the bound material eluted by exposing the cultures for 5 min at 37 °C to elution buffer (in mmol/l: 5 EDTA, 10 HEPES, pH 7.5, 137 NaCl, 11 glucose, 4 KCL and 1 mg/ml BSA). Radioactivity of the samples was measured using a gamma counter (1261 Multigamma, LKB Wallac). The experiments were performed in triplicate.

Western blot analysis. Membrane fraction was prepared from the cells by ultracentrifugation. Between 5 and 10×10^7 cells were suspended in PBS and washed three times with PBS by repeated resuspension and centrifugation at 200 *g* for 5 min. A cell membrane fraction was then prepared. Briefly, cells were suspended in 5 ml of ice-cold 25 mmol/l Tris-HCL, 290 mmol/l sucrose, pH 7.4 and disrupted by 25 strokes of a Dounce homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min at 4 °C to remove unbroken cells, nuclei and cell debris and this fraction was retained as a crude nuclear fraction. The supernatant was removed and centrifuged at 105000 *g* for 20 min at 4 °C. The pellet was suspended in 5 ml of 25 mmol/l Tris-HCL, 0.25 mmol/l EDTA, pH 7.4 and then centrifuged at 105000 *g* for 20 min at 4 °C. The supernatant was then discarded and the pellet was dissolved in non-reducing sample buffer (0.06 mol/l Tris-HCL, pH 6.8; 0.1 % v/v glycerol; 2 % SDS; 0.1 % bromophenol blue).

Protein concentration was determined and the membrane fraction from BRP, BREC, BAEC and human monocytes separated by SDS-PAGE on a 10 % acrylamide gel [22]. Proteins were transferred to Hybond-ECL (Amersham, Aylesbury, UK) and the presence of 60 kDa AGE-binding receptor detected by immunoblotting using a polyclonal antibody specific to the NH_2-terminal peptide of the p60 component of the AGE-receptor as reported [12, 13]. Membrane was blocked with 5 % BSA in 20 mmol/l Tris-HCL pH 7.5, 0.05 mol/l NaCl, and 0.05 % Tween 20 (Tris buffered saline TBST). All antibody incubations were performed in 1 % BSA in TBST. Anti-p60 antiserum was used at a 1 : 1000 dilution. Primary antibody-antigen complex was detected with anti-rabbit IgG-horseradish peroxidase conjugate (1 : 10,000, Sigma) with enhanced chemiluminescence detection reagent (ECL) (Amersham). The intensity of the immunoreactive bands on autoradiograms was determined by scanning densitometry (Image Quant, Molecular dynamics, Kemsing, UK).

Retinal digest preparation and indirect immunofluorescence. Microcirculatory vessels of the human retina were freed of adherent cell layers according to the method of Kuwabara and Cogan [23]. Briefly, retinas were removed from enucleated eyes, fixed in 3.7 % formalin for 30 min and digested first with 3 % pepsin (Sigma) and after washing with 3 % trypsin (1/250; Sigma). Both of these digestions were for 30 min at 37 °C. Subsequent incubation with distilled water (24 hours) removed adhering remnants of nervous tissue. The remaining vascular tree was transferred on glass slides and allowed to dry.

The microvessels were blocked with 3 % BSA in PBS for 1 h at room temperature. After washing the microvessels were incubated with the primary antibody (diluted 1 : 500) for 1 h at room temperature. The microvessels were then washed and incubated with diluted biotinylated anti-rabbit IgG (diluted 1 : 100) for 1 h at room temperature. After washing with PBS, extrAvidin labelled with fluorescein isothiocyanate (ExtrAvidin-FITC, diluted 1 : 30, Sigma) was added for 1 h. The slides were then washed, mounted and observed with an Olympus fluorescence microscope.

Protein measurement. Total protein was determined using bicinchioninic acid (Sigma).

Statistical analysis

The results are presented as means ± SEM. Differences between groups in the toxicity data were sought using paired Student's *t*-test.

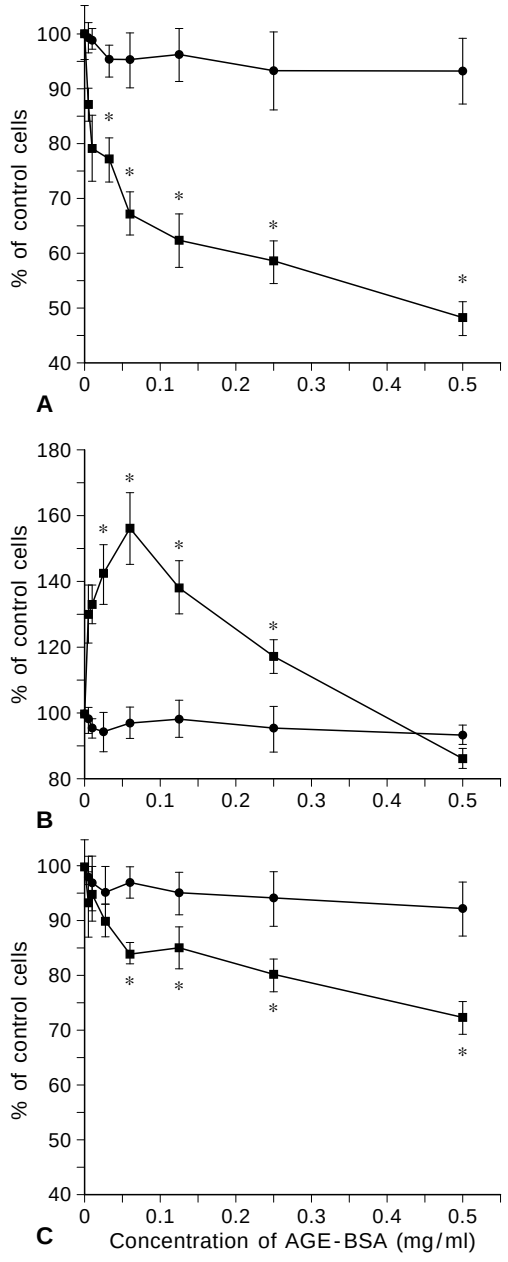

Fig. 1. A–C The effect of AGE-modified BSA (■) and BSA (●) on the proliferation of, (A) BRP, (B), BREC, (C) BAEC. Approximately 1×10^4 cells were exposed to various concentrations of AGE-BSA and BSA. After 4 days the cells were trypsinized and the number of viable cells counted using haemocytometer. The results are presented as percentage of untreated controls and each point represents the mean ± SEM of three replicate cultures repeated on four separate occasions. * $p < 0.05$ vs untreated cultures

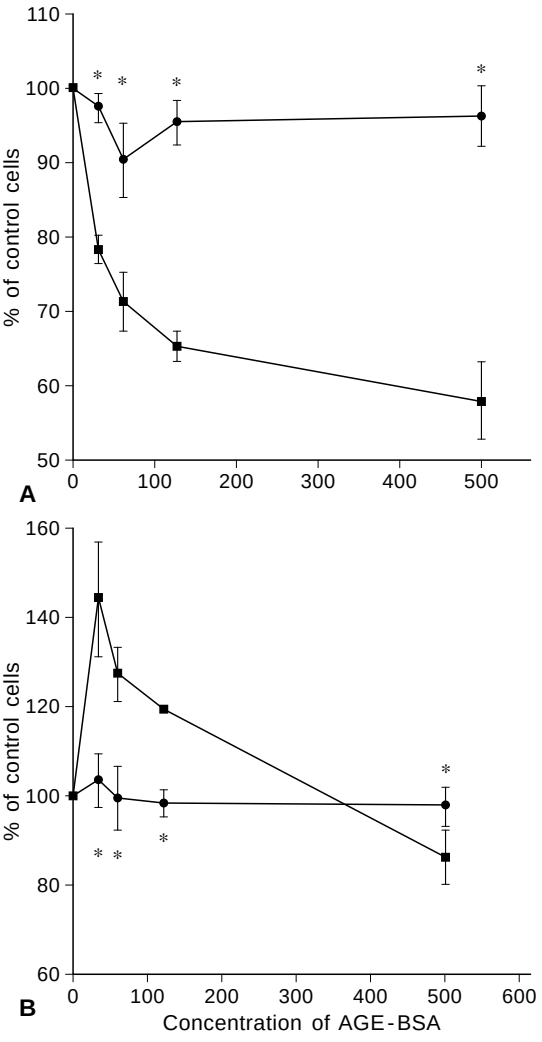

Fig. 2. A, B Reversal of AGE-induced toxicity in BRP (A) and BREC (B) by the addition of the antiserum to the p60 AGE-receptor. Cells were exposed to increasing concentrations of AGE-BSA for 4 days in the presence (●) or absence (■) of 0.25 μg/ml of p60 antibody and nonimmune IgG. Cells were counted and the results are presented as percent of untreated controls. Each point represents the mean ± SEM of four separate determinations. * $p < 0.05$ vs control

Results

Toxicity of AGE-BSA. AGE-modified BSA was found to be toxic to BRP in a near dose-dependent manner, reducing the cell number to 48 ± 3% ($p < 0.05$) of untreated controls at the highest concentration of 500 μg/ml (Fig. 1A). Under similar conditions, low concentrations of AGE-BSA were found to be significantly mitogenic to BREC. At 62.5 μg/ml AGE-BSA increased the cell number to 156 ± 11% of controls ($p < 0.05$) (Fig. 1B). This mitogenic effect was gradually lost as the concentration of AGE-BSA was increased in the medium to a maximum of 500 μg/ml where it actually reduced BREC proliferation to 85 ± 6% of untreated controls ($p < 0.05$). AGE-BSA was also found to be slightly toxic to BAEC and at a high concentration of 500 μg/ml it reduced the proliferation of BAEC to 84 ± 3.1% (Fig. 1C). At similar concentrations unmodified BSA was found to have no significant effect on the proliferation of BRP, BREC, and BAEC (Fig. 1).

The addition of 0.25 μg/ml of the p60 AGE-receptor antibody to the cells almost completely blocked

Table 1. Effect of various concentrations of AGE-BSA on the release of lactate dehydrogenase by BRP and BAEC

Concentration of AGE-BSA (mg/ml)	LDH activity (Units/l ± SEM)	
	BRP	BAEC
0.000	209.3 ± 9.1	229.7 ± 17.6
0.008	234.7 ± 19.5	218.0 ± 7.2
0.015	221.7 ± 12.4	225.7 ± 0.9
0.030	213.3 ± 13.3	228.7 ± 18.5
0.060	204.7 ± 11.7	213.0 ± 12.5
0.125	192.0 ± 8.2	231.3 ± 19.9
0.500	189.3 ± 3.8	218.0 ± 14.1
1.000	210.7 ± 6.7	214.3 ± 7.5

Values are the mean ± SEM

the effect of various concentrations of AGE-BSA on BRP, BREC and BAEC (Fig. 2).

The possibility that AGE-BSA causes direct cellular toxicity was investigated by measuring the release of LDH from damaged cells. It was assumed that LDH would be released rapidly after damage to the cell membrane. No significant release of LDH was observed from BRP and BAEC exposed to various concentrations of AGE-BSA for 2 days (Table 1). The level of LDH in MEM containing 0.1 % FCS was found to be 225 IU/l of medium. The complete lysis of 1×10^5 BRPs increased the LDH activity in the medium by almost 252 %.

Immunoblot analysis and immunofluorescence. A polyclonal antibody raised against the p60 component of the AGE-receptor was used to detect immunoreactive material on Western blot (Fig. 3) of membrane fraction from BRP (lane 1), BREC (lane 2), and BAEC (lane 3). Membrane fraction from human monocytes (lane 4) was used as the positive control. An equal amount of protein (15 μg) from the different cell types was loaded per lane. A band corresponding to a relative mass of 60 kDa was observed in cell extracts from all three cell types (Fig. 3). For equal amounts of cell protein the relative intensity of the immunoreactive bands determined (volume analysis) by scanning densitometry was 10.1, 7.5, 8.5 for BAEC, BRP and BREC, respectively.

Specific staining for the p60 AGE-receptor was also observed in the human capillary bed prepared by retinal digest preparation (Fig. 4 A). Negative control stained without the p60 AGE-receptor antibody is presented in Figure 4 B.

Binding, internalization and degradation of [^{125}I]-labelled AGE-BSA. Radioligand binding experiments were carried out by incubating cells with [^{125}I] AGE-BSA for various times at 37 °C. A significant amount of radioactivity was eluted from the surface of BRP, BREC and BAEC by exposing the cells for 5 min to 2 mg/ml of heparin (Fig. 5 A–C). The level of cell surface-associated pool of [^{125}I] AGE-BSA was low but significant and comparable among the three cell

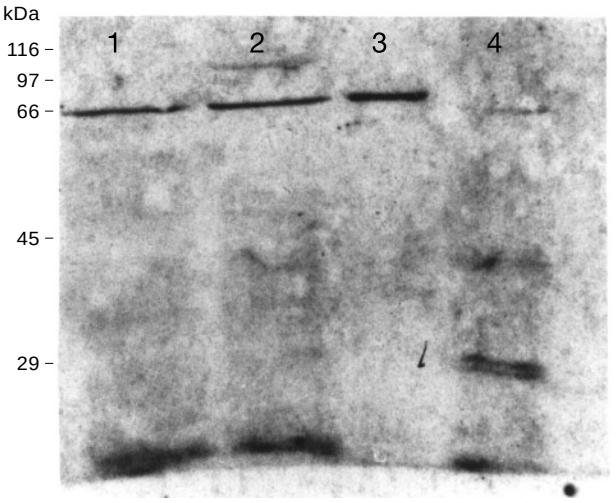

Fig. 3. Western immunoblot of cell proteins of (lane 1) BRP, (lane 2) BREC, (lane 3) BAEC, (lane 4) human monocytes showing cross-reactivity of antibody directed against the p60 component of the AGE-receptor. Equal amount (15 μg) of membrane protein was loaded per lane, separated on 10 % acrylamide gel and after transfer to hybond-ECL membrane the presence of AGE-receptor was detected as described in the text. Molecular weight markers (kDa): 116, β-galactosidase; 97, phospholipase; 66, bovine serum albumin; 45, ovalbumin; 29, carbonic anhydrase

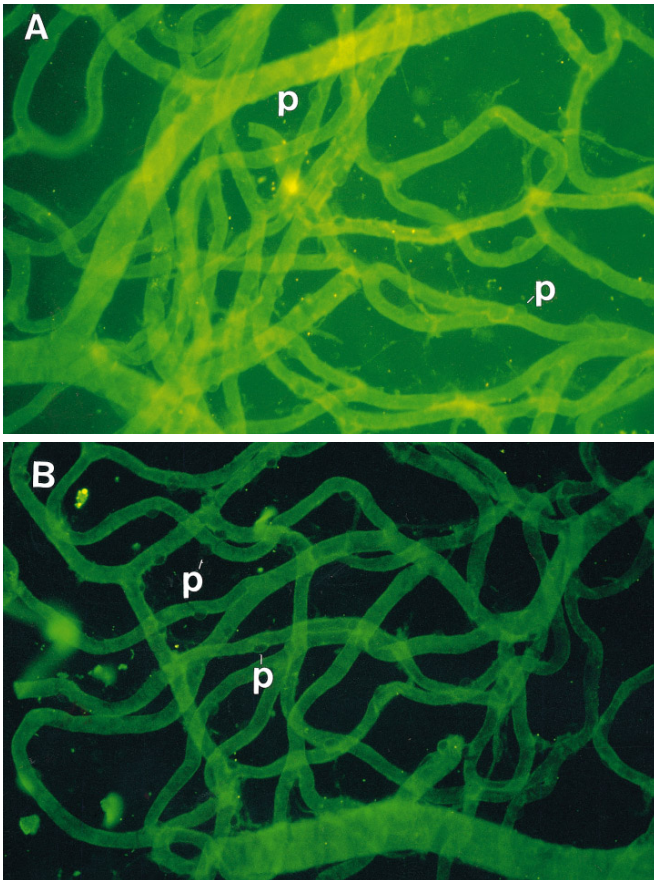

Fig. 4. A, B Immunofluorescence of isolated piece of human retinal microvasculature stained with (A) immune IgG to the p60 component of the AGE-receptor and (B) nonimmune IgG. p, Pericyte, magnification × 200

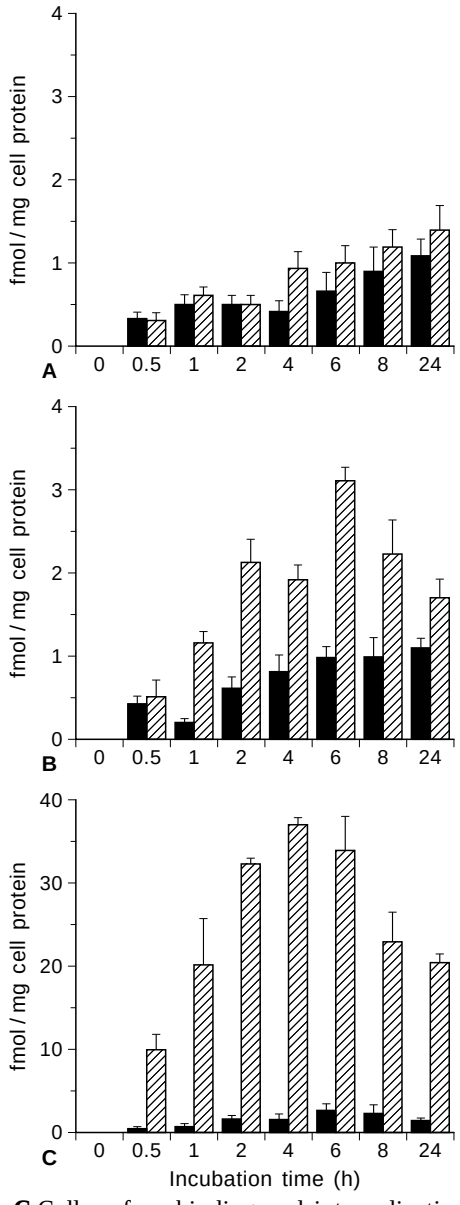

Fig. 5. A–C Cell surface binding and internalization of [^{125}I] AGE-BSA by (A) BRP, (B) BREC and (C) BAEC. Confluent monolayers of the cells were incubated 37 °C with 3 μg/ml of [^{125}I] AGE-BSA. At the indicated time points the amount of surface-bound material eluted with heparin/EDTA (■) and internalized material (▨) were determined by measuring the level of radioactivity. The results presented as fmol/mg of cell protein are the mean ± SEM of duplicate dishes repeated on at least six separate occasions. Note, results for BAEC are presented on a different scale to BRP and BREC

types. In BAEC there was a rapid build-up of an internalized pool of radioligand that remained cell-associated after elution with heparin (Fig. 5C). At similar incubation times, there was a significant increase in the internal pool of radioligand in BRP (Fig. 5A) and BREC (Fig. 5B) but this was very much lower than that in BAEC.

Using TCA solubility as a criterion for degradation of [^{125}I] AGE-BSA, it was found that BAEC failed to release any degradation products of AGE-BSA into the media (data not shown). This was observed despite the rapid internalization of [^{125}I] AGE-BSA by BAEC. Under the same conditions BRP and BREC also failed to release degradation products into the media over the course of the experiment. The capacity of the cells to degrade [^{125}I] AGE-BSA was also investigated using a second protocol which involved incubating the cells with radiolabelled AGE-BSA for 40 min at 37 °C. The cells were then incubated with fresh medium without AGE-BSA. When the medium was treated with TCA it was shown that no significant radioactivity was released into the medium during the 24 h of incubation.

The antiserum against the p60 component of the AGE-receptor prevented the binding of [^{125}I]-labelled AGE-BSA to the surface of BRP and BREC (Fig. 6).

Discussion

The results from the present study demonstrate that AGE-modified bovine serum albumin (AGE-BSA) is toxic to cultured retinal capillary pericytes but increases the proliferation of endothelial cells at low concentrations. Although we have used AGE-BSA for the experiments it is possible that other AGE-modified proteins may have the same effect since immunochemical studies using antibodies against AGE suggest a common structure among the different AGE-modified proteins [24]. The finding that AGE-BSA decreases the proliferation of retinal capillary pericytes is consistent with a recent report by Yamagishi et al. [25] but our results showing that AGE-BSA does not cause direct toxicity are in conflict with their observations that it can exert an immediate toxicity in pericytes. One explanation for these discrepant results may be the nature of the tests used in the two studies to assess direct toxicity of AGE-BSA in pericytes [25]. The finding that AGE-BSA is also toxic to aortic endothelial cells but only at the high concentration of 62.5 μg/ml is in agreement with Tezuka et al. [26] showing that AGE-BSA at 50 μg/ml inhibits the proliferation of cultured human umbilical cord vein endothelial cells (HUVEC). Despite the low level of AGE-mediated toxicity in BAEC, it is possible that exposure to AGE-BSA may have resulted in changes to the cytoskeleton and cell permeability as reported by Esposito et al. [10]. These changes were not investigated in the present study. In an in vitro study, Hogan et al. [27] have reported that AGE can block the antiproliferative effect of nitric oxide on aortic smooth muscle cells and renal mesangial cells.

Previous studies have reported that AGE-modified proteins have a number of biological effects

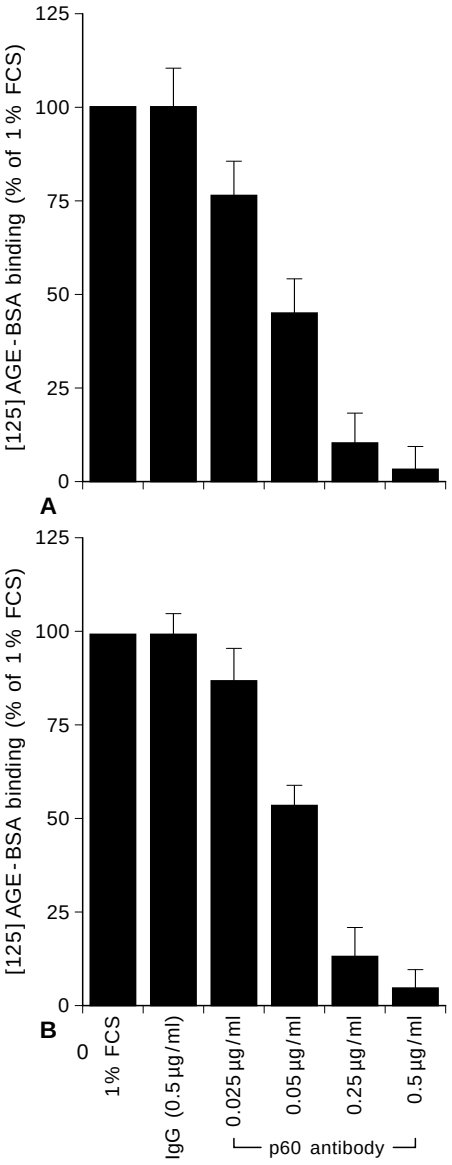

Fig. 6. A, B Inhibition of [^{125}I]-labelled AGE-BSA binding on BRP (A) and BREC (B) surface by anti-p60 antibody. Near confluent cultures in 3 cm tissue culture dishes were preincubated with nonimmune IgG and various concentrations of antiserum against the p60 AGE-receptor. Cultures were then washed and radioligand binding assay was carried out by incubating the cells with 3 µg/ml of radiolabelled ligand in the presence or absence of 200-fold excess of unlabelled AGE-BSA for 4 h at 4°C. Results are presented as percent of specific binding in the presence of 1% FCS. Results represent the mean ± SEM of triplicate determinations

on cultured cells, including stimulation of smooth muscle cell proliferation [28], endothelial cell migration and tube formation [26], alteration in cell shape/cytoskeleton along with perturbation of barrier and coagulant function [10]. Most of these effects are suggested to be mediated by the interaction of AGE with AGE-specific receptors which are localized on various cell types, including smooth muscle cells, monocytes, macrophages and mesangial cells [29]. The immunoreactivity data presented here using a well-characterized antibody [30] confirm that the 60 kDa AGE-specific receptor, also known as AGE-R1 [31] is localized on the surface of pericytes and retinal endothelial cells. Although not known at the time of the present study, retinal pericytes and endothelial cells also express the previously described p90 component of the AGE-receptor (H. Vlassara, personal communication). Taking into account the differences in culture environment, passage number, and protein loading, BAEC appear to have slightly higher numbers of the p60 AGE-receptor on their surface than pericytes and retinal endothelial cells. Our observation that the p60 component of the AGE receptor is also localized in human retinal capillaries is consistent with the recent findings of Stitt et al. [32] showing the presence of both the p60 and p90 AGE-receptors in the retinal microvessels of rats. Consistent with previous reports [30] human monocytes also express the p60 component of the AGE-receptor. Two other AGE-binding proteins (termed receptors for AGEs or RAGE), a 35 kDa and a 46 kDa, have recently been isolated and characterised from extracts of bovine lung by Schmidt et al. [31]. Both of these proteins have been shown to be present on endothelial cells [33].

As expected [9] cell surface binding of radiolabelled AGE-BSA by aortic endothelial cells is followed by receptor-mediated endocytosis and the accumulation of the material inside the cells. In contrast to previous reports we failed to observe the release of any degradation products into the media over the 24-h incubation. The reason for this discrepancy is at present unclear but one explanation could be that degraded material released by the confluent monolayer may become associated with the extracellular matrix components [10]. The finding that the pool of cell surface-associated AGE-BSA in BAEC is much lower than the pool of internalized ligand is consistent with the observation made by Schmidt et al. [31] with macrophages. Compared to aortic endothelial cells the extent of surface binding and receptor-mediated endocytosis of [^{125}I] AGE-BSA is lower in pericytes and endothelial cells. These results would argue against the possibility that actual differences in the intracellular pool of AGE-BSA is directly responsible for AGE-mediated toxicity in pericytes, retinal endothelial cells and aortic endothelial cells. Despite having a higher intracellular pool of AGE-BSA than pericytes the aortic endothelial cells are less sensitive to low concentrations of AGE-BSA. The effect of AGE-BSA on the pericytes and endothelial cells appears to be mediated via its interaction with the AGE-receptors since it can be blocked by the addition of the antibody against the p60 AGE-receptor. This would suggest that AGE-modified BSA mediates its biological effects on pericytes and endothelial cells mostly thorough its interaction with the p60 component of the AGE-receptor. From the binding

studies carried out by Yang et al. [30] it was suggested that the function of the p60 may be to bind AGE-modified proteins while the p90 may serve to stabilise the resulting interaction. It has been suggested that the p60 and p90 proteins represent structurally distinct subunits of a single receptor complex, each with different affinities to bind AGE molecules [30]. In their study Yamagishi et al. [25] were also able to reverse the AGE-induced toxicity in pericytes with antisense oligonucleotides complementary to mRNA coding for RAGE.

Recent studies have demonstrated that the interaction of AGE with the AGE-receptor can mediate directly or indirectly the production of various cytokines and growth factors [29]. The binding of AGE-modified proteins with RAGE has been shown to mediate monocyte migration and activation [31], induce oxidative stress on endothelial cells [34], and increase the expression of the vascular cell adhesion molecule-1 (VCAM-1) [35]. The binding of AGE to the p60 and p90 component of the AGE-receptors on mesangial cells can also induce the synthesis of basement membrane collagen IV, laminin and heparan sulphate proteoglycan [9]. This upregulation of collagen IV by AGE appears to be mediated by platelet derived growth factor (PDGF) since the AGE-mediated effect could be blocked by the addition of PDGF antibody [9].

The data on the toxic action of AGE-BSA on cultured retinal vascular cells are particularly interesting since early diabetic retinopathy is characterized by the selective loss of pericytes and increased proliferation of endothelial cells [13, 36]. Since AGE-modified proteins are reported to accumulate to excessive levels in tissues and serum of diabetic patients it is tempting to speculate that these AGEs may have the same effect on pericytes and endothelial cells in the diabetic milieu as we observed in this in vitro study. Results from numerous investigations have already suggested a strong link between the deposition of AGE in tissues and the pathogenesis of diabetic complications [29]. The work of Hammes et al. [37] demonstrating, (i) the presence of AGE products in retinal capillaries of 26-week diabetic rats, and (ii) significant reduction in the number of diabetes-induced acellular capillaries and pericyte loss when the formation of AGE is inhibited with aminoguanidine, seems to suggest a possible link between excessive accumulation of AGE and the pathogenesis of diabetic retinopathy. There does appear to be a good correlation between the extent to which AGE accumulates on patients' dermal collagen and the degree of diabetic retinopathy [38]. More recently, Stitt et al. [32] have demonstrated that AGE-modified albumin co-localizes with the p60 and p90 component of the AGE-receptors in the retinal vasculature of both diabetic rats and AGE-infused rats suggesting that progressive accumulation of AGE may well be the underlying mechanism for the loss of pericytes and endothelial cells in early diabetic retinopathy.

In conclusion, the in vitro studies on the toxic action of AGE-modified BSA in pericytes and endothelial cells suggest that formation of AGE in diabetes may play an important role in the development of diabetic retinopathy. This is supported by the finding that the p60 AGE-receptor is localized on the surface of cultured retinal capillary pericytes and endothelial cells and also in human and rat [32] retinal microvessels. The hypothesis that excessive accumulation of AGE in diabetes may cause retinopathy is currently being tested further by examining the development of retinal changes characteristic of early retinopathy after both intravitreal and tail vein infusion of AGE-modified albumin in rats.

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