Albumin-derived advanced glycation end-products trigger the disruption of the vascular endothelial cadherin complex in cultured human and murine endothelial cells

Karel OTERO[1], Fernando MARTÍNEZ, Amada BELTRÁN, Deyarina GONZÁLEZ, Beatriz HERRERA, Gypsy QUINTERO, René DELGADO and Armando ROJAS

Laboratorio de Farmacología, Centro de Química Farmacéutica, Apartado Postal 6990, La Habana, Cuba

Endothelial cell (EC) junctions regulate in large part the integrity and barrier function of the vascular endothelium. Advanced glycation end-products (AGEs), the irreversibly formed reactive derivatives of non-enzymic glucose–protein condensation reactions, are strongly implicated in endothelial dysfunction that distinguishes diabetes- and aging-associated vascular complications. The aim of the present study was to determine whether AGEs affect EC lateral junction proteins, with particular regard to the vascular endothelial cadherin (VE-cadherin) complex. Our results indicate that AGE-modified BSA (AGE-BSA), a prototype of advanced glycated proteins, disrupts the VE-cadherin complex when administered to ECs. AGE-BSA, but not unmodified BSA, was found to induce decreases in the levels of VE-cadherin, β-catenin and γ-catenin in the complex and in total cell extracts, as well as a marked reduction in the amount of VE-cadherin present at the cell surface. In contrast, the level of platelet endothelial cell adhesion molecule-1 (PECAM-1), which is located at lateral junctions, was not altered. Supplementation of the cellular antioxidative defences abolished these effects. Finally, the loss of components of the VE-cadherin complex was correlated with increases in vascular permeability and in EC migration. These findings suggest that some of the AGE-induced biological effects on the endothelium could be mediated, at least in part, by the weakening of intercellular contacts caused by decreases in the amount of VE-cadherin present.

Key words: adherens junctions, AGEs, diabetes, permeability, VE-cadherin.

INTRODUCTION

Endothelial cells (ECs) constitute an important interface lining the internal vascular surface, and regulate the passage of solutes and circulating cells from blood to tissues and vice versa [1]. This property is regulated in large part by EC junctions, which are considered to be responsible for several of the structural and functional characteristics specific to this cell type [2,3]. Molecules at junctions are required for endothelial cell–cell anchorage and for vascular remodelling, but they are also signalling structures [4].

The endothelium expresses a cell-specific member of the cadherin family, known as cadherin-5 or vascular endothelial cadherin (VE-cadherin) [5,6]. This molecule is the only cadherin identified so far to be consistently organized at inter-endothelial adherens junctions (AJ) of essentially all types of endothelia [6]. Like the other members of the cadherin family, VE-cadherin has adhesive properties and mediates homotypic cell adhesion [7]. The cytoplasmic domain interacts with a group of submembranous proteins called catenins [8] that transmits the adhesion signal and contributes to the anchorage of the protein to the actin cytoskeleton.

The distribution of VE-cadherin at intercellular junctions is altered markedly after treatment of the cells with agents known to increase endothelial permeability, suggesting that the VE-cadherin–catenin complex is involved in the regulation of vascular permeability [6,9,10]. However, none of these agents is capable of affecting the cellular level of the complex. *In vitro*, antibodies directed to this molecule increased the permeation of macromolecules across endothelial monolayers in the absence of any obvious changes in cell morphology [6]. VE-cadherin transfectants showed a significant decrease in permeability compared with control cells [7].

It has been demonstrated that the transendothelial migration of tumour cells involves a localized disassembling of AJ. The adhesive interaction of tumour cells and endothelium leads to the disappearance of VE-cadherin from the retracting endothelial junction until it is re-formed [11,12]. *In vivo*, an antibody against VE-cadherin was found to increase vascular permeability and accelerate neutrophil recruitment [13]. Taken together, these observations suggest that the opening up of VE-cadherin-mediated cell–cell contacts is an important step during the permeation of macromolecules and cell extravasation.

Reducing sugars such as glucose can react non-enzymically with the amino groups of proteins to form, via a cascade of events, irreversible structures known collectively as advanced glycation end-products (AGEs) [14]. The accumulation of AGEs *in vivo* has been found to increase with age, and occurs at an accelerated rate in subjects with diabetes. AGEs have been strongly implicated in the initiation and acceleration of multiple-organ damage in pathological conditions of diabetic and non-diabetic aetiology [15–17], especially microvascular and macrovascular complications [18].

Circulating, as well as subendothelial, AGEs interact directly with ECs through a specific binding system [19,20]. The functional consequences of AGE–endothelial interactions include increased

Abbreviations used: AGE, advanced glycation end-product; AGE-BSA, AGE-modified BSA; AJ, adherens junctions; EC, endothelial cell; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; mAb, monoclonal antibody; NAC, *N*-acetyl-L-cysteine; N-cadherin, neural cadherin; PDTC, pyrrolidine dithiocarbamate; PECAM-1, platelet endothelial cell adhesion molecule-1; RAGE, receptor for AGEs; TBS, Tris-buffered saline; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor.

[1] To whom correspondence should be addressed, at: IRF 'Mario Negri', Via Eritrea 62, 20157 Milan, Italy (e-mail otero@marionegri.it).

permeability of the monolayer to intravascular solutes [18], the induction of monocyte transendothelial migratory activity [21] and the stimulation of angiogenesis [22].

Given the fact that VE-cadherin is an important molecule in EC biology, contributing to the regulation of a number of known AGE-influenced cellular properties, we therefore analysed whether this molecule is, at least in part, responsible for the effects of AGEs on ECs. To this end, we studied the effects of the interaction of endothelium with AGE-modified BSA (AGE-BSA), as a prototype of this class of non-enzymically glycosylated proteins, in the VE-cadherin complex.

EXPERIMENTAL

All reagents were purchased from Sigma unless indicated otherwise.

Antibodies

Mouse monoclonal antibody (mAb) to human VE-cadherin (clone BV9) [8], mouse mAb to human platelet endothelial cell adhesion molecule-1 (PECAM-1) (clone 5F4) [23], and rat mAb to mouse VE-cadherin (clone BV13) [24] were kindly donated by Dr E. Dejana (Mario Negri Institute, Milan, Italy). Mouse mAbs to β-catenin and γ-catenin were purchased from Transduction Laboratories (Lexington, KY, U.S.A.). Mouse mAb to human vinculin was from Sigma.

Cell cultures

Human umbilical vein ECs (HUVEC) were routinely cultured and characterized as described in detail elsewhere [6]. The murine microvascular EC line H5V [25] was grown in Dulbecco's modified Eagle's medium containing 10 % (v/v) fetal calf serum. All studies on HUVEC were performed on early (up to the third passage) cultures that were 48–72 h post-confluent at the time of the study (long-confluent HUVEC). The fetal calf serum content was lowered to 2 % (v/v) when the cells were exposed to AGE-modified or unmodified BSA. For antioxidant treatment, cells were exposed for 1 h to 1 μmol/l *N*-acetyl-L-cysteine (NAC) or 50 μmol/l pyrrolidine dithiocarbamate (PDTC) prior to the addition of AGE-BSA. Cell viability and cellular protein levels were assayed by the Trypan Blue exclusion and Bradford [25a] methods respectively.

Preparation of AGE-BSA

Unmodified BSA, AGE-BSA, reduced AGE-BSA and heat-inactivated AGE-BSA were prepared and characterized as described previously in detail [26].

Immunoprecipitation and immunoblotting

Triton X-100-soluble fractions, as well as whole-cell extracts, were obtained from long-confluent ECs as described previously [6]. Long-confluent EC monolayers were washed twice with Ca^{2+}- and Mg^{2+}-containing PBS and twice with serum-free medium. Cells were extracted for 20 min on ice in lysis buffer, comprising Tris-buffered saline (TBS; 10 mM Tris/HCl and 150 mM NaCl, pH 7.5), 1 mM PMSF, 20 units/ml aprotinin, 15 μg/ml leupeptin, 2 mM $CaCl_2$, 1 % Nonidet P40 and 1 % Triton X-100. Cell extracts were then centrifuged at 14000 ***g*** for 5 min (4 °C). The supernatant was defined as the Triton X-100-soluble fraction. For total cell lysates, monolayers were extracted in lysis buffer containing 0.5 % SDS.

Immunoprecipitation was performed as described previously [8], with some modifications. Briefly, cell extracts [representing $(0.5–1.0) \times 10^6$ ECs] were precleared on Protein A–Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) for 1 h at room temperature. The supernatant, separated by centrifugation, was incubated with Protein A–Sepharose coupled to mAb for 3 h at 4 °C on a rocking platform. This was followed by three washes with TBS containing 1 mM PMSF, 20 units/ml aprotinin and 2 mM $CaCl_2$. Immunocomplexes were collected on the resin and finally resuspended in Laemmli sample buffer and boiled for 5 min.

For immunoprecipitation of culture supernatants, they were collected and centrifuged at 14000 ***g*** for 5 min at 4 °C, and immunoprecipitated as described above.

For immunoblotting, the entire supernatant from samples was electrophoresed by SDS/PAGE on a 7.5 % (w/v) polyacrylamide gel. The gel was then incubated for 30 min in 50 mM Tris/HCl, pH 8.9, 95 mM glycine and 1 mM $CaCl_2$. Separated proteins were electrotransferred on to a nylon membrane (NY 13-N; Schleicher & Schuell, Dassel, Germany), which was blocked with 10 % (w/v) skimmed milk (Oxoid, Basingstoke, U.K.) in Ca^{2+}- and Mg^{2+}-containing TBS overnight. The membrane was incubated for 1 h with the appropriate antibody at the optimal dilution in blocking buffer (either BV9 or BV13 or 5F4 hybridoma culture supernatant diluted 1:2 in blocking buffer, or 0.5 μg/ml mAb to β-catenin or γ-catenin diluted in blocking buffer). This was followed by a 1 h incubation with goat anti-(mouse IgG) or goat anti-(rat IgG) conjugated to horseradish peroxidase (HRP), and developed with an ECL® Western blotting detection kit (Amersham, Little Chalfont, Bucks., U.K.). Between the various incubation steps, the nylon membranes were washed several times with Ca^{2+}- and Mg^{2+}-containing TBS/0.05 % Tween-20.

Cellular ELISA

Cell surface expression of VE-cadherin was determined as reported for other cell adhesion molecules by a cellular ELISA [27,28]. HUVEC were seeded at 1×10^4 cells/cm^2 on 96-well plates and were cultured to reach a long-confluent state. After treatment, cells were washed twice with Ca^{2+}- and Mg^{2+}-containing TBS and fixed with 0.025 % glutaraldehyde for 5 min at room temperature. Primary binding was carried out with 100 μl of specific mAb BV9 (5 μg/ml; 1 h incubation). After washing, 100 μl of goat anti-(mouse IgG) conjugated to HRP at the optimal concentration was dispensed into wells and incubated for 1 h. The level of surface antigen was then estimated colorimetrically, subsequent to the addition of *o*-phenylenediamine dihydrochloride as chromogen substrate according to the manufacturer's instructions. Controls included wells with ECs incubated only with substrate, ECs stained with secondary antibody only, and ECs stained with an irrelevant mAb.

Immunofluorescence microscopy

Indirect immunofluorescence microscopy analysis was performed as described previously [6]. Briefly, HUVEC were grown on glass coverslips (13 mm diameter) coated with human plasma fibronectin (7 μg/ml) set in a well of a 24-well plate. Monolayers were treated and fixed with 3 % paraformaldehyde, permeabilized with 0.5 % Triton X-100 and labelled with mAbs against VE-cadherin, β-catenin, PECAM-1 or vinculin, followed by rhodamine-conjugated secondary antibody (Dakopatts). Fluorescence was detected with a Zeiss Axiophot microscope and photographed using T-Max 3200 film.

In vitro permeability assay

This assay was carried out in Transwell units, as described previously [7]. HUVEC (1.5×10^4 at seeding) were cultured for 5 days in Transwell units (0.4 μm pore size polycarbonate filters; Costar) coated previously with fibronectin (2 μg/well). At the start of the experiment, the culture medium in the lower and upper compartment was replaced with medium containing AGE-BSA or control BSA. Elastase (0.8 μM) was used as a positive control. After incubation, HRP conjugated to goat immunoglobulin (8 μg/ml initial concentration in the upper chamber; minimal calculated molecular mass 200 kDa; specific activity 28 units/ml) was added to the upper compartment. After 1 h of additional incubation at 37 °C, the medium in the lower compartment was assayed photometrically for the presence of HRP activity with *o*-phenylenediamine dihydrochloride as the chromogenic substrate.

Cell migration

The migration assay in wounded monolayers was performed as described previously [7]. HUVEC were cultured for 5 days in 24-well plates to obtain a confluent cell layer. The culture medium was aspirated and areas of confluent cells were removed using a plastic tip. The remaining cells were washed twice with culture medium to remove cell debris and were incubated at 37 °C in culture medium containing AGE-BSA or other stimuli. The cells were fixed with Fast Green (0.02 % in methanol) and stained with Crystal Violet (0.5 % in methanol/water, 20:80, v/v). The migration distance was measured using a micrograduated scale adapted to the optics of a Zeiss inverted-phase contrast microscope (100 × magnification). Twelve observations, obtained in three adjacent wells, were recorded for each treatment.

Statistical analysis

Data are reported as means ± S.E.M. Statistical analysis was performed by using one-way ANOVA followed by a parametric Dunnett's test. A P value of < 0.05 was taken as significant.

RESULTS

Effects of AGE-BSA on the VE-cadherin complex

To evaluate whether albumin-derived AGEs had any effects on the VE-cadherin complex, AGE-BSA or control preparations were incubated with long-confluent cell cultures. Immunoprecipitation with an anti-VE-cadherin mAb of Triton X-100-

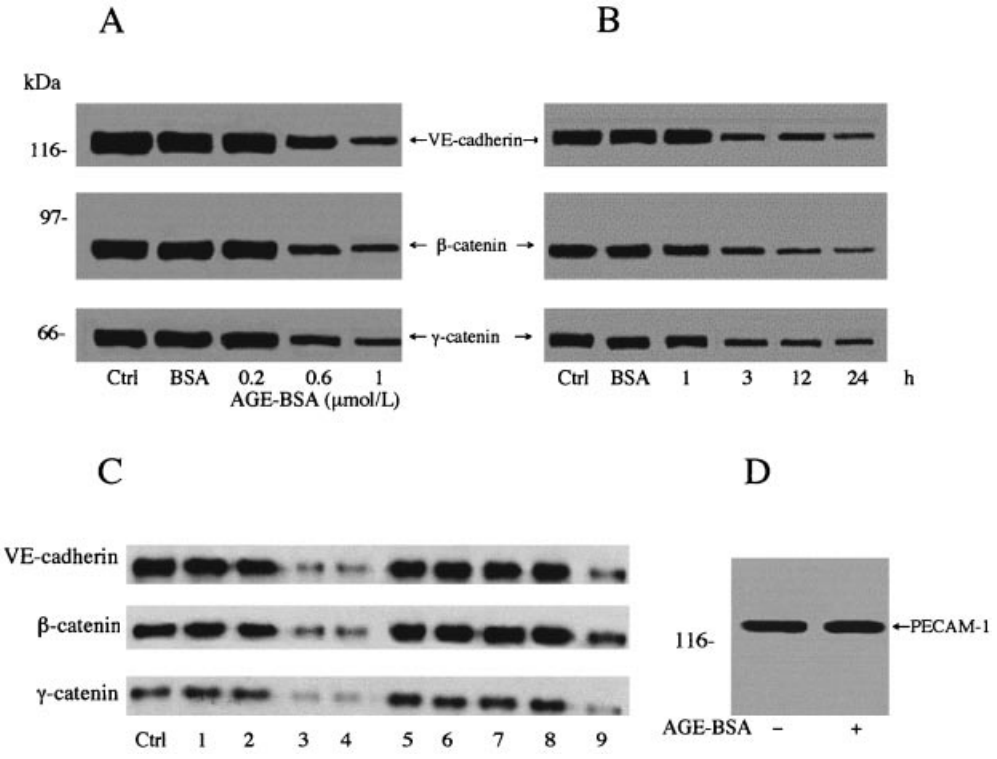

Figure 1 Effects of AGE-BSA on the endothelial VE-cadherin complex in HUVEC

Long-confluent HUVEC were incubated with medium alone (Ctrl), 1 μmol/l unmodified BSA (BSA), other controls or AGE-BSA in the presence or absence of NAC or PDTC. Triton X-100-soluble fractions from whole cells were obtained and aliquots were immunoprecipitated with anti-VE-cadherin or anti-PECAM-1 mAbs and analysed by immunoblotting for the presence of VE-cadherin, β-catenin, γ-catenin and PECAM-1, as described in the Experimental section. The migration of molecular-mass markers (kDa) is shown on the left. Experiments were done in triplicate and typical results are shown. (**A**) Dose–response study of the decreases in VE-cadherin, β-catenin and γ-catenin in response to incubation with AGE-BSA for 24 h. (**B**) Time course of protein levels when incubated with 1 μmol/l AGE-BSA for up to 24 h. Controls (medium alone and unmodified BSA) were incubated for 24 h. (**C**) Specificity, reversible character and oxidative-stress-dependence of the AGE-BSA-induced decrease in VE-cadherin levels. HUVEC were incubated for 3 h (unless otherwise stated) before analysis with the following agents: Ctrl, medium alone; lane 1, 1 μmol/l heat-inactivated AGE-BSA; lane 2, 5 mmol/l EGTA for 30 min; lane 3, 1 μmol/l AGE-BSA; lane 4, 1 μmol/l reduced AGE-BSA; lane 5, 1 μmol/l AGE-BSA + 1 mmol/l NAC; lane 6, 1 μmol/l AGE-BSA + 50 μmol/l PDTC. A 1 h pretreatment with antioxidant was performed before AGE-BSA treatment. Lanes 7 and 8 show the reversal of the effect of a 3 h incubation with 1 μmol/l AGE-BSA at 8 and 24 h respectively after replacement with fresh medium, and lane 9 shows the reversal at 8 h in the presence of 0.3 μmol/l cycloheximide. (**D**) Treatment with 1 μmol/l AGE-BSA for 24 h does not modify the level of PECAM-1 protein.

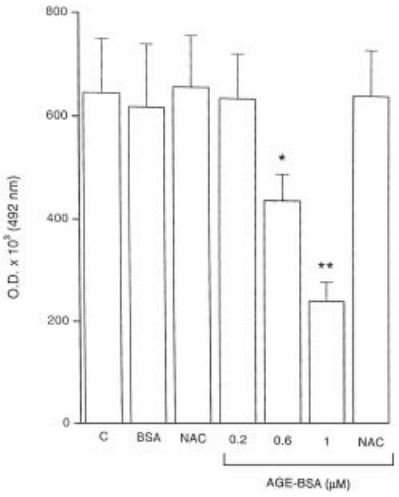

Figure 2 AGE-BSA induces a decrease in cellular surface VE-cadherin content in HUVEC that is blocked by NAC, as measured by ELISA

Long-confluent HUVEC were treated for 3 h with medium alone (C), 1 μmol/l unmodified BSA (BSA), 1 mmol/l NAC, AGE-BSA at different doses or AGE-BSA + 1 mmol/l NAC. Co-incubation with NAC was at the maximal dose of AGE-BSA (1 μmol/l) following a 1 h NAC pretreatment. Cell surface expression of VE-cadherin was determined by ELISA, with primary binding with specific mAb BV9 followed by secondary binding with an HRP-tagged goat anti-(mouse IgG), as described in detail in the Experimental section. O. D., absorbance. All data points represent the means ± S.E.M. of quadruplicate ELISA wells from two independent experiments. Significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with C.

soluble lysates and immunoblotting with specific mAbs to VE-cadherin, β-catenin or γ-catenin revealed that the level of immunoreactive VE-cadherin was markedly reduced, in a dose-dependent manner, when HUVEC were exposed for 24 h to AGE-BSA (at doses of 0.6 and 1 μmol/l), but not to unmodified BSA (Figure 1A). Similarly, levels of β- and γ-catenins bound to VE-cadherin were also decreased. Moreover, changes in levels of components of the VE-cadherin complex were time-dependent. Significant decreases were observed as early as 3 h after exposure to AGE-BSA, and an additional slight decrease was detected at 12 and 24 h (Figure 1B). On the other hand, the content of PECAM-1, an adhesive molecule located at cell–cell contacts in the endothelium [29], remained essentially unaffected by AGE-BSA treatment (Figure 1D). When AGE-BSA was removed after 3 h of incubation by extensive washing and replacement with fresh medium, VE-cadherin and catenin levels were completely restored within 8 h (Figure 1C). EC monolayers were also incubated with 0.3 μmol/l cycloheximide after 3 h of AGE-BSA treatment and medium replacement. Blockage of protein synthesis prevented the increases in VE-cadherin and catenin content observed previously. Cycloheximide alone did not modify the VE-cadherin content, due to the slow turnover of this protein [30]. The specificity of the observed response of HUVEC to AGE-BSA was confirmed by using unmodified BSA and heat-inactivated AGE-BSA (10 min at 90 °C) as controls; neither had any significant effects compared with untreated controls. The AGE-BSA preparation becomes inactive after heat treatment [19].

Because early glycation products are known to be converted into glucitol-lysine by reduction with sodium borohydride, we ruled out an effect of early glycation products by using a reduced AGE-BSA preparation [26]. Similar decreases in VE-cadherin and catenin levels were observed when reduced AGE-BSA was used (Figure 1C), suggesting that the effects were not mediated by reversible BSA adducts such as Amadori products.

In preliminary experiments, we verified that concentrations of AGE-BSA up to 1 μmol/l did not have any effects on total protein content or cell viability throughout the experiments (results not shown).

To determine if the AGE-BSA-induced effect was due, at least in part, to oxidant-sensitive mechanisms, we performed experiments in the presence of 1 mmol/l NAC or 50 μmol/l PDTC, administered extracellularly. As shown in Figure 1(C), the decrease in VE-cadherin levels was prevented by both antioxidant treatments.

As a control to discriminate between the observed degradation effect and a diffusion effect, the composition of the complex after treatment with 5 mmol/l EGTA was assessed. The levels of the proteins of the VE-cadherin complex (Figure 1C), as well as the total levels of each protein (results not shown), did not change after 30 min of treatment.

When supernatants from AGE-BSA-treated HUVEC were immunoprecipitated with anti-VE-cadherin mAb and immunoblotted with the same mAb, no immunoreactive bands to VE-cadherin were observed (results not shown).

In confluent ECs, VE-cadherin is located at cell–cell contacts. To determine directly whether AGE-BSA modifies the surface expression of VE-cadherin, an ELISA was carried out. AGE-BSA, but not unmodified BSA, reduced the cell surface expression of VE-cadherin in HUVEC in a dose-dependent manner, by 31 % and 60 % at the concentrations of 0.6 and 1 μmol/l AGE-BSA respectively (Figure 2). Co-treatment with NAC abolished this effect.

The localization of the VE-cadherin complex in untreated and AGE-BSA-treated HUVEC was studied by immunofluorescence. The corresponding images, taken 3 h after the addition of 1 μmol/l AGE-BSA to long-confluent cultures, revealed reduced immunostaining of VE-cadherin and β-catenin, but not of PECAM-1 (Figure 3). In contrast, AGE-BSA at the same concentration did not perturb the staining of VE-cadherin or β-catenin when 1 mmol/l NAC was also applied. NAC alone did not have any effect. Under these conditions, no substantial cell retraction was observed by vinculin staining.

To explore the spectrum of the AGE-BSA-induced disruption of the VE-cadherin complex, a murine EC line of microvascular origin, H5V, was treated with AGE-BSA, and its Triton X-100-soluble lysate was obtained and analysed as above. AGE-BSA induced dramatic decreases in VE-cadherin complex components in a dose-dependent manner, whereas unmodified BSA had no effect (results not shown).

AGE-BSA-induced loss of components of the VE-cadherin complex

The disruption of the VE-cadherin complex by AGE-BSA was accompanied by significant loss of individual components. Total cell lysates from AGE-BSA-treated cultures were immunoprecipitated with specific mAbs directed against each component. Immunoprecipitates were detected subsequently by immunoblotting with the same mAb. The presence of SDS in the lysis buffer dissociates the complex itself and also dissociates it from the cytoskeleton, and thus allows for determination of the total cellular content of each component. As shown in Figure 4, decreases in the levels of VE-cadherin, β-catenin and γ-catenin were observed in total cell extracts. This effect was again suppressed when NAC was added at the same time as AGE-BSA. PDTC had the same effect (results not shown). These results rule out the possibility that components of the VE-cadherin complex shift their distribution from the Triton X-100-

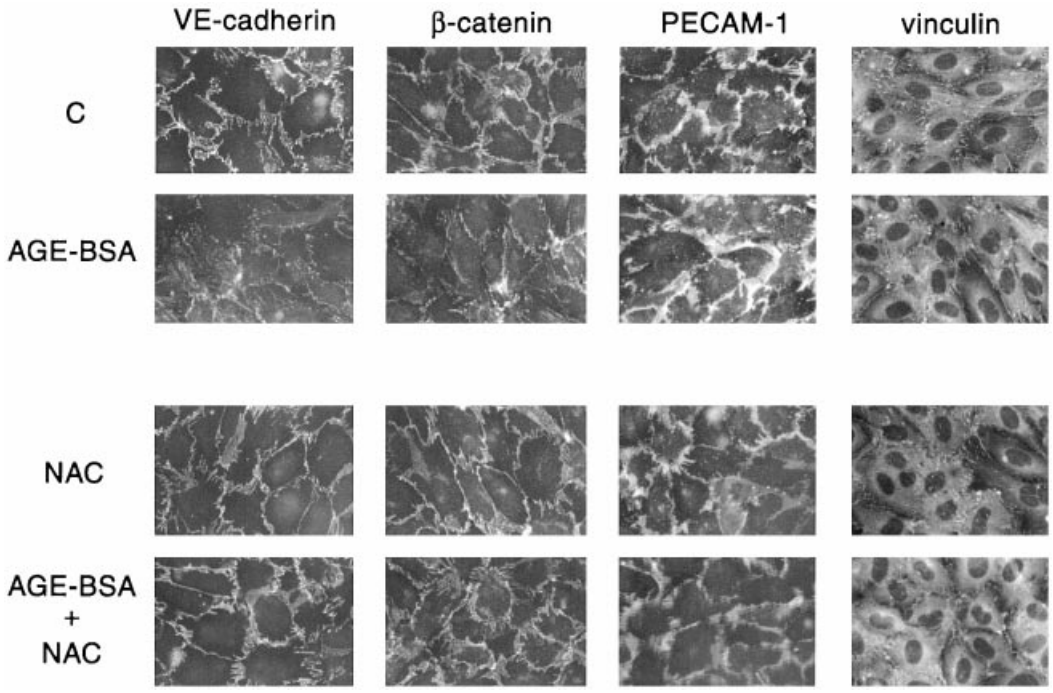

Figure 3 Effects of AGE-BSA on the distribution of VE-cadherin, β-catenin and PECAM-1 in confluent HUVEC monolayers, measured by indirect immunofluorescence

Long-confluent HUVEC grown on glass coverslips were treated with medium alone (C), AGE-BSA, NAC or AGE-BSA + NAC. After 3 h, cells were fixed and processed for immunofluorescence analysis of VE-cadherin, β-catenin, PECAM-1 or vinculin, as described in the Experimental section. VE-cadherin and β-catenin disappeared from junctions after AGE-BSA treatment (1 μmol/l), whereas junctional staining for PECAM-1 remained unchanged compared with medium alone. NAC alone did not have any effect on any protein, whereas co-incubation with AGE-BSA prevented the disappearance of AJ proteins VE-cadherin and β-catenin.

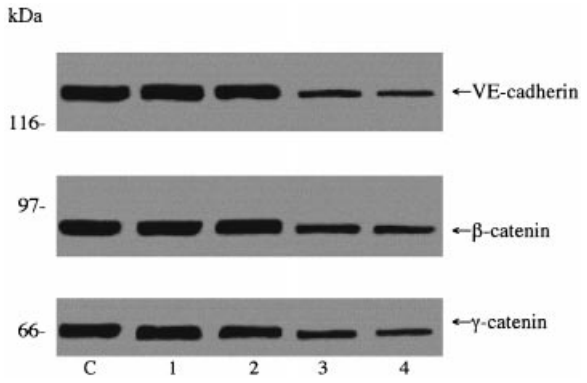

Figure 4 AGE-BSA decreases the total cellular levels of VE-cadherin and of β- and γ-catenins

Long-confluent HUVEC were treated for 3 h with AGE-BSA or controls. After washing, monolayers were extracted with lysis buffer containing 0.5% SDS (total cell lysate). Proteins were immunoprecipitated with specific mAbs against VE-cadherin and β- and γ-catenins, and subsequently detected by immunoblotting with the same antibody. Lanes correspond to: C, medium alone; 1, 1 μmol/l unmodified BSA; 2, 1 μmol/l AGE-BSA + 1 mmol/l NAC; 3, 1 μmol/l AGE-BSA; 4, 1 μmol/l reduced AGE-BSA. The migration of molecular-mass markers (kDa) is shown on the left. Studies were performed in triplicate and a typical result is shown.

soluble fraction to the insoluble fraction (i.e. cytoskeleton-associated). No immunoreactive signals were detectable with any of the mAbs apart from the bands with apparent molecular masses of 130, 93 and 83 kDa, corresponding to VE-cadherin, β-catenin and γ-catenin respectively, as reported previously [8].

Biological consequences of disruption of the VE-cadherin complex by AGE-BSA

We next studied whether the observed changes in the VE-cadherin complex were correlated with functional changes in endothelial properties regulated in large part by intercellular AJ. In view of the relevant role of the VE-cadherin complex in the control of vascular permeability, perturbation of endothelial barrier function by our AGE-BSA preparation was assessed. In preliminary experiments, we verified that treatment with AGE-BSA did not cause significant cell retraction or detachment from the substratum (results not shown). Using this *in vitro* system, cultures incubated with AGE-BSA demonstrated an increase in their permeability to a high-molecular-mass marker, whereas treatment with unmodified BSA or heat-inactivated AGE-BSA did not change this parameter (Figure 5). Perturbation of endothelial barrier function was dependent on the concentration of AGE-BSA, being significant from a concentration of 0.6 μmol/l. In addition, the time course of AGE-BSA-induced permeability was correlated with loss of the VE-cadherin complex, as detected by Western-blot analysis (results not shown). The increased permeability of monolayers in response to AGE-BSA was reversible, with barrier function being restored by 24 h after removal of AGE-BSA and replacement with fresh medium. Co-treatment with NAC or PDTC kept permeability at basal levels. Overall, these data indicate that the observed changes in the VE-cadherin complex induced by AGE-BSA could lessen the

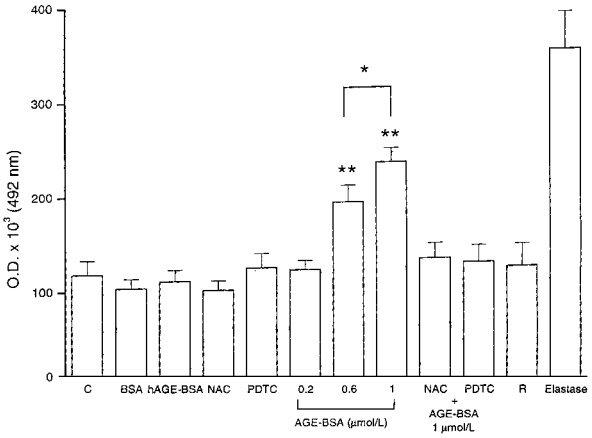

Figure 5 AGE-BSA perturbs the endothelial barrier function of cultured HUVEC monolayers

Long-confluent HUVEC on Transwell inserts were incubated for 3 h with AGE-BSA at various doses or with controls. AGE-BSA increased the permeability of monolayers to a high-molecular-mass marker (HRP conjugated to goat immunoglobulin). Unmodified BSA (BSA), heat-inactivated AGE-BSA (hAGE-BSA), NAC or PDTC alone had no effects on monolayer permeability. A 1 h antioxidant pretreatment with NAC or PDTC abolished the rise in permeability induced by subsequent incubation with 1 μmol/l AGE-BSA. The AGE-BSA-induced increase in permeability was reversed 24 h after the removal of AGE-BSA and replacement with fresh medium (R). Elastase (0.8 μmol/l) was used as a positive control. The results are presented as means $\pm$ S.E.M. ($n = 6$); O. D., absorbance. Significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with C.

capacity of endothelial AJ to control the permeability of the endothelial monolayer.

As an additional indication of the cohesion of intercellular contacts, mediated in part by VE-cadherin, we tested the capacity of AGE-BSA-treated HUVEC to detach from the neighbouring cells and migrate into a wounded area produced in the cultured monolayer. The distance covered by the migration front was measured at 12 and 24 h after the lesion. As reported in Figure 6, treatment with AGE-BSA, but not unmodified BSA, increased cell migration in the wounded area in a dose-dependent manner. Again, antioxidant co-treatment abolished the AGE-BSA effect (results not shown). The observed changes in cell migration could not be attributed to differences in proliferation rates, because the number of treated cells was not significantly different from the number of control cells within the time course of the experiment (results not shown).

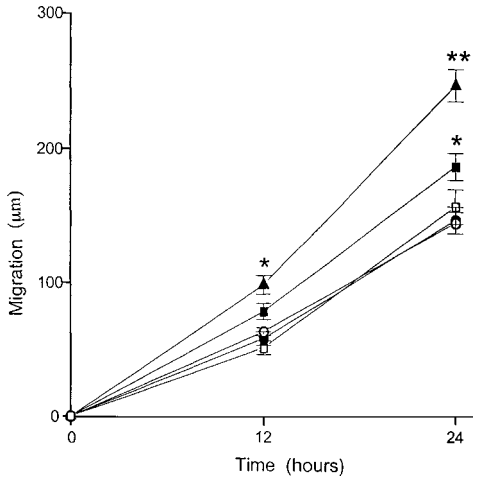

Figure 6 AGE-BSA increases EC motility

Time course of the migration of HUVEC treated with medium alone (○), unmodified BSA (●), or AGE-BSA at 0.2 μmol/l (□), 0.6 μmol/l (■) or 1 μmol/l (▲). Monolayers were wounded, washed and treated for up to 24 h. Migration of the cells out of the wounded edge was recorded at 12 and 24 h. The values shown are the means $\pm$ S.E.M. of 12 observations obtained in three adjacent wells, each in triplicate. Significance of differences: $^{*}P < 0.05$, $^{**}P < 0.01$ compared with medium alone.

DISCUSSION

VE-cadherin is a cell–cell adhesion molecule that plays a fundamental biological role in ECs, as it is essential for the assembly and integrity of the vascular structure [3,30]. On the other hand, the formation of AGEs has been strongly implicated in the endothelial dysfunction associated with microvascular and macrovascular complications that accompany diabetes and normal aging [15]. Although it is known that AGEs exert their cellular effects mainly via specific cellular receptors expressed by the endothelium [31], little is known about the specific target proteins responsible for the perturbed endothelial functions. We therefore postulated that the AGE-induced cellular effects might be associated with structural alterations to endothelial junction organization, specifically in endothelial AJ.

Our results indicate that AGE-BSA greatly alters the organization of the endothelial VE-cadherin complex; this, in turn, could induce perturbations to properties of the endothelium and thereby contribute to vascular dysfunction. Exposure of long-confluent HUVEC to AGE-BSA induced a decrease in the amount of VE-cadherin present at the cellular surface, as well as marked decreases in the cellular content of VE-cadherin and two major components of the VE-cadherin complex that are linked directly to the cytoplasmic tail of VE-cadherin, i.e. the β- and γ-catenins. This effect was not a generalized EC junctional event, as shown by the stable levels of PECAM-1 after AGE-BSA treatment. Diminution of VE-cadherin was also observed in a murine EC line of microvascular origin, supporting its relevance to the endothelium. Biochemical analysis of each molecule revealed that these proteins were extensively lost. This effect was not associated with cell toxicity, and appeared to be specific for advanced glycation.

Decreases in the levels of cellular catenins may affect other cadherins present in ECs, namely neural (N)-cadherin. This is a classical cadherin present in significant amounts in ECs [3]. It interacts with catenins and the actin cytoskeleton to promote homotypic endothelial cell–cell adhesion. However, its function in this cell type is controversial. In ECs, N-cadherin is not located at cell–cell contacts, but remains diffuse on the cell membrane, and this exclusion from junctions is due to competition with VE-cadherin. VE-cadherin appears to play a predominant role over N-cadherin in promoting homotypic endothelial cell–cell adhesion and barrier properties, while N-cadherin may be involved in the interaction of ECs with other cell types in the vasculature [32].

Supplementation of the cellular antioxidative defences with NAC or PDTC prevented the AGE-BSA-dependent decrease in VE-cadherin content. Accordingly, several reports have indicated that the interaction of AGEs with their cellular receptor results in the generation of reactive oxygen intermediates [33,34].

Several AGE receptors have been identified on a wide range of cells and tissues [35]. While AGE-binding receptor systems contribute to the removal of AGE-modified molecules and therefore limit their harmful effects, they mediate important

cellular functions that are tissue-specific. Vascular endothelium is known to bind and internalize AGEs through a receptor-mediated mechanism [19]. Some of the AGE receptors described so far have been demonstrated to occur on HUVEC plasma membranes, where they are able to bind AGEs specifically [36,37]. These include the three AGE receptor complex components oligosaccharyl transferase-48 (AGE-R1), 80K-H phosphoprotein (AGE-R2) and galectin-3 (AGE-R3), and the receptor for AGEs (RAGE). The best characterized AGE receptor is RAGE, which is a multi-ligand member of the immunoglobulin family [37]. Interaction of AGEs with RAGE has been shown to accelerate intracellular oxidative stress and subsequent activation of the redox-sensitive transcription factor nuclear factor-κB, resulting in the perturbation of a variety of vascular homoeostatic functions. The interaction plays a key role in cellular responses such as hyperpermeability [31] and the up-regulation of vascular cell adhesion molecule and tissue factor [38]. The AGE–RAGE interaction thus has been thought to play a central role in the development of diabetic vasculopathy. Although the exact molecular mechanism underlying the AGE-induced disruption of VE-cadherin has not been addressed in the present study, our data suggest that intracellular redox imbalance is central to the development of the reported AGE-induced disruption of the VE-cadherin complex.

An important question relates to the biological significance of decreases in the levels of the VE-cadherin complex. Recently, a case report showed reduced VE-cadherin expression in diabetic retinal vasculature [39]. In our hands, loss of VE-cadherin was correlated with increases in permeability and cell migration. Moreover, after withdrawal of AGE-BSA, the barrier properties returned to baseline, with a concomitant increase in VE-cadherin complex content. This suggests that AGEs must be constantly present in order to exert the observed effects. This is in accordance with AGE physiology, as AGE proteins accumulate in the vasculature with aging and at an accelerated rate in diabetes. The effects exerted by AGEs thus should be related to their chronic presence in the diabetic milieu.

A comparison of the dose responses and time courses for the AGE-BSA-induced effects in our studies suggests a correlation between the decrease in VE-cadherin content and the modulation of permeability and cell motility. Notably, antioxidant co-treatment abolished the effects of AGE-BSA. Increasing evidence associates AGEs with vascular damage leading to microvascular complications such as retinopathy, neuropathy, nephropathy and macrovascular disease [35]. Our *in vitro* studies are consistent with the possibility that junctional proteins, especially VE-cadherin, are altered in diabetic vascular complications.

Other vascular functions may be affected by the decrease in the content of VE-cadherin and the associated catenins in a diabetic microenvironment. VE-cadherin has been identified as a molecule critical for EC tube formation (*in vitro* angiogenesis) [40,41]. Angiogenesis is the principal vascular derangement of diabetic microangiopathy [15], and AGEs have been implicated in eliciting angiogenesis through the induction of autocrine vascular endothelial growth factor (VEGF) [22]. It has been demonstrated that VEGF induces VE-cadherin phosphorylation in HUVEC [42], and it was suggested that this might somehow mediate increased vascular permeability and endothelial migration during angiogenesis through impairment of VE-cadherin function. Nevertheless, these authors showed that VEGF had no significant effects on the overall levels of individual components of the complex, or on their expression at the membrane. Therefore it seems that the AGE-induced effects seen by us are mediated by a mechanism distinct from downstream autocrine production of VEGF.

As yet, little is known about the functional regulation of AJ in ECs. Some reports indicate that tyrosine phosphorylation is an important regulatory mechanism for AJ. Alterations in the tyrosine phosphorylation status of proteins within the cadherin complex have been linked *in vitro* to the confluence state of cells [23], VEGF stimulation [42] and tumour-cell–EC interactions [11]. In general, phosphorylation has been associated with loosening of cell–cell adhesion properties, even in the absence of changes in VE-cadherin complex organization or cellular distribution. On the other hand, the disappearance of cell adhesion components from the cell–cell contact area is thought to destabilize endothelial AJ intercellular contacts. In fact, destruction of the homotypic interactions between VE-cadherin molecules by a polyclonal anti-VE-cadherin antibody disrupts confluent EC monolayers and increases monolayer permeability. Restoration of endothelial cell–cell contacts is achieved by the expression of new molecules of VE-cadherin [30].

In summary, this work provides evidence that AGE-BSA, as a prototype of advanced glycated proteins, can induce the disruption and loss of the VE-cadherin complex, and suggests that it might be responsible, at least in part, for the weakening of intercellular contacts that subserve the AGE-induced biological effects on the endothelium. Further work is necessary to elucidate the molecular events involved in the modulation of the VE-cadherin complex by AGE-BSA.

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