

ENDOTHELIAL RECEPTOR-MEDIATED BINDING OF
GLUCOSE-MODIFIED ALBUMIN IS ASSOCIATED WITH
INCREASED MONOLAYER PERMEABILITY AND
MODULATION OF CELL SURFACE
COAGULANT PROPERTIES

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Endothelium regulates vascular homeostasis, including coagulation and permeability, by modulating the expression of cell surface molecules and secreted products in response to blood-borne mediators and those emanating from the vessel wall. Recent studies have emphasized the responses of endothelium to cytokines, such as TNF/cachectin and IL-1, which perturb a range of homeostatic endothelial functions (20, 46, 48).

The progressive accumulation of irreversible advanced glycosylation end products (AGE)¹ (8, 10, 11) on both plasma and subendothelial tissues, such as vascular basement membrane proteins, may constitute a potentially significant perturbant of endothelial cell function. Recent studies have focussed on these biologically important covalent products of the nonenzymatic glycosylation reaction, which derive slowly from the early Amadori product after a sequence of further reactions and rearrangements (8, 10, 11, 33). These adducts are characteristically fluorescent, yellow-brown pigments that can crosslink proteins and nucleic acids (8). Accumulation of AGEs on long-lived proteins *in vivo* has been found to increase linearly with age (33), is accelerated in patients with diabetes, and is thought to be an important contributor to the development of a host of complications, such as vascular and renal disease (11). The identification and isolation of a receptor on human and murine macrophages that selectively recognizes AGE adducts on proteins (41) support the concept that these modified macromolecules may exert specific effects on tissue or cellular targets (50, 51, 53). In this context, interaction of AGE-BSA with macro-

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¹ *Abbreviations used in this paper:* AGE, advanced glycosylation end products.

phages has been shown to induce the elaboration of TNF/cachectin and IL-1, thereby initiating a sequence of events by which macrophages remove and orchestrate the replacement of senescent proteins (54). Under pathologic conditions, such as in diabetes or atherosclerosis, AGEs may contribute to the pathogenesis of proliferative vascular lesions (10).

These considerations, and the fact that endothelium is in direct contact with subendothelial, as well as circulating AGEs, led us to examine the interaction of endothelium with AGE-BSA, as a prototype of this class of nonenzymatically glycosylated proteins. The results indicate that AGE-BSA binds in a saturable manner to the cell surface with subsequent uptake by endocytosis, which leads to either transcytosis or eventual transfer to a lysosomal compartment. Functional consequences of AGE-BSA-endothelial interaction include increasing permeability of the monolayer and alteration of cell surface coagulant properties, potentially promoting clot formation. These data indicate that AGE derivatives of proteins, ubiquitous components of the vascular milieu in diabetes (11, 33) and also in normal aging (29), interact directly with the endothelium, potentially contributing to the pathogenesis of subsequent vascular dysfunction.

Materials and Methods

Cell Culture. Bovine aortic endothelial cells were grown from aortas of newborn calves in MEM supplemented with penicillin-streptomycin (50 U/ml, 5 μ g/ml), glutamine, and FCS (10%) as described (6, 44). Cultures were characterized as endothelial based on morphologic criteria, immunofluorescence for protein S, von Willebrand factor, and thrombomodulin (17, 25, 47). Cells were separated for subculture with trypsin/EDTA. For experiments, cells from different aortas were grown to confluence in 9.6-, 2-, or 0.32-cm² wells ($1.1\text{--}1.5 \times 10^5$ cells/cm²) and used from passages 3–16, within 48 h of having achieved confluence. For permeability studies, cultures were grown as described previously (7) on 6.5-mm diameter polycarbonate membranes (pore size 0.4 μ m), mounted on polystyrene inserts (Transwell plates; Costar, Cambridge, MA), and used for studies 10 d after plating.

Preparation and Radiolabeling of AGE-BSA. AGE-BSA was prepared by incubating BSA (fraction V; Boehringer-Mannheim Biochemicals, Indianapolis, IN) in 10 mM PBS buffer, pH 7.4, with 50 mM glucose at 37°C for 6 wk in the presence of protease inhibitors (1.5 mM PMSF and 0.5 mM EDTA) and antibiotics (100 U/ml penicillin and 40 μ g/ml gentamicin) as described previously (41). The amount of AGE formed on all BSA preparations was assessed by characteristic absorption and fluorescence spectra (29) and quantitated by RIA using 2-furoyl-4-(5)-(2-furanyl)-1H-imidazole (FFI, a chemically synthesized AGE) (12, 39), which has been shown to be recognized by the macrophage AGE receptor (51–53), as a standard. According to this assay, our routinely prepared AGE-BSA conjugates have ~ 360 pmol FFI equivalents/mg BSA. SDS-PAGE showed a single major band with $M_r \sim 67,000$ (data not shown). Radiolabeling was accomplished by the lactoperoxidase method (15) using Enzymobeads (Bio-Rad Laboratories, Sacramento, CA) by incubating AGE-BSA (50 μ g) with 50 μ l of beads for 3 min at 4°C. The reaction was stopped by the addition of azide, beads were pelleted by centrifugation, and free iodine was removed by gel filtration on a Sephadex G-25 column pre-equilibrated with MEM containing 1% FCS. The specific radioactivity of the tracer was 1.5×10^3 cpm/ng over four iodinations and autoradiograms prepared from SDS-polyacrylamide gels (30) (10%; nonreduced) demonstrated a single band corresponding to $M_r \sim 67,000$. Nonglycosylated albumin, used for controls, was prepared in an identical fashion except that glucose and glucose 6-phosphate were omitted from the incubation buffer. Murine ribonuclease and hemoglobin (Sigma Chemical Co., St. Louis, MO) were glycosylated and characterized as described for AGE-BSA (51, 53–54).

Preparations of AGE-BSA used for specific experiments were prepared under endotoxin-free conditions, and passed over an affinity PAK column (Detoxigel; Pierce Chemical Co.,

Rockford, IL). All solutions were assessed for contaminating endotoxin by a standard Limulus amoebocyte assay (Sigma Chemical Co.) and found to be negative (54). In some experiments polymyxin B (100 ng to 10 $\mu\text{g}/\text{ml}$) was added to determine whether any responses reflected trace amounts of LPS undetectable by Limulus assay.

Binding and Internalization Studies. The binding of ^{125}I -AGE-BSA to endothelial cell monolayers was studied after washing cells four times in a balanced salt solution (10 mM Hepes, pH 7.45, 137 mM NaCl, 4 mM KCl, 11 mM glucose) over 15 min and the addition of MEM containing 5% FCS. Radiolabeled AGE-BSA alone or in the presence of a 100-fold molar excess of unlabeled material, each in a volume of 5 μl , was then incubated with the cells for the indicated times at 4°C. Where indicated, other competitors were added to the incubation mixture. Binding was terminated by 10 rapid washes (over ~ 30 s) with MEM containing 1% FCS, and bound material was then eluted by exposing the cultures to buffer containing EDTA (5 mM), heparin (10 mg/ml), and BSA (1 mg/ml) in a balanced salt solution for 5 min at 37°C. Where indicated, cells were eluted by incubation with NP-40 (1%) for 5 min at 37°C. Specific binding was defined as the difference of binding observed in wells incubated with ^{125}I -AGE-BSA alone and binding observed in wells incubated with ^{125}I -AGE-BSA in the presence of a 100-fold molar excess of unlabeled AGE-BSA (51). No specific binding was observed in wells without cells. Data from binding experiments were fit to the equilibrium binding equation $B = nKA/(1 + KA)$, where B is the amount of specifically bound ligand, n is the number of binding sites per cell, A is the concentration of free radioligand, and K is the association constant (28). A nonlinear least-squares program was used to obtain the best fit curve, to solve for n and K , and to determine the standard error.

Dissociation studies used the method of infinite dilution (31); after binding of ligand to the cell surface, cultures were washed 10 times and then fresh MEM with FCS was added. At the indicated times, wells were washed again and bound radioactivity was determined. In pilot studies, similar results were observed using either this method or by studying dissociation in the presence of excess unlabeled ligand.

Endocytosis experiments were performed at 37°C as described previously (37) by incubating the cells with ^{125}I -AGE-BSA alone or in the presence of excess unlabeled material. Cell-associated radioligand was divided into two pools: surface-bound material, eluted with heparin (10 mg/ml)/EDTA (5 mM)-containing buffer (5 min at 37°C), and internalized material, eluted after solubilizing the cells in buffer containing NP40 (1%; 5 min at 37°C). Degraded radioligand present in the culture supernatant was assessed by serial precipitation of aliquots of culture medium in trichloroacetic acid (5%) during the incubation period.

Assessment of Endothelial Monolayer Permeability after Exposure to AGE-BSA. Confluent endothelial monolayers plated on Transwell inserts (see above) were washed with Earle's balanced salt solution and MEM containing FCS (5%) and either AGE-BSA, heat-treated AGE-BSA (10 min at 90°C), or normal BSA was added for the indicated times. At the end of this incubation period, permeability of the endothelial monolayer was assessed by studying the passage of [^3H]inulin (271 mCi/ μg ; New England Nuclear, Boston, MA) across the membranes as described previously (7, 16, 45). In brief, fresh medium was added to both the inner and outer (corresponding to upper and lower) chambers such that the oncotic and hydrostatic pressures in the two chambers were equal. A radiolabeled marker, [^3H]inulin, was then added in trace amounts (~ 3 $\mu\text{g}/\text{ml}$) to the upper chamber. Transfer of tracer across the monolayer was assessed by serial sampling of the lower chamber (the volume in the chambers was not changed significantly, $<5\%$, as a result of sampling) and was expressed as the quotient of radioactivity emerging in the outer well divided by radioactivity remaining in the inner well.

Assessment of Endothelial Coagulant Properties after Exposure to AGE-BSA. Cultures were incubated with AGE-BSA in MEM containing 5% FCS and polymyxin B (1 $\mu\text{g}/\text{ml}$) for the indicated times at 37°C and cell surface thrombomodulin and tissue factor were assessed. Where indicated, nonglycosylated BSA or heat-treated AGE-BSA (10 min at 90°C) replaced AGE-BSA. Purified recombinant human TNF- α ($\sim 10^8$ U/mg, generously provided by Hoffman-LaRoche, Nutley, NJ) was also added to the cultures in certain experiments. For thrombomodulin functional assays (38), cultures were washed four times in balanced salt solution and then incubated for 15 min at 37°C in 10 mM Hepes, pH 7.45, 137 mM NaCl, 11 mM glucose, 4 mM KCl, 2 mM CaCl_2 , and 2 mg/ml BSA containing protein C (100

$\mu\text{g/ml}$) and thrombin (0.1 U/ml). Formation of activated protein C was terminated by the addition of antithrombin III (100 $\mu\text{g/ml}$) and the amount of enzyme formed was determined using a chromogenic assay, hydrolysis of the substrate Lys-Pro-Arg-*p*-nitroanilide (Spectrozyme; American Diagnostica, New York, NY). Enzyme concentration was determined by comparison with a standard curve made in the presence of known amounts of activated protein C. Thrombomodulin antigen was determined by a RIA with a monospecific polyclonal antbovine thrombomodulin antibody. This antibody was produced in rabbits by standard methods (22). RIA was carried out by a modification of the procedure described for human factor IX (49). In brief, the assay was performed in 1.5-ml microcentrifuge tubes by adding ^{125}I -thrombomodulin (5 μl), antithrombomodulin antiserum (10 μl), and incubation buffer (20 μl ; Tris, 20 mM, pH 7.4, NaCl, 0.1 M, and NP-40, 1%). Selected tubes contained either samples or thrombomodulin standard diluted in the assay buffer (20 μl). All dilutions were made with incubation buffer. Tubes were incubated overnight at 4°C after which 50 μl of a 10% suspension of *Staphylococcus* protein A (IgGSorb; Enzyme Center, Inc., Walden, MA) was added to each tube for 30 min at 21°C. Incubation buffer (500 μl) was added next, and the tubes were centrifuged for 3 min at 13,000 rpm. The supernatant was aspirated and discarded, the bottom of each tube containing the pellet was cut off with a hot wire, and counted. Bovine thrombomodulin was purified from lungs by the method of Jakubowski et al. (26) and radiolabeling of preparations was carried out by the lactoperoxidase procedure (15), as described above for ^{125}I -AGE-BSA. The sensitivity of this assay was 10 ng/ml, which corresponded to 80% binding on the standard curve. The thrombomodulin content of untreated endothelial cultures was $\sim 40\text{--}60$ ng/ 10^6 cells.

Tissue factor activity of endothelial cell monolayers was determined using a coagulant assay after detaching cells from the growth surface by scraping. In brief, after washing monolayers, cells were scraped with a rubber policeman and suspended in barbital-buffered saline. A two-stage coagulant assay was then carried out as described previously (36). Where indicated, cells were preincubated with a mAb to bovine tissue factor at 10 $\mu\text{g/ml}$ for 30 min at 37°C (this antibody was generously provided by Dr. R. Bach, Mt. Sinai Medical Center, New York, NY). Quantification of tissue factor was accomplished by comparing clotting times with a standard curve generated with known amounts of purified tissue factor (2). In certain experiments, endothelium was incubated with AGE-BSA in the presence of the indicated concentration of TNF.

Microscopy. AGE-BSA colloidal gold conjugates were prepared and used by the general method described previously (37). In brief, suspensions of colloidal gold were prepared (18) with an average diameter of 12 nm (1) and conjugated to albumin at pH 6.0. Complete surface labeling was achieved, as determined by the serial electrolyte test (24) and labeled colloids were evaluated for stability as previously described (21). Experiments with gold-labeled AGE-BSA were carried out according to the same general protocols outlined above for radiolabeled AGE-BSA. After binding of gold particles to the surface, unbound particles were removed by washing and samples were prepared for electron microscopy; samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 min, postfixed in 2% osmium tetroxide, dehydrated in ethanol, and embedded in EPON. Sections were viewed in a Phillips 300 electron microscope.

The effects of AGE-BSA on the endothelial actin-based cytoskeleton and cell shape were assessed by fluorescence microscopy, using rhodamine phalloidin staining to display F-actin. These methods have been described previously (7). Indirect immunofluorescence for thrombomodulin was carried out by procedures described previously (37, 47).

Results

Binding of Cellular Processing of AGE-BSA by Endothelium. At 4°C, specific binding of ^{125}I -AGE-BSA to cultured bovine aortic endothelial cells (the difference between binding of ^{125}I -AGE-BSA to endothelium incubated with tracer alone and binding in the presence of excess unlabelled ligand) was observed when cell-bound material was eluted with a buffer containing NP-40 (1%) (Fig. 1 A). Since detergent-treatment of cell monolayers removes both cell surface and internalized ligand, a method

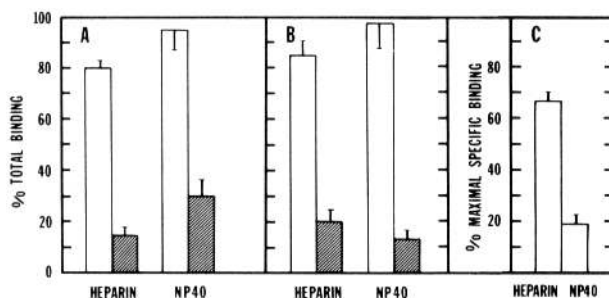


FIGURE 1. Binding of ^{125}I -AGE-BSA to endothelial cell monolayers: methods of elution. Binding was studied at 4°C (A) and at 37°C (B). Endothelium was incubated with ^{125}I -AGE-BSA (100 nM) alone (open bars) or in the presence of a 100-fold molar excess of unlabeled AGE-BSA (shaded bars) for 4 h at the indicated temperature. Cultures were then washed 10 times and eluted with one of the following agents: heparin-heparin (10 mg/ml)/EDTA (5 mM); NP-40 = 1%

NP-40. Data shown in A–B are a percent of total ^{125}I -AGE-BSA binding elutable with NP-40 when the binding experiment was carried out at 37°C. (C) Cultures were incubated for 4 h at 37°C with ^{125}I -AGE-BSA (80 nM) alone or in the presence of unlabeled AGE-BSA (10 μM), washed 10 times and eluted first with heparin/EDTA as above and then with NP-40. The data shown represent specifically cell-bound ^{125}I -AGE-BSA (the difference of radioligand eluted from cultures incubated with tracer alone and radioligand eluted from cultures incubated with tracer in the presence of unlabeled AGE-BSA) after sequential elution: first with heparin/EDTA (heparin) and then with NP-40. The mean \pm SD is shown in each case.

for selective elution of cell surface-bound material was developed. Based on reports that heparin could prevent the binding of AGE-BSA to murine peritoneal macrophages (52), a buffer containing heparin (10 mg/ml)/EDTA (5 mM) was used to elute cell-associated ^{125}I -AGE-BSA. Specifically bound ^{125}I -AGE-BSA eluted in the presence of heparin/EDTA was the same as that observed after elution with detergent at 4°C (Fig. 1 A), and probably represented selective elution from the cell surface. Studies in which endothelium with bound ^{125}I -AGE-BSA was exposed to trypsin (5 $\mu\text{g}/\text{ml}$ for 5 min at 21°C) confirmed that elution with heparin/EDTA represented complete removal of specifically bound cell surface material (data not shown). At 37°C, endocytosis of AGE-BSA occurred and in addition to the surface-bound material observed at 4°C, an internalized pool formed (see below, 37°C studies, and Fig. 1 B–C).

To complement the radioligand binding experiments, interaction of AGE-BSA colloidal gold conjugates with endothelium was studied at 4°C. Incubation of AGE-BSA-gold particles with endothelium resulted in binding of gold particles to the cell surface which could be eluted by brief exposure to heparin/EDTA (Fig. 2, A–B). Furthermore, a 100-fold molar excess of AGE-BSA not bound to gold particles blocked binding of AGE-BSA colloidal gold conjugates by >95%. Since these studies were carried out in the presence of FCS (5%), which contains BSA but not AGE adducts of proteins (data not shown), the observed binding does not represent BSA-endothelial cell interaction. Furthermore, identical binding was observed in the presence of excess normal albumin (1%; data not shown). These results suggested that radioligand binding studies at 4°C could be done to assess parameters of ^{125}I -AGE-BSA binding to endothelial cell surface.

Binding of ^{125}I -AGE-BSA to endothelium at 4°C was time dependent, reaching an apparent maximum by 3 h, even at the lowest concentrations of tracer used (Fig. 3 A). The apparent second order rate constant for association, calculated from the data in Fig. 3 A, was $\sim 0.13 \times 10^7/\text{M}/\text{min}$. The concentration of binding sites used in this calculation was taken from the data in Fig. 3 C. Dissociation studies (Fig.

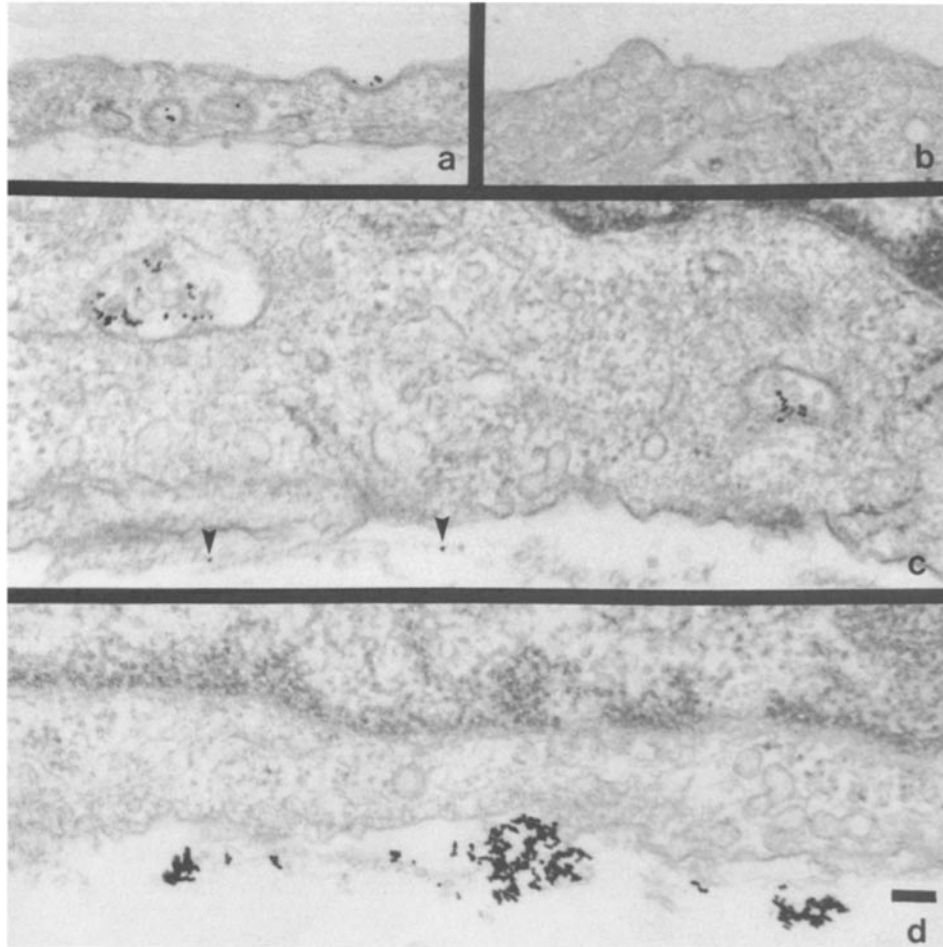


FIGURE 2. Binding of AGE-BSA-colloidal gold conjugates to endothelial monolayers. (*A-B*) Endothelial cell monolayers were incubated at 4°C with AGE-BSA-gold (100 nM) for 2 h, washed, and then either fixed for electron microscopy (*A*) or eluted with heparin (10 µg/ml)/EDTA (5 mM) and then prepared for electron microscopy (*B*). (*C-D*) Monolayers were incubated with AGE-BSA-gold (100 nM) for 2 h at 4°C, washed, further incubated at 37°C for 2 h (*C*) or 20 h (*D*), and then prepared for electron microscopy. After the 4°C incubation, AGE-BSA-colloidal gold particles were bound to the cell surface (*A*). Cells treated with heparin/EDTA did not display gold particles on the cell surface (*B*). After 2 h at 37°C, AGE-BSA gold particles were primarily associated with multivesicular bodies within the cytoplasm and bound to subcellular matrix components (*C*). By 20 h at 37°C, large accumulations of AGE-BSA gold particles were found in association with the extracellular matrix (*D*). Bar, 0.1 µm.

3 *B*) indicated that the interaction of ^{125}I -AGE-BSA with endothelium was reversible with an apparent first-order dissociation constant of $\sim 0.07/\text{min}$. Although ~ 90 min was required for complete dissociation, during this time cell-associated AGE-BSA remained on the cell surface, since a 3-min exposure to buffer containing heparin/EDTA resulted in rapid elution of bound ^{125}I -AGE-BSA, but did not disrupt the intact monolayer. SDS-PAGE of cell-bound radioligand eluted with hepa-

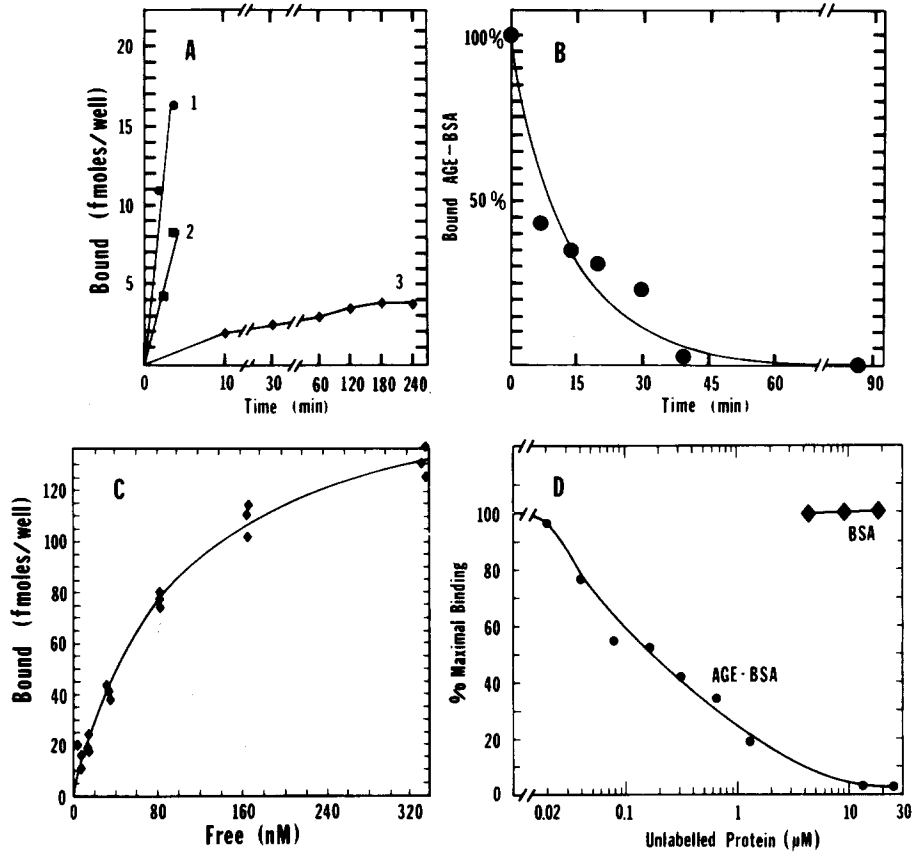


FIGURE 3. Binding of ^{125}I -AGE-BSA to endothelial cell monolayers at 4°C . (A) Time course. Monolayers were incubated with ^{125}I -AGE-BSA (1, 25 nM; 2, 10 nM; 3, 1 nM) alone or in the presence of a 100-fold molar excess of unlabeled AGE-BSA for the indicated times. Cultures were then washed 10 times and eluted with heparin/EDTA as described in the text. Specific binding, total binding (observed in the presence of ^{125}I -AGE-BSA alone) minus nonspecific binding (observed in the presence of ^{125}I -AGE-BSA and excess unlabeled ligand) is shown (the mean of duplicates). Low concentrations of radioligand were used in this study to allow determination of incubation times necessary to achieve apparent maximal binding with these amounts of ^{125}I -AGE-BSA (this information was necessary to carry out the experiment in C). (B) Reversibility. Monolayers were incubated with ^{125}I -AGE-BSA (100 nM) alone (total binding) or in the presence of a 100-fold molar excess of unlabeled AGE-BSA (nonspecific binding) for 4 h and washed 10 times. At this point (time 0), cultures were incubated for the indicated additional times at 4°C . Cultures were then washed and eluted with heparin/EDTA-containing buffer. Maximal specific binding (time 0) was 85 fmol per well. The average of duplicate determinations of specific binding is shown as a percent of maximal specific binding and is plotted versus time. (C) Saturability of binding. Endothelial cell monolayers were incubated with the indicated concentrations ^{125}I -AGE-BSA alone or in the presence of a 100-fold molar excess of unlabeled AGE-BSA for 4 h at 4°C . Cultures were then washed and eluted with heparin/EDTA. Specific binding versus the concentration of free tracer is plotted. Data were analyzed by the nonlinear least squares program and the curve indicates the best fit line. (D) Competitive binding studies. Endothelial monolayers were incubated with ^{125}I -AGE-BSA (100 nM) alone or in the presence of the indicated concentration of either unlabeled AGE-BSA (circles) or normal BSA (diamonds) for 4 h at 4°C . Maximal binding (90 fmol/well) was defined as the amount of ^{125}I -AGE-BSA bound when ^{125}I -AGE-BSA was incubated with cultures alone minus the amount of ^{125}I -AGE-BSA bound in the presence of a 100-fold molar excess of unlabeled AGE-BSA. The average of duplicate determinations is shown. Details of experimental procedure are described in the text.

rin/EDTA demonstrated a single band that migrated identically to the initial tracer with $M_r \sim 67,000$ (data not shown). This indicates that cell surface-bound ^{125}I -AGE-BSA has not formed covalent complexes with cellular proteins and has not been cleaved during or as a consequence of binding, based on comparison with the initial tracer by electrophoretic analysis.

Using these conditions for equilibrium binding, the association of ^{125}I -AGE-BSA with endothelium was found to be saturable (Fig. 3 C). Binding was half-maximal at 99 ± 18 nM, and at saturation there were $2.6 \pm 0.3 \times 10^6$ molecules bound/cell. Experiments in which ^{125}I -AGE-BSA was diluted with unlabeled AGE-BSA showed comparable binding parameters, indicating that unlabeled and labeled AGE-BSA interacted with endothelium similarly. Consistent with this interpretation, unlabeled AGE-BSA was an effective competitor for ^{125}I -AGE-BSA-endothelial cell binding, whereas unmodified BSA was not (Fig. 3 D). These results suggested that the cell surface binding sites for AGE-BSA were specific for the AGE adduct of the protein. This led us to examine if AGE adducts of other proteins could compete with ^{125}I -AGE-BSA for occupancy of the binding sites (Fig. 4). Although nonglycosylated ribonuclease and hemoglobin failed to inhibit the binding of ^{125}I -AGE-BSA to endothelium, the AGE-modified forms of these proteins were competitors. Further evidence emphasizing the importance of AGE moieties for interaction with the cellular receptor is derived from experiments indicating that neither addition of glucose (at a concentration of 45 or 90 mM) nor albumin incubated with glucose or glucose 6-phosphate for 1, 3, or 6 d, at which time little, if any, AGE is present (29), inhibited binding of ^{125}I -AGE-BSA to endothelium (Fig. 4). However, after incubation of albumin with glucose or glucose 6-phosphate for 16 d or more, which results in sufficient AGE adduct formation (9), this modified form of the protein became a competitor of ^{125}I -AGE-BSA binding to the cell surface. These results suggested that the cell surface binding site recognizes the AGE-modified derivatives of a variety of proteins and led us to examine subsequent processing of surface bound ligand.

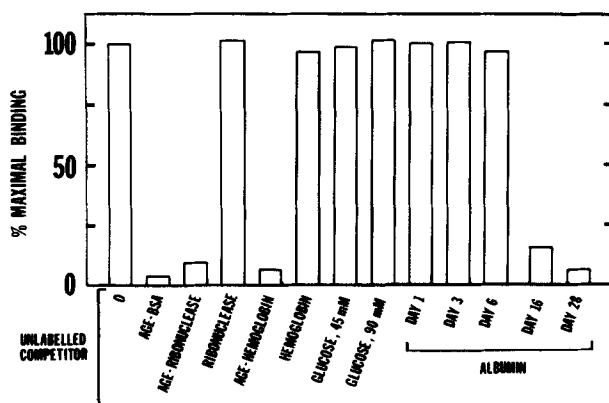


FIGURE 4. The effect of AGE-ribonuclease, AGE-hemoglobin, and glucose on the binding of ^{125}I -AGE-BSA to endothelium. Endothelial monolayers were incubated for 4 h at 4°C with ^{125}I -AGE-BSA alone (100 nM; first bar) (0) or in the presence of one of the following unlabeled competitors: AGE-BSA (10 μM), AGE-ribonuclease (10 μM), ribonuclease (10 μM), AGE-hemoglobin (10 μM), hemoglobin (10 μM), or BSA (albumin) incubated with glucose for the indicated number of days (each at a concentration of 20 μM). Where indicated (glucose), the incubation medium for cell binding studies was supplemented with additional glucose, either 45 or 90 mM. Data shown, the average of duplicate determinations, are expressed as percent maximal binding of ^{125}I -AGE-BSA.

Several pieces of evidence indicated that at 37°C, binding of ^{125}I -AGE-BSA to the cell surface was followed by entry into an intracellular compartment, presumably by endocytosis. First, in contrast to the complete elution of radiolabeled AGE-BSA specifically associated with endothelium by heparin/EDTA at 4°C, at the higher temperature sequential treatment of cultures with heparin/EDTA followed by NP-40 demonstrated an additional pool of cell-associated ^{125}I -AGE-BSA only elutable with detergent (Fig. 1, B-C). Second, dissociation studies demonstrated only incomplete dissociation of ^{125}I -AGE-BSA at 37°C. Residual bound ligand was not accessible on the cell surface; brief exposure of cultures to trypsin, which removes surface-bound ^{125}I -AGE-BSA (see above), only achieved partial elution at 37°C. Taken together, these data pointed to the formation of an intracellular pool of ^{125}I -AGE-BSA at 37°C, which followed interaction of the ligand with a limited number of receptors since the presence of excess unlabeled AGE-BSA prevented its appearance.

Based on these results, further studies with ^{125}I -AGE-BSA were performed to characterize the nature of cellular processing of the ligand at 37°C. For these experiments, cell-associated ^{125}I -AGE-BSA was divided into two pools; surface-bound radioligand, eluted by brief (3 min) exposure of the cells to buffer containing heparin/EDTA, and internalized ^{125}I -AGE-BSA, eluted by detergent solubilization of monolayers with NP-40 (1%) after surface-bound material had been removed. At 37°C, ^{125}I -AGE-BSA (160 nM) was bound to the cell surface, reaching a maximum by about 4 h and was also in an internalized pool. Formation of the internalized pool correlated with evidence of endocytosed ligand based on visualization of AGE-BSA colloidal gold conjugates (see below and Fig. 2, C-D). In addition, ^{125}I -AGE-BSA in culture supernatants at 37°C was apparently becoming degraded, evidenced by decreased precipitation of radiolabeled material in trichloroacetic acid (Fig. 5). Appearance of degraded material in culture supernatants could be prevented by the addition of excess unlabeled AGE-BSA, indicating that uptake of the ligand occurred by a limited number of cell surface binding sites. Taken together, these data indicate that endocytosis of AGE-BSA with subsequent lysosomal-mediated degradation is one pathway by which intracellular processing occurs. However, degradation could also be occurring in other compartments, such as at the basal cell surface after transcytosis. Studies with chloroquine, an inhibitor of intracellular acidification

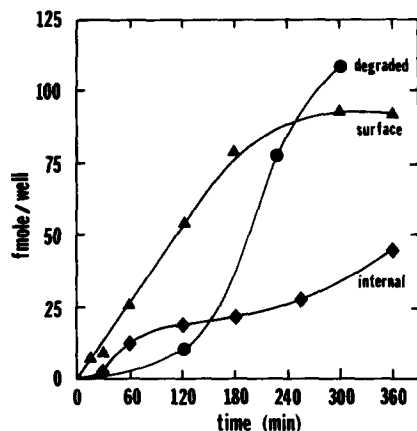


FIGURE 5. Binding and cellular processing of ^{125}I -AGE-BSA by endothelial cell monolayers at 37°C. Endothelial cell monolayers were incubated with ^{125}I -AGE-BSA (160 nM) for the indicated times at 37°C. The amount of surface-bound (heparin/EDTA elutable) (triangle), internalized (diamond; NP-40 elutable after previous heparin/EDTA elution), and degraded (circle; material in culture supernatants which remained in the soluble phase after precipitation of proteins in trichloroacetic acid) ligand was determined as described in the text. Each point is the average of duplicate wells.

that blocks degradation of a variety of internalized ligands (13) were carried out next to provide additional support for a mechanism of AGE-BSA processing by a lysosomal-dependent mechanism. The results were inconclusive, however, since this agent altered AGE-BSA binding to the cell surface and thus perturbed formation of the intracellular pool of ligand. Thus, although the radioligand and morphologic studies both support a role for intracellular degradation of AGE-BSA, ligand may also be degraded in other locations after receptor-dependent uptake by the cells, such as the subendothelial space.

Ultrastructural studies also indicated that entocytosis was occurring at 37°C (Fig. 2, C-D). AGE-BSA colloidal gold conjugates bound to the cell surface were internalized with transfer of ligand to a vesicular compartment. This was followed by accumulation of AGE-BSA gold particles in multivesicular bodies, lysosomal-like structures, and/or transfer of gold particles through the cell with deposition on the subcellular matrix. After 2 h at 37°C, AGE-BSA gold particles had accumulated primarily in multivesicular bodies although small clusters and individual particles were associated with components of the extracellular matrix (Fig. 2 C). By 20 h, large aggregates of AGE-BSA gold particles were associated with matrix components. AGE-BSA gold particles were never observed within intercellular junctions. Unlabeled AGE-BSA could block cellular processing of AGE-BSA gold particles, presumably by blocking its binding to the cell surface. In addition, gold particles conjugated to native BSA did not demonstrate similar intracellular uptake and deposition in the matrix (data not shown).

Modulation of Endothelial Cell Barrier Function by AGE-BSA. In view of the association of advanced glycosylation endproducts with vascular complications, in which increased vascular permeability is often evident, especially in diabetes, perturbation of endothelial barrier function by AGE-BSA was assessed (Fig. 6). Confluent en-

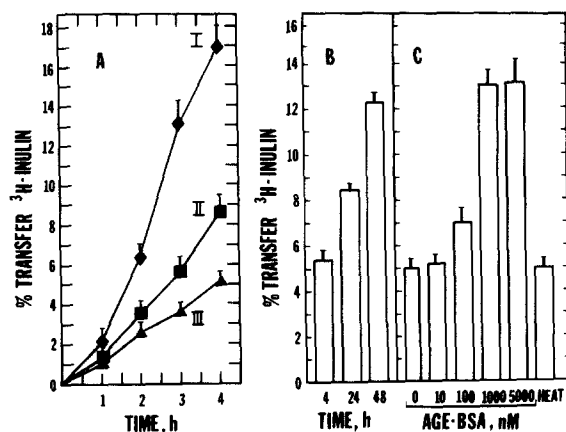


FIGURE 6. Effect of AGE-BSA on the permeability of endothelial monolayers. (A) Endothelial monolayers on filters were incubated in 1% FCS alone (III) or supplemented with AGE-BSA (250 nM; II) for 48 h and then transfer of [³H]inulin across the monolayer over 4 h was assessed as described in the text. Passage of [³H]inulin across a filter without cells is shown (I). (B) Time course. Endothelial monolayers on filters were incubated in growth medium containing 1% FCS supplemented with AGE-BSA (500 nM) for the indicated times. Then, cultures were washed and percent transfer of [³H]inulin was determined at 4 h. Permeability after a 4-h incubation with AGE-BSA was not significantly differ-

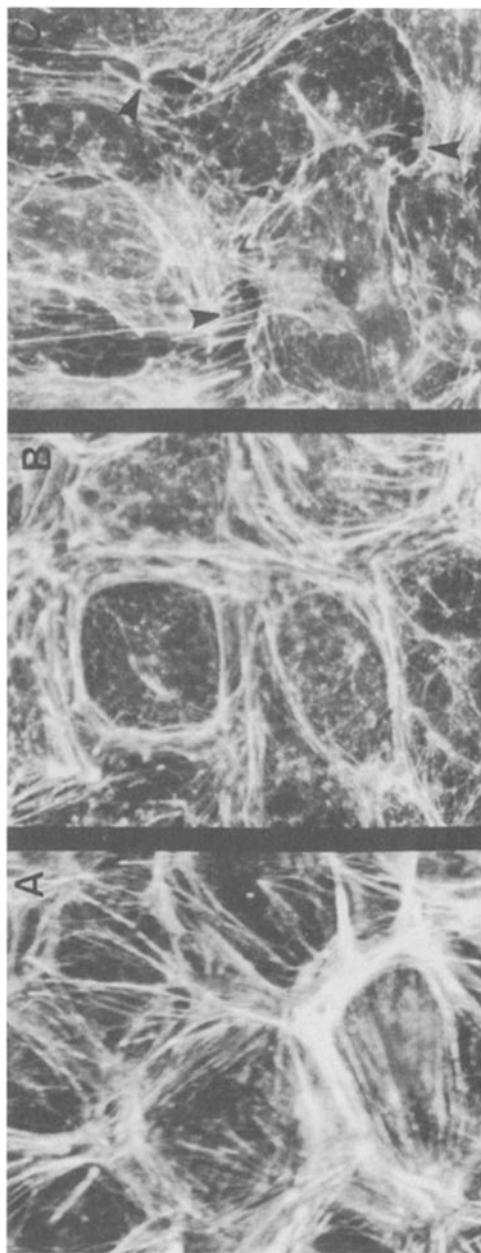
ent from untreated cultures. (C) Dose-response. Endothelial monolayers on filters were incubated for 48 h with growth medium containing 1% FCS alone (0) or medium supplemented with AGE-BSA as indicated (AGE-BSA) or heat-treated AGE-BSA (500 nM; heat). Cultures were then washed and permeability to [³H]inulin was studied as described in the text. Data were obtained at a time point 4 h after the addition of [³H]inulin. The mean \pm SD of [³H]inulin transfer from the compartment above (inner well) to below the monolayer (outer well) is shown.

endothelial monolayers on filters form a barrier restricting the passage of macromolecules and lower molecular weight solutes (Fig. 6 A) (7, 16, 45). Using this experimental system, cultures incubated with AGE-BSA demonstrated an increase in their permeability; after a 48-h incubation with AGE-BSA (250 nM) there was a time-dependent increase in the passage of the inert macromolecular tracer [³H]inulin evident by 3 h (Fig. 6 A). Perturbation of endothelial barrier function depended on the amount of time cultures were exposed to AGE-BSA, requiring ~24 h to be significantly increased above the baseline (Fig. 6 B). Increased permeability of monolayers in response to AGE-BSA was reversible, with barrier function being restored by 72 h after removal of AGE-BSA and addition of fresh medium (data not shown). The effect of AGE-BSA on permeability was also dose dependent, being half-maximal at 100–300 nM over several experiments, which approximately corresponds to AGE-BSA concentrations resulting in half-maximal occupancy of the cell surface binding sites. In contrast, addition of excess normal BSA (in addition to that present in the serum-containing medium present in the incubation mixture) had no effect on monolayer permeability. These data suggest that AGE-BSA-induced increase in endothelial permeability is a consequence of AGE-BSA interaction with the cell surface receptor.

Since endotoxin can also modulate endothelial permeability (23), and this agent could potentially be present as a contaminant in the AGE-BSA preparations, studies were carried to exclude this possibility (Fig. 6 B). Heat treatment of AGE-BSA prevented its effect on endothelial permeability under conditions that we have previously found not to affect endotoxin-induced alterations in a range of endothelial functions (38; data not shown). In addition, the LPS content of AGE-BSA preparations was found to be negative, as monitored by a standard limulus amoebocyte assay, and in the presence of polymyxin B (3).

Concomitant with AGE-BSA-induced perturbation of monolayer barrier function, there was an alteration in cell shape/cytoskeletal organization (Fig. 7). Confluent endothelial cultures form a contiguous monolayer with actin-based cytoskeletons characterized by variable central stress fiber arrays and prominent circumferential stress fiber bundles (Fig. 7 A). After exposure to AGE-BSA at 10 nM for 48 h, a dose that results in minimal alteration of barrier function, cells were contiguous and tightly apposed, but both peripheral and central stress fiber arrays were reduced, and punctate actin foci appeared in the perinuclear cytoplasm (Fig. 7 B). At a higher dose of AGE-BSA (100 nM for 48 h), organized actin structures were almost completely disrupted, remnants of circumferential and central stress fiber arrays, represented by actin aggregates, were scattered throughout the cytoplasm (Fig. 7 C). Concomitant with these advanced changes in the actin-based cytoskeleton, retraction of cellular margins resulted in formation of intercellular gaps between contiguous cells. These gaps provide a paracellular pathway for escape of intravascular contents into the vessel wall.

Modulation of Endothelial Cell Coagulant Properties by AGE-BSA. Alterations in vascular permeability are closely linked to perturbation of the coagulation mechanism, since access of plasma proteins to subendothelium, such as would result from retraction of endothelial cells, upregulates the procoagulant response. Conversely, activation of coagulation on the endothelial surface leads to the formation of proteases and fibrin that can alter endothelial cell shape and increase vascular permeability



are still closely apposed, but display reduction in the integrity of circumferential stress fibers and altered central stress fibers. At higher concentrations of AGE-BSA (*C*), gaps at the margins of contiguous cells are evident (*arrows*), and there is considerable disruption of the actin-based cytoskeleton with loss of circumferential stress fibers. Bar, 10 μm .

FIGURE 7. Effect of AGE-BSA on endothelial morphology. Rhodamine phalloidin staining of the actin-based cytoskeleton in control cultures (*A*) and cultures exposed for 2 d to AGE-BSA at a concentration of 10 nM (*B*) or 100 nM (*C*). Control monolayers display typical endothelial cytoskeletal morphology with close cell-cell apposition and prominent circumferential stress fibers (*A*). After incubation with a low concentration of AGE-BSA (*B*), cells

(16, 27). These considerations led us to examine if exposure of endothelium to AGE-BSA could alter certain cell surface anti- and procoagulant properties.

Since the central anticoagulant protein C/protein S pathway is closely linked to endothelium, we began our studies by examining modulation of thrombomodulin by AGE-BSA. Thrombomodulin is an endothelial cell surface cofactor promoting thrombin-mediated formation of the anticoagulant enzyme activated protein C (17). Incubation of endothelial cultures with AGE-BSA at 37°C led to a time-dependent decline in cell surface thrombomodulin activity (Fig. 8 *A*). Suppression of thrombomodulin activity was evident by 1 h, reached a maximum by 20 h, and persisted up to the longest time points tested (48 h). AGE-BSA-induced suppression of thrombomodulin activity was also dependent on the dose of AGE protein added, being half-maximal at ~70–100 nM (Fig. 8 *B*). In contrast, prolonged incubations or high concentrations of native BSA did not depress thrombomodulin activity (Fig. 8, *A* and *B*). This indicates that the effect of AGE-BSA on this cellular anticoagulant property was due to the AGE form of the protein and paralleled occupancy of cell surface binding sites (Fig. 3 *C*), suggesting that the cell-bound ligand is mediating the cellular effect.

In contrast to the fall in thrombomodulin activity on the cell surface, total cellular thrombomodulin antigen was not suppressed (Fig. 8 *C*), suggesting that redistribution, possibly making it inaccessible to thrombin, rather than degradation or shedding, had occurred. Visualization of thrombomodulin by immunofluorescence during the incubation with AGE-BSA (under conditions in which the antithrombomodulin antibody was incubated with nonpermeabilized cells so that only cell surface ligand

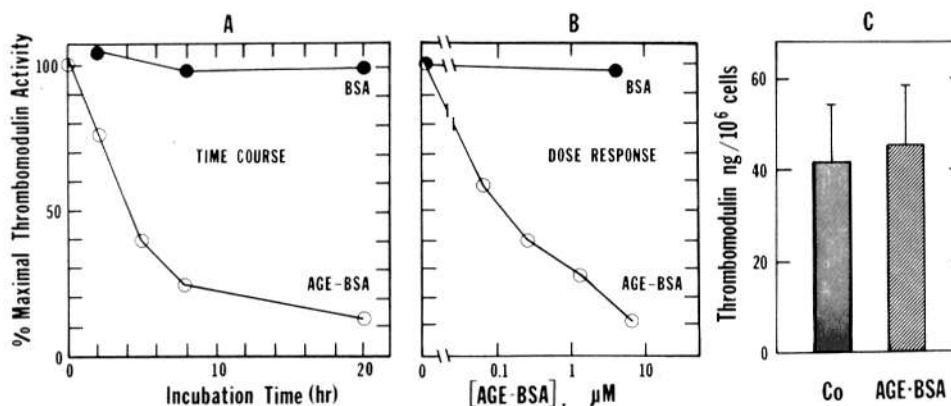


FIGURE 8. Effect of AGE-BSA on endothelial thrombomodulin. (*A*) Time course. Cultures were incubated with AGE-BSA (200 nM) (open circles) or medium supplemented with normal BSA (200 nM) (closed circles) at 37°C for the indicated times and then thrombomodulin activity was determined by assessing thrombin-mediated activation of protein C. (*B*) Dose response. Cultures were incubated with the indicated concentrations of AGE-BSA (open circles) or normal BSA (closed circles) and after 5 h at 37°C, thrombomodulin activity was assessed. Data in *A–B* are expressed as percent maximal thrombomodulin activity. (*C*) RIA for thrombomodulin antigen. Cultures were incubated in medium supplemented with normal BSA (250 nM; Co) or AGE-BSA (250 nM; AGE-BSA) for 24 h. Monolayers were then solubilized with NP-40 in the presence of inhibitors and thrombomodulin antigen was assessed by radioimmunoassay. Thrombomodulin antigen per 10⁶ cells, the mean \pm SD, is shown in each case, and details of experimental methods are described in the text.

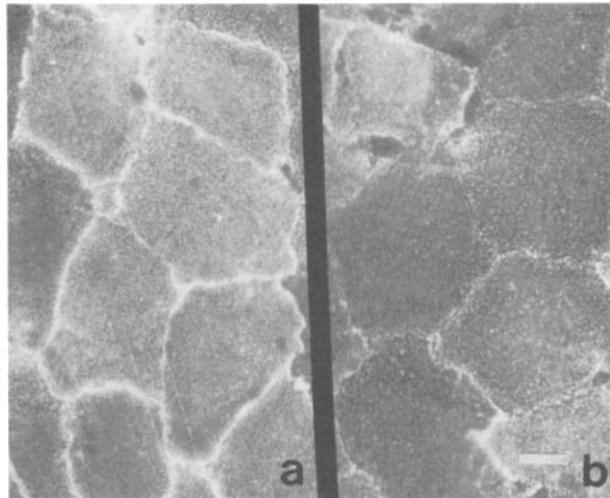


FIGURE 9. Immunofluorescence study of thrombomodulin: effect of AGE-BSA. Endothelial cell monolayers were incubated in normal medium (*A*) or medium supplemented with AGE-BSA (100 nM) (*B*) for 48 h and thrombomodulin was visualized by immunofluorescence as described in the text. The micrograph of the treated cultures in *B* demonstrates reduced fluorescence intensity compared with the untreated controls in *A*. Bar, 10 μ m.

was accessible), demonstrated a decrease in fluorescence intensity (Fig. 9). No evidence of increased thrombomodulin shedding was detectable by immunofluorescence or RIA of supernatants from AGE-BSA-treated endothelial cultures. Furthermore, AGE-BSA did not have a direct effect on thrombomodulin activity when experiments were performed with purified thrombomodulin. These data suggest that sequestration of thrombomodulin at an inaccessible site, with respect to enzyme and substrate present in the extracellular compartment, may be the predominant mechanism of AGE-BSA-mediated downregulation of this cell surface anticoagulant.

Although LPS has been reported to downregulate endothelial cell thrombomodulin (34), polymyxin B (which was present in the incubation buffer for studies of endothelial coagulant function) had no effect on AGE-BSA-mediated suppression of thrombomodulin and heat-treated AGE-BSA was inactive (data not shown). Furthermore, as described above, no LPS was detectable in the AGE-BSA preparations used.

In parallel with suppression of the cell surface activity of the anticoagulant cofactor thrombomodulin, exposure of endothelium to AGE-BSA led to an increase in endothelial cell procoagulant activity, which was tissue factor, based on its inhibition in the presence of a neutralizing monoclonal antibody (Fig. 10). Induction of tissue factor occurred steadily over several days and could be inhibited in the presence of cycloheximide, indicating the importance of de novo protein synthesis for expression of this procoagulant activity (Fig. 10*A*). The effect of AGE-BSA on tissue factor was also dependent on the dose of AGE-BSA added, being half-maximal at \sim 100–150 nM (Fig. 10*B*). As with AGE-BSA-mediated suppression of thrombomodulin, heat-treated AGE-BSA was ineffective and polymyxin B (the latter was added to culture medium in all experiments examining endothelial tissue factor expression) had no

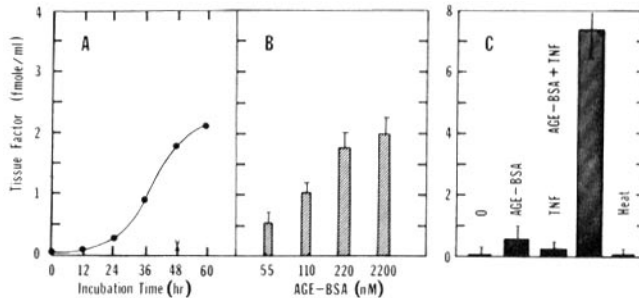


FIGURE 10. Effect of AGE-BSA on endothelial cell tissue factor activity. (A) Time course. Endothelial monolayers were incubated for the indicated times in medium supplemented with AGE-BSA (220 nM). Where indicated, cycloheximide was added to cultures simultaneously with AGE-BSA (x). Tissue factor activity was then assessed as described in the text. (B) Dose response. Endothelial cell monolayers were incubated

for 48 h in medium supplemented with the indicated amount of AGE-BSA and tissue factor activity was assessed. (C) Effect of TNF on AGE-BSA-induced tissue factor activity. Endothelial cell monolayers were incubated with normal medium or medium supplemented with AGE-BSA (55 nM). Then the incubation period was continued for 6 h using either the same medium or medium supplemented with TNF (0.03 nM). Where indicated, AGE-BSA was replaced by heat-treated AGE-BSA (0.5 μ M) (*heat*). Tissue factor activity of monolayers was then assessed. Details of experimental procedure are described in the text. The average of duplicate determinations is shown. 0, cultures incubated in normal medium alone; AGE-BSA, cultures incubated in medium supplemented with AGE-BSA; TNF, cultures incubated in normal medium for 48 h followed by a 6-h incubation with TNF; AGE-BSA + TNF, cultures incubated with medium supplemented with AGE-BSA for 48 h followed by a 6-h incubation with TNF. Data shown are the means \pm SD.

effect. Furthermore, native BSA had no effect on endothelial tissue factor activity. Taken together, these data indicate that AGE-BSA bound to its specific binding sites was the active species for induction of tissue factor in these studies.

In view of the sustained induction of low amounts of tissue factor in endothelium by AGE-BSA, we examined whether AGE-BSA could prime the cells to respond to a challenge with TNF, an agent that results in the induction of greater amounts of tissue factor activity, but within hours (5, 38). Addition of TNF (0.03 nM) to cultures incubated with AGE-BSA (55 nM) gave enhanced induction of procoagulant activity compared with either agent alone (Fig. 10 C). Experiments carried out with endothelial cultures preincubated for 3 d with AGE-BSA and then exposed to several low concentrations of TNF (0.05–0.2 nM) also demonstrated increased tissue factor induction, compared with that observed when either agent was present alone.

Discussion

The results of this study indicate that endothelium expresses a binding site that selectively interacts with glucose-modified BSA (AGE-BSA), and probably other AGE-modified proteins as well, in a manner distinct from normal albumin (32, 42, 43). Albumin, which has a half-life in the circulation of \sim 19 d, has detectable AGEs within 3 d of exposure to high glucose concentrations (12) and has sufficient AGEs within 16 d to interact with the endothelial cell binding site (Fig. 4). It is therefore likely that small amounts of circulating glucose-modified (AGE) plasma proteins (such as Igs, albumin, and lipoproteins) are bound by the vascular endothelium *in vivo*. The presence of these binding sites or putative receptors for AGE adducts of proteins on the vessel surface suggests that their interaction with endothelium could result in clearance from the intravascular space and/or modulation of vascular function. Consistent with this concept, cell surface binding of radiolabeled AGE-BSA

is followed by endocytosis with subsequent degradation and/or transcytosis. Of larger *in vivo* significance, however, may be the interaction of endothelial AGE-binding sites with subendothelial AGE proteins in the matrix, which accumulate due to their slow turnover rate (8, 10, 11, 33). Although endothelium may play a role in the clearance of these AGEs, our data indicate that occupancy of the endothelial AGE binding site results in perturbation of endothelial barrier and coagulant function, potentially contributing to the pathogenesis of diabetic microvascular hyperpermeability and hypercoagulability.

The binding of AGE-BSA to endothelium reported here shares several features in common with that previously reported for the interaction of ^{125}I -AGE-BSA with macrophages (52). In both cases a limited number of binding sites specific for the AGE moiety of the protein were present with a K_d of ~ 70 – 100 nM. Structurally, it is possible that different binding sites exist on macrophages and endothelial cells that recognize only some of the large family of AGE adducts. For example, the chemically defined AGE adduct FFI (39) is not recognized by the endothelial AGE-binding site, although it binds to the macrophage with comparable affinity to AGE-BSA and can elicit functional alterations, such as cytokine elaboration (54).

Exposure of endothelial cultures to AGE-BSA resulted in alterations in cell shape/cytoskeleton along with perturbation of barrier and coagulant function. In contrast to the effect of TNF on endothelial permeability, which was maximal within several hours (7), AGE-BSA-induced perturbation of endothelial permeability required several days. Increased permeability to [^3H]inulin occurred concomitantly with AGE-BSA-induced formation of intercellular gaps in the monolayer, presumably due to this paracellular pathway. Enhanced vascular permeability could increase access of plasma proteins from the intravascular space to the subendothelium, potentially providing a basis for the accumulation of protein in the vessel wall, such as has been observed in diabetic and aging vasculature (8, 10, 11, 33). In addition, after binding to the cell surface, AGE-BSA was apparently transcytosed and deposited within hours on the endothelial-derived extracellular matrix. Thus, transcytosis of AGE-BSA was probably also contributing to the permeability defect. The presence of these mechanisms delivering AGEs to the basement membrane could explain, at least in part, their deposition in this locus *in vivo* and their potential contribution as long-term regulators of endothelial function by altering the composition of the extracellular matrix.

Although our findings with AGE-BSA and cultured bovine aortic endothelium and those recently reported by Predescu et al. (40) with glycoalbumin and native murine coronary endothelium are in general agreement, there are multiple differences, as well. Predescu et al. demonstrated binding of gold-labeled glycoalbumin to endothelium that was partially inhibitable by native albumin and glucose, and almost completely blocked by the combination of these two. In contrast, our data indicate that neither of these agents affected AGE-BSA binding to the surface of cultured endothelial cells. There are several possible explanations for these differences. On the one hand, the source of endothelium in the two studies was quite different, i.e., cultured large vessel bovine endothelium and native microvascular endothelium. More importantly, the glycoalbumin used by Predescu et al. was prepared by incubating albumin with glucose (at 90 mM) for 14 d, and was distinguished from nonglycosylated albumin by the presence of an abundance of early glycosylation products (Amadori; reference 11). We have previously found that detectable

amounts of AGE form under these conditions (9), thus accounting for the competitive inhibition shown at 16 d using our preparations (Fig. 4). However, our glycosylated albumin preparations have been shown to be maximally modified by AGE after 6 wk of incubation with glucose (unpublished observation). Future studies of the binding of AGE-BSA and other forms of glycosylated albumin to the vessel surface *in vivo*, and to different types of endothelium *in vitro*, will be required to resolve these questions.

In addition to increasing permeability of the endothelial monolayer to intravascular solutes, AGE-BSA modulated endothelial coagulant function, potentially promoting activation of the clotting mechanism on the vessel surface; expression of thrombomodulin was suppressed and induction of tissue factor was enhanced. The effects of AGE-BSA on these endothelial coagulant properties were slower in their onset and more sustained, compared with the more rapid effects of cytokines such as TNF (4, 7, 14, 19, 35, 38). Furthermore, in contrast to TNF, which decreases total thrombomodulin antigen in parallel with the fall in cell surface thrombomodulin activity (14, 19, 35), AGE-BSA did not depress the pool of total thrombomodulin antigen, but rather exerted its effect more selectively on accessibility of the cell surface receptor. Induction of tissue factor in response to AGE-BSA was also quite different than the response to cytokines, such as TNF or IL-1 (4, 7, 38, 45). At least 2–3 d were required for induction of maximal tissue factor activity with AGE-BSA and that activity was maintained for several days when AGE-BSA was allowed to remain in the culture medium. In contrast, the cytokines induce maximal tissue factor activity by ~6 h, which subsequently decays to the baseline by 24 h, even when present at lower concentrations (7, 38, 45). These findings, taken together with the results described above, support our conclusion that the effects of AGE-BSA on endothelium are mediated, at least initially, by mechanisms involving AGE-BSA interaction directly with its cell surface receptor. The possible role of AGE-mediated induction of endothelial cytokine elaboration in explaining longer-term effects of these modified proteins on endothelium remains to be examined. In this context, in pilot studies we were unable to demonstrate induction of IL-1 synthesis during exposure of endothelial cultures to AGE-BSA.

These studies represent an initial characterization of the interaction of AGE-proteins, with AGE-BSA as a prototype, with endothelium. Many important issues remain to be examined, such as comparison of the endothelial cell receptor (of micro- and macrovascular-derived cells) with its counterpart recently described on macrophages, the ability of the receptor to interact with a spectrum of AGE-proteins, and the full range of cellular properties modulated by AGE-BSA endothelial interaction. Since AGE-proteins accumulate in the basement membrane, modulation of endothelial cell function by an abnormal extracellular matrix must be examined. In this context, preliminary studies indicate that modulation of coagulant properties can occur with endothelial cells seeded on AGE matrix. Although the *in vivo* physiologic relevance of these findings in cell culture is not yet clear, they may provide a basis for the initiation of early vascular pathology as manifested by increased permeability, as well as widespread coagulopathy.

Summary

Advanced glycosylation end products (AGE) of proteins accumulate in the vasculature with diabetes and aging, and are thought to be associated with vascular

complications. This led us to examine the interaction of AGE-BSA as a prototype of this class of nonenzymatically glycosylated proteins subjected to further processing, with endothelium. Incubation of ^{125}I -AGE-BSA with cultured bovine endothelium resulted in time-dependent, saturable binding that was half-maximal at a concentration of ~ 100 nM. Although unlabeled normal BSA was not a competitor, unlabeled AGE-BSA was an effective competitor of ^{125}I -AGE-BSA-endothelial cell interaction. In addition, AGE modification of two alternative proteins, hemoglobin and ribonuclease, rendered them inhibitors of ^{125}I -AGE-BSA binding to endothelium, although the native, unmodified forms of these proteins were not. At 37°C , binding of ^{125}I -AGE-BSA or gold-labeled AGE-BSA was followed by internalization and subsequent segregation either to a lysosomal compartment or to the endothelial-derived matrix after transcytosis. Exposure of endothelium to AGE-BSA led to perturbation of two important endothelial cell homeostatic properties, coagulant and barrier function. AGE-BSA downregulated the anticoagulant endothelial cofactor thrombomodulin, and induced synthesis and cell surface expression of the procoagulant cofactor tissue factor over the same range of concentrations that resulted in occupancy of cell surface AGE-BSA binding sites. In addition, AGE-BSA increased endothelial permeability, resulting in accelerated passage of an inert macromolecular tracer, [^3H]inulin, across the monolayer. These results indicate that AGE derivatives of proteins, potentially important constituents of pathologic vascular tissue, bind to specific sites on the endothelial cell surface and modulate central endothelial cell functions. The interaction of AGE-modified proteins with endothelium may play an important role in the early stages of increased vascular permeability, as well as vessel wall-related abnormalities of the coagulation system, characteristic of diabetes and aging.

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