Advanced Glycation Endproducts Interacting with Their Endothelial Receptor Induce Expression of Vascular Cell Adhesion Molecule-1 (VCAM-1) in Cultured Human Endothelial Cells and in Mice

A Potential Mechanism for the Accelerated Vasculopathy of Diabetes

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Abstract

Vascular cell adhesion molecule-1 (VCAM-1), an inducible cell–cell recognition protein on the endothelial cell surface (EC), has been associated with early stages of atherosclerosis. In view of the accelerated vascular disease observed in patients with diabetes, and the enhanced expression of VCAM-1 in diabetic rabbits, we examined whether irreversible advanced glycation endproducts (AGEs), could mediate VCAM-1 expression by interacting with their endothelial cell receptor (receptor for AGE, RAGE). Exposure of cultured human ECs to AGEs induced expression of VCAM-1, increased adhesivity of the monolayer for Molt-4 cells, and was associated with increased levels of VCAM-1 transcripts. The inhibitory effect of anti-RAGE IgG, a truncated form of the receptor (soluble RAGE) or N-acetylcysteine on VCAM-1 expression indicated that AGE-RAGE-induced oxidant stress was central to VCAM-1 induction. Electrophoretic mobility shift assays on nuclear extracts from AGE-treated ECs showed induction of specific DNA binding activity for NF-kB in the VCAM-1 promoter, which was blocked by anti-RAGE IgG or N-acetylcysteine. Soluble VCAM-1 antigen was elevated in human diabetic plasma. These data are consistent with the hypothesis that AGE-RAGE interaction induces expression of VCAM-1 which can prime diabetic vasculature for enhanced interaction with circulating monocytes. (J. Clin. Invest. 1995: 96:1395–1403.)

Key words: hyperglycemia • atherosclerosis • adhesion molecule • endothelium • oxidation

Introduction

Accelerated atherosclerosis and microvascular disease are the major vascular complications of diabetes, and constitute the principal cause of morbidity and mortality in this ubiquitous disorder (1–2). Many underlying factors could contribute to this outcome, including abnormalities in plasma lipoproteins, blood pressure, and renal function. A final common pathway in the development of vascular pathology is the expression of inducible adhesion molecules rendering the vasculature a selective target for circulating peripheral blood cells. In this context, vascular cell adhesion molecule-1 (VCAM-1) is of particular interest as its expression has been linked to the early phase of experimental hypercholesterolemia-induced atherosclerosis (3–4), and enhanced vascular VCAM-1 expression has been demonstrated in the vasculature of alloxan-treated diabetic rabbits (5) as well as in human atherosclerotic lesions (6).

To identify a sustained stimulus for enhanced expression of VCAM-1 in diabetes, we have considered a potentially etiologic role for advanced glycation endproducts (AGEs). Exposure of proteins and lipids to aldoses results in nonenzymatic glycation; initially forming reversible early glycation products, Schiff bases, and Amadori products (7, 8), which, after complex molecular rearrangements, become irreversible AGEs (8–9). The latter constitute a class of heterogeneous structures of yellow-brown color, characteristic fluorescence, which form cross-links, generate reactive oxygen intermediates (ROI), and interact with particular cellular receptors (8–9). The endothelial receptor for AGEs, termed RAGE, is a newly identified member of the immunoglobulin superfamily of cell surface molecules and has been shown to mediate interactions with these glycated proteins in vitro and in vivo (10). RAGE is expressed both in normal (15) and diabetic vasculature (A. M. Schmidt and D. Stern, unpublished observations).

Since AGEs are found in the plasma and accumulate in the vessel wall in diabetes, the potential role of interaction of AGEs with endothelial RAGE in induction of VCAM-1 expression was examined. Our data indicate that AGEs, either those prepared in vitro or isolated from patients with diabetes, led to VCAM-1 expression in cultured ECs by a mechanism involving AGE binding to RAGE likely as a consequence of induction of cellular oxidant stress and activation of the transcription factor NF-kB. Infusion of AGEs into mice also enhanced vascular VCAM-1 expression, and soluble VCAM-1 antigen was ele-
vated in plasma of patients with diabetes, suggesting increased VCAM-1 production. These data support the concept of a role for AGEs in enhanced expression of VCAM-1 in diabetes, and suggest that this could provide a mechanism for targeting mononuclear phagocytes (MPs) to diabetic vasculature.

Methods

**Endothelial cell culture and RAGE blocking reagents.** Human ECs were prepared from umbilical cords, grown in culture as described (16, 17), and experiments utilized confluent cultures (passages 1–3) grown in Medium 199 supplemented with fetal bovine serum (15% Gemini, Calabasas, CA), human serum (5%; Gemini), endothelial cell growth supplement (Boehringer Mannheim, Indianapolis, IN), heparin (90 μg/ml; Sigma Chemical Co., St. Louis, MO) and antibiotics. In most experiments, human umbilical vein endothelial cells (ECs) were used, but specifically indicated, certain experiments were performed with human aortic endothelial cells (Clonetics, San Diego, CA). Confluent cultures of human aortic endothelial cells were used for experiments at passages 4–6. RAGE cultures were prepared by immunohistochemistry using previously published methods (15, data not shown). Blockade of EC RAGE used soluble(s) bovine RAGE and monospecific antibody to sRAGE. Bovine RAGE was purified to homogeneity from bovine lung acetone powder (Sigma Chemical Co.) by the methods previously described (12). This material represents the amino-terminal two-thirds of the molecule based on analysis of tryptic peptides and is called soluble RAGE or sRAGE. Purified bovine RAGE was used to immunize rabbits, according to previously described methods (18). IgG was purified by affinity chromatography with immobilized protein A, and nonimmune rabbit IgG was similarly prepared. Immunoblotting studies showed that anti-RAGE IgG was monospecific; it bound only RAGE and binding was blocked completely by pre-absorption with purified RAGE. F(ab’)_2 was prepared from bovine RAGE (Affi-Gel 10, Bio-Rad, Hercules, CA) to the manufacturer’s instructions. The final F(ab’)_2, which was homogeneous on SDS PAGE, was then dialyzed extensively versus phosphate-buffered saline.

**Preparation of glycated proteins and antibodies.** Bovine serum albumin (fatty acid free; Sigma Chemical Co.) was glycated by incubation with glucose (0.5 M) at 37°C for 4 wk. Glycated proteins were characterized by native PAGE (which showed that the final preparation contained less than 1% AGE). Nonglycated albumin was affinity-purified from concentrated material, or placed in Medium 199 containing N-acetylcysteine (200 μM) for 30 min, followed by 200 μM N-acetylcysteine for 1 h at 37°C. The final preparation was dialyzed extensively against 20 mM tris (pH 7.4), 0.1 M NaCl, and 1 mM phenylmethylsulfonyl fluoride. 2A + 0.5% NP40. Where indicated, EC monolayers were pretreated with cycloheximide (50 μg/ml for 60 min), anti-RAGE IgG (30 μg/ml for 30 min), nonimmune IgG (30 μg/ml for 30 min), or N-acetylcysteine (30 mM for 60 min). Determination of soluble ICAM-1 was determined by ELISA using the monoclonal antibody to ICAM-1 NC/16 (21). The limit of detection in this assay is < 2.5 ng/ml.

**Northern blotting.** RNA prepared from cultured ECs (10^6 cells) using the guanidinium thiocyanate procedure (23) was applied to agarose-formaldehyde gels (0.8%; 30 μg), and transferred to nylon filters. Filters were prehybridized for 1 h at 68°C with QIAGEN Hyb buffer.
(Stratagene, La Jolla, CA) and hybridized in QIUK Hyb buffer for 3 h at 68°C in the presence of 32P-labeled full-length human VCAM-1 cDNA (24) labeled by the random primer procedure. Filters were then washed with SSC (2X) containing SDS (0.1%) twice for 15 min each at room temperature, washed again with SSC (0.1X) containing SDS (0.1%) for 30 min at 55°C, dried and subjected to autoradiography. To assess RNA loading, filters were also hybridized with random primer labeled 32P-CDNA for glyceraldehyde phosphate dehydrogenase (GAPDH) using the same washing and hybridization procedure.

**Nuclear run-on transcription assay.** Nuclear run-on transcription assay was performed using a modification of the methods as described by Santisteban et al. (25). ECs were incubated with AGE albumin (100 μg/ml) or native albumin (100 μg/ml) for 6 h at 37°C. Monolayers were then washed twice with cold phosphate-buffered saline, scraped, and cell pellets prepared. Pellets were then resuspended in 10 ml of lysis buffer containing Tris (0.01 M), pH 7.4, NaCl (10 mM), MgCl2 (3 mM), Nonidet P-40 (0.5% [vol/vol]), leupeptin (50 μg/ml), dithiothreitol (1 mM), phenylmethylsulfonfluoride (0.5 mM), and aprotonin (500 μg/ml), sodium azide (0.1%), and centrifuged at 5 min at 4°C. The nuclear pellets were washed once with lysis buffer as above (4 ml) and centrifuged at 500 g for 5 min at 4°C. Supernatants were discarded and nuclei resuspended in 0.2 ml reaction buffer containing Tris (10 mM, pH 8.0), MgCl2 (5 mM), KCl (100 mM), dithiothreitol (1 mM), ATP (1 M), CTP (0.5 M), GTP (0.5 M), and 200 micromolar of [α-32P]UTP (3,000 Ci/mmol; Amersham, Arlington Heights, IL), and reacted at 37°C for 1 h. RNA was then extracted as above and samples resuspended in hybridization buffer containing PIPES (50 mM, pH 6.8), EDTA (10 mM), NaCl (600 mM), SDS (0.2%), and denatured salmon test DNA (100 μg/ml). Hybridization to denatured human VCAM-1 and β-actin DNA slot-blotted on nylon filters was performed at 42°C for 72 h after prehybridization at 80°C for 2 h in hybridization buffer as above containing 0.1% SDS. After hybridization, the filters were washed in SSC (2X) containing SDS (0.5%), rinsed once in 2X SSPE/0.1% SDS, and air-dried and exposed to autoradiographic film for 4 d. For binding to the nylon filter, the human VCAM gene DNA and β-actin gene DNA were denatured by incubation with NaOH (0.3 N) for 30 min at 65°C. DNA was spotted onto the nylon filter and cross-linked with a UV cross-linker (Stratagene).

**Electrophoretic mobility shift assays (EMSA).** Nuclear extracts were prepared from ECs by the method of Schreiber et al. (26). Protein concentration was determined using the Bradford reagent as above. Double-stranded oligonucleotides representing a portion of the human VCAM-1 promoter containing an NF-kB site or a mutant NF-kB site were synthesized (GenSet, Paris, France) (24). Oligonucleotides were used as probes as follows: NF-kB: 5'CCTTTGAAAGGATTCCTCCT3' and a mutated, inactive form 5'CCTTTGAAATTTATTCCTCCT3' (24). Probes were 5' end-labeled with γ-32P ATP and purified as above. Each reaction contained 10 fmol of each probe, 2–5 μl (1.5 μg protein) of nuclear extract. Binding reactions were performed in HEPES 50 mM (pH 7.9), glycerol (5%), KCl (5 mM), MgCl2, dithiothreitol (1 mM), and poly (dl-dC; 1 μg) in a reaction volume of 20 μl for 20 min at room temperature. Complexes were resolved on nondenaturing polyacrylamide gels (6%) in Tris (50 mM), borate (45 mM), and EDTA (0.5 mM). 0.5X TBE buffer; final pH 7.5. Samples were subjected to electrophoresis for 2 h at 10 V/cm. Gels were then dried and subjected to autoradiography. For supershift assays, complexes were preincubated for 2 h at 4°C with 7.5 μg/ml of anti-p65 IgG, anti-p50 IgG (Santa Cruz Biotechnology, Santa Cruz CA), both together, or nonimmune IgG, in a total reaction volume of 20 μl.

**Immunostaining.** Immunostaining for VCAM-1 was performed on lung tissue which had been placed in freezing medium (Polysciences, Inc., Warrington, PA) and snap frozen in dry ice/ methanol for sectioning. Sections were incubated in blocking buffer (phosphate-buffered saline/bovine serum albumin [1%]/normal rabbit serum [4%]), and then exposed for 45 min at 37°C to monoclonal rat anti–murine VCAM-1 IgG (20 μg/ml; Southern Biotechnologies, Birmingham, AL). Primary antibody was revealed with rabbit anti–rat Extra Avidin (Sigma Chemical Co.) using acetylated carbazol as the chromogen.

**Results**

**Exposure of cultured human ECs to AGEs increases their adhesivity for Molt-4 cells and expression of VCAM-1.** EC monolayers incubated with AGE albumin exhibited time-dependent enhancement of Molt-4 binding (these cells bear the counterligand for VCAM-1, very late antigen 4 [VLA-4]; 22), which was maximal at 2 h (Fig. 1 A) and gradually fell to the baseline by 12 h (data not shown). AGE-enhanced binding of Molt-4 cells to endothelium was prevented by blocking with Fab’2 against VCAM-1, suggesting a central role for VCAM-1 as the EC adhesion molecule mediating this interaction (Fig. 1 B). De novo endothelial protein synthesis was required to support Molt-4 binding, as preincubation of endothelium with cycloheximide was inhibitory (Fig. 1 B). Molt-4 binding to AGE-treated ECs was not due to passive adsorption of the glycated protein to the endothelial surface because affinity-purified antibody to AGEs, which prevents their cellular interactions (18, 19), had no effect when added during the adherence assay (data not shown).

In parallel with increased VCAM-1–dependent binding of Molt-4 cells to AGE-treated endothelium, expression of VCAM-1 antigen by ECs was enhanced as measured by ELISA (Fig. 2 A); this occurred in a time-dependent manner similar to that observed for Molt-4 binding to AGE-treated endothelium (data not shown). Enhancement of cellular VCAM-1 expression by AGEs was prevented by addition of cycloheximide (Fig. 2 A), consistent with results of cell adherence experiments (Fig. 1 B). AGEs obtained from diabetic serum (18) also enhanced expression of VCAM-1 (Fig. 2 A), indicating that AGE-adducts generated in vivo had similar properties, with respect to endothelial VCAM-1 expression, compared to AGE albumin prepared in vitro. In contrast, AGE-depleted serum was without effect (Fig. 2 A). The effect of AGEs on VCAM-1 expression was selective, as there was no change in EC levels of ICAM-1 as measured by ELISA after treatment with AGE albumin (Fig. 2 B), whereas tumor necrosis factor-α, added as a positive control, significantly increased ICAM-1 expression (27). Human aortic vein endothelial cells were also tested for their response to AGEs since they more closely approximate the endothelial cells likely to be relevantly affected in vivo models of atherosclerosis. When human aortic endothelial cells were exposed to AGE albumin, expression of cellular VCAM-1 antigen was increased as measured by ELISA (Fig. 2 C) whereas native albumin was without effect (Fig. 2 C).

Consistent with possible AGE-induced de novo synthesis of VCAM-1, levels of VCAM-1 mRNA were elevated in ECs exposed to AGE albumin, whereas transcripts for GAPDH were unchanged (Fig. 2 D). Nuclear run-on transcription assay indicated that this was due to increased transcription of mRNA for VCAM-1 in AGE-treated ECs (Fig. 2 E).

**Mechanisms underlying AGE-induced EC expression of VCAM-1.** Previous studies have shown that RAGE mediates the expression of VCAM-1 in vitro, in both ECs and in vivo, and that it is the principal binding site on endothelium for AGEs (11). To determine if AGE-RAGE interaction-mediated VCAM-1 induction, access to RAGE was blocked with monoclonal antibody to RAGE, and with sRAGE, the latter a truncated form of full-length.
RAGE comprising the extracellular domain (11–13). Pre-incubation of ECs with anti-RAGE F(ab’)2 largely prevented AGE-mediated increased endothelial adherence of Molt-4 cells (Fig. 3 A) and anti-RAGE IgG blocked the increase in VCAM-1 antigen completely (Fig. 3 B), whereas nonimmune reagents had no effect (Fig. 3, A and B). sRAGE had a similar, though even more complete (>95%) inhibitory effect on the binding of Molt-4 cells to AGE-treated ECs and their expression of VCAM-1 (Fig. 3, A and B). These data indicated that AGE-RAGE interaction had a central role in AGE induction of endothelial VCAM-1.

AGEs tethered to cell surface RAGE induce cellular oxidant stress; this assertion is based on several lines of evidence, including generation of thiobarbituric acid-reactive substances, expression of heme oxygenase type I mRNA, and activation of the transcription factor NF-kB (19). NF-kB sites in the VCAM-1 promoter have been shown to participate in regulation of VCAM-1 expression in response to cytokines, mediated, at least in part, by an oxidant mechanism impacting on activation of NF-kB (20, 24, 28). These data led us to examine if AGE-induced cellular oxidant stress was involved in up-regulating VCAM-1 expression. Pretreatment of endothelium with the antioxidant N-acetylcysteine inhibited AGE-enhanced Molt-4 binding (Fig. 3 A) as well as enhanced expression of VCAM-1 antigen (Fig. 3 B). In addition, AGE binding to endothelial RAGE triggered events resulting in expression of DNA binding activity for the NF-kB site in the VCAM-1 promoter. Activation of NF-kB, by incubating the ECs with TNF-α, served as a positive control in this experiment (Fig. 3 C, lane 1). After exposure of ECs to AGE albumin, EMSA demonstrated a gel shift band (Fig. 3 C, lanes 3, 6, and 10) not observed with cultures exposed to native albumin (Fig. 3 C, lane 2). AGE-induced DNA binding activity was sequence specific, as its appearance was blocked by excess unlabelled NF-kB probe, but was not affected by a mutationally inactivated NF-kB probe (Fig. 3 C, lanes 4 and 5, respectively). DNA binding activity was probably dependent on p50 and p65 proteins of the NF-kB family in the nucleus, as indicated by the distinct retardation patterns of AGE-induced gel shift bands in the presence of anti-p65 IgG (Fig. 3 C, lane 6), anti-p50 IgG (Fig. 3 C, lane 7) or in the presence of both antibodies (Fig. 3 C, lane 8), suggesting the involvement of both the p50 and p65 proteins of the NF-kB family. The same concentration of nonimmune IgG was without effect (data not shown). NF-kB activation was largely inhibited by the higher concentration of anti-RAGE IgG, 70 μg/ml (Fig. 3 C, lane 11) whereas a lower concentration of anti-RAGE IgG, 0.7 μg/ml had no effect (Fig. 3 C, lane 12). In contrast, nonimmune IgG did not alter intensity of the AGE-induced gel shift band (Fig. 3 C, lane 13). NF-kB activation by AGE albumin was also inhibited by addition of N-acetylcysteine (Fig. 3 C, lane 14). These data indicate that AGE binding to endothelial RAGE activates nuclear binding activity for the VCAM-1 NF-kB site, likely via an oxidant mechanism. Consistent with these data, elevated levels of VCAM-1 transcripts in AGE-treated ECs were blocked in the presence of anti-RAGE IgG, but not with nonimmune IgG (Fig. 3 D, lanes 2 and 3, respectively), and by N-acetylcysteine (data not shown).

Infusion of AGEs induces VCAM-1 in mice and patients with diabetes display increased expression of soluble VCAM-1. In view of the association of AGEs and oxidant stress with diabetes (8), we considered whether induction of VCAM-1 might occur in diabetic vasculature. To approach this question,
AGE albumin was infused into normal mice and expression of VCAM-1 in the vasculature was assessed. After administration of AGE albumin, VCAM-1 was identified in murine vasculature by immunostaining (Fig. 4 B), but was absent in animals infused with native albumin (Fig. 4 A).

These data led us to inquire whether patients with diabetes might also exhibit increased expression of VCAM-1. In pilot studies, incubation of AGE albumin with ECs not only led to increased cell-associated VCAM-1 (Fig. 2 A), but also to increased soluble VCAM-1 in culture supernatants based on ELISA (Fig. 4 C); elaboration of sVCAM-1 occurred in a time-dependent manner paralleling expression of cellular VCAM (data not shown). Of note, pilot studies revealed that EC supernatants containing sVCAM-1 did not antagonize binding of Molt-4 cells to stimulated ECs expressing VCAM-1 (data not shown). These data suggested that sVCAM-1 might serve as a marker for AGE-induced (and probably the capacity of other mediators to induce) VCAM-1 expression. sVCAM-1 antigen was measured in patients with a spectrum of diabetic complications at different stages in the severity of their vascular disease (Fig. 4 D). Only patients with diminished glomerular filtration rate were excluded, as sVCAM-1 levels have been shown to be elevated with renal insufficiency (29). Patients with diabetes (n = 18) displayed higher sVCAM-1 levels (mean, 1.11 ng/ml) compared to age-matched controls (mean, 0.82 ng/ml) (n = 20; P < 0.001 using the student’s unpaired t test). Consistent with these data, diabetic patients demonstrated higher circulating levels of plasma AGEs as measured by ELISA (244 ng/ml AGE albumin equivalents) than age-matched controls (157 ng/ml AGE albumin equivalents, P < 0.001 using the student’s unpaired t test).

Discussion

Our results indicate that AGEs, aldose-modified proteins present within the intravascular space and which accumulate in the vessel wall and other tissues especially in diabetes (7–10), interact with endothelium to induce VCAM-1, an adhesion molecule for MPs associated with development of atherosclerosis (3–6). These data add to a growing body of evidence concern-
samples obtained from: ECs incubated for 6 h at 37°C with TNF-α (10 nM, lane 1) nonglycated albumin (100 μg/ml, lane 2) or with AGE albumin (100 μg/ml, lanes 3, 6, and 10); nuclear extract from ECs incubated with AGE albumin preincubated with anti-p50 IgG (lane 7) or both anti-p50 IgG and anti-p65 IgG together (lane 9). ECs were either preincubated with anti-RAGE IgG (70 μg/ml or 0.7 μg/ml for 3 h, lanes 11 and 12, respectively) or nonimmune IgG (70 μg/ml for 3 h, lane 13) or N-acetylcycteine (30 mM for 1 h, lane 14), followed by treatment with AGE albumin (100 μg/ml) for 6 h at 37°C. After treatment with AGE albumin for 6 h at 37°C, EC nuclear extracts were prepared and incubated with 32P-labeled NF-κB probe in the presence of an 100-fold molar excess of either unlabelled NF-κB (lane 4) or the mutated NF-κB probe (lane 5). (D) ECs were incubated with AGE albumin (100 μg/ml) (lane 1) for 6 h in the presence or absence of anti-RAGE IgG (30 μg/ml for 3 h) or nonimmune IgG (30 μg/ml for 3 h) (lanes 2 and 3, respectively) and then RNA extracted and Northern blotting performed as above.

Figure 3. AGE albumin-mediated induction of endothelial VCAM-1: effect of blocking access to RAGE on enhanced Molt-4 binding (A), enhanced expression of VCAM-1 antigen (B), activation of NF-κB (C), and enhanced VCAM-1 transcription (D). (A) ECs were incubated with AGE albumin (100 μg/ml) alone for 6 h at 37°C or in the presence of nonimmune F(ab')2 (2 μg/ml), anti-RAGE F(ab')2 (2 μg/ml), sRAGE (1500 μg/ml) or N-acetylcycteine (30 mM) as above. A binding assay was then performed with 32Cr-labeled Molt-4 cells as described. The mean±SEM of at least triplicate determinations is shown. In all cases, ** denotes P < 0.001 compared with treatment with AGE albumin alone using the student’s unpaired t test. (B) ECs were incubated with AGE albumin alone or in the presence of anti-RAGE IgG (30 μg/ml for 3 h), nonimmune IgG (30 μg/ml for 3 h) or N-acetyl cysteine (30 mM for 60 mins) and cellular ELISA for VCAM-1 was performed. In each case, the mean±SEM of at least triplicate determinations is shown. ** denotes P < 0.001 compared with treatment with AGE albumin alone using the student’s unpaired t test. (C) Binding of EC nuclear proteins to NF-κB binding site in the VCAM-1 promoter: EMSA. All lanes contain the labelled probe. Lanes refer to the following agonists: lane 1, ECs alone; lane 2, ECs treated with AGE albumin (30 μg/ml); lane 3, ECs treated with AGE albumin (30 μg/ml) and anti-RAGE IgG (70 μg/ml); lane 4, ECs treated with AGE albumin (30 μg/ml) and N-acetylcycteine (30 mM), lane 5, ECs treated with AGE albumin (30 μg/ml) and N-acetylcycteine (30 mM) and anti-RAGE IgG (70 μg/ml).

The expression of VCAM-1 by ECs in response to AGE albumin probably underlines the enhanced monocyte binding followed by transmigration through EC monolayers grown on filters with AGES in the lower compartment (18, 31). This suggests the possibility that AGES within the vessel wall (33) could stimulate overlying endothelium to produce VCAM-1, thereby targeting MP to the vessel surface. Such AGES, which are present for long times within the vessel wall, might produce increased permeability of EC monolayers (30); changes in cell surface coagulant properties with induction of low levels of tissue factor procoagulant activity (30); and receptor-mediated transcytosis and delivery of the glycated ligand to the subendothelium (14) where it permits interaction with smooth muscle cells. AGE-mediated induction of VCAM-1 also elucidates an evolving picture in which the glycated ligand stimulates cytokine and growth factor production, and modulates macrophage migration. There is accelerated chemotaxis of macrophages in response to a gradient of soluble AGES and inhibition of migration when a deposit of immobilized AGES is encountered (18, 31, 32). Expression of VCAM-1 by ECs in response to AGE albumin probably underlies the enhanced monocyte binding followed by transmigration through EC monolayers grown on filters with AGES in the lower compartment (18, 31). This suggests the possibility that AGES within the vessel wall (33) could stimulate overlying endothelium to produce VCAM-1, thereby targeting MP to the vessel surface. Such AGES, which are present for long times within the vessel wall, might
have a more sustained effect on EC expression of VCAM-1 and/or could magnify effects of other stimuli which modulate endothelial VCAM-1 production, such as cytokines (27). Thus, measurement of circulating (plasma) AGE levels may only be partially relevant given the likely diffuse distribution of pathogenic irreversibly-glycated proteins in the vasculature, only some of which may be readily amenable to isolation and analysis pre-mortem.

Although AGEs comprise a class of heterogeneous structures, their interactions with endothelium appear to be mediated mostly via RAGE (11–12). In the current studies, either blocking antibody/F(ab')2 or sRAGE inhibited induction of VCAM-1 at the same concentrations that blocked binding of AGE albumin to the EC surface. Interaction of AGE albumin with MPs, as well as their interaction with other AGE-modified proteins (34), has been shown similarly to involve RAGE, suggesting that this receptor is a major site for cell surface AGE-mediated events although other AGE cellular binding sites may also be present (35, 36). VCAM-1 expression appears to be induced by generation of cellular oxidant stress consequent on cell surface AGE-RAGE interaction. This could arise from ROIs produced by the glycated proteins themselves (8, 37–40) and/or receptor-mediated triggering of signal transduction mechanisms leading to low levels of oxygen-free radical production (20, 24, 41–43). Specific signal transduction mechanisms activated by AGE-RAGE interaction have not been mapped in detail, but based on the presence of a short, highly charged cystolic tail at the receptor carboxy terminus, we hypothesize that ligand-receptor interaction allows the cytosolic tail to bind intracellular polypeptides which participate in signaling. These observations support the concept that RAGE is not a simple scavenger receptor which rapidly endocytoses and degrades AGES, thereby clearing tissues of aldose-modified polypeptides or lipids; rather, engagement of the ligand sets in motion events which lead to a spectrum of changes in cellular properties. In view of the potentially injurious nature of subsequent cellular events following AGE-RAGE interaction, it seems probable that AGES are accidental ligands of RAGE, a receptor meant to recognize other ligands in quite different settings. Such a role for RAGE other than binding to AGES is supported by evidence of enhanced RAGE expression in the central nervous system in early development and in some types of mature neurons, and its strong constitutive expression in smooth muscle cells (15; data not shown).

The observation that the AGE-RAGE interaction induces cell-associated VCAM-1, and also releases sVCAM-1 antigen into culture supernatants, suggests a means of monitoring this event in vivo. Furthermore, increased levels of sVCAM-1 were observed in diabetic patients compared with a range of normal controls. Although the functional significance of sVCAM-1 is at present unclear (pilot studies have shown lack of antagonism of Molt-4 binding to EC surface VCAM-1), sVCAM-1 could serve as a marker of vascular perturbation in diabetes. Since endothelial expression of VCAM-1 occurs in response to diverse stimuli, including cytokines (27), and could result from the oxidant stress consequent on hyperglycemia itself (44),

**Figure 4.** AGE albumin infusion induces vascular expression of VCAM-1 (A and B), release of sVCAM-1 from cultured ECs (C), and sVCAM-1 is present in diabetic plasma (D). (A–B) Mice were infused with either nonglycated albumin (500 μg/animal; A) or AGE albumin (500 μg/animal; B) and 6 h later lung was harvested and immunostaining with anti-murine VCAM-1 antibody was performed. Magnification: ×340. (C) ECs were incubated with AGE albumin (100 μg/ml) or nonglycated albumin (100 μg/ml) for 6 h at 37°C, culture supernatant was collected, concentrated 10-fold, and assayed for VCAM-1 antigen by ELISA. The mean±SEM of at least triplicate determinations is shown. **P < 0.001 using the student’s unpaired t test. (D) Plasma from patients with diabetes (n = 18) or normal controls (n = 20) was obtained and assayed for VCAM-1 antigen or AGE albumin equivalent by ELISA. For sVCAM-1, values are 1,115 ng/ml (SD = 469 ng/ml) for diabetic patients and 632 ng/ml (SD = 115 ng/ml) for age-matched controls. For AGE levels, values are 244 ng/ml AGE albumin equivalents (SD = 27 ng/ml AGE albumin equivalents) for diabetic patients and 157 ng/ml AGE albumin equivalents (54 ng/ml AGE albumin equivalents). In both cases, **P < 0.001 using the student’s unpaired t test.

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**Table 1.** Mean±SEM VCAM-1 expression of cultured ECs

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<tr>
<th>Condition</th>
<th>VCAM-1 expression (ng/ml)</th>
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<tr>
<td>Normal control</td>
<td>157±42</td>
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<tr>
<td>Diabetic patients</td>
<td>378±74</td>
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*P < 0.001 using the student’s unpaired t test.*

Endothelium, Receptors, and Glycation

1401
plasma VCAM-1 levels are likely to reflect the complexity of events which occur within the vasculature in vivo. Even with these diverse stimuli for induction of VCAM-1 in diabetes, we hypothesize that serial sVCAM-1 levels could be of benefit in identifying patients at high risk for the development of clinically apparent vascular complications (as a result of EC perturbation due to AGEs or other agents) and might provide a surrogate marker for assessment of therapy designed to attenuate the progression of vascular disease.

Taken together with our recent demonstration that AGEs induce expression by smooth muscle cells of the chemotactic polypeptide JE/MCP-1 (45), these data suggest a pathway through which AGEs attract monocytes to bind to the vessel surface, followed by their migration into the vessel wall and subsequent stimulation with production of mediators potentially contributing to the development of vascular lesions. The recent observation of increased VCAM-1 expression in diabetic rabbits (5) and our finding of elevated sVCAM-1 levels in diabetic patient plasma suggests that this cell adherence molecule might have a important role in the pathogenesis of diabetic vascular disease.

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