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Expression of Receptors for Advanced Glycation End Products in Peripheral Occlusive Vascular Disease

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The cellular interactions of advanced glycation end products (AGEs), which have been hypothesized to contribute to the development of vascular lesions, occur, at least in part, through their binding to a novel integral membrane protein, the receptor for AGEs (RAGE). Studies of human vascular segments show that endothelial RAGE expression at the antigen and mRNA level was variable and usually at low levels in samples from healthy individuals. In contrast, patients with a range of peripheral occlusive vascular diseases, with or without underlying diabetes, demonstrated prominent enhancement of endothelial RAGE expression. Smooth muscle cells and nerves in the vessel wall showed constitutively high levels of RAGE expression that were unchanged with aging (from 1 to 92 years) or by the presence of vascular disease. These data suggest that RAGE is likely to have ligands other than AGEs, and that multiple factors in addition to AGEs impact on its expression. Taken together, our findings suggest that RAGE may contribute to the pathogenesis of a range of vascular disorders. (Am J Pathol 1995, 146:688–694)

The accumulation of advanced glycation endproducts (AGEs), the ultimate result of nonenzymatic glycation of proteins and lipids,[1–3] in tissues has been correlated with the development of complications in diabetes.[1,4–8] One critical means through which AGEs interact with cellular elements of the vessel wall is through binding to specific receptors initially identified on endothelial cells and mononuclear phagocytes.[9,10] Engagement of these receptors results in modulation of functions important in vascular homeostasis, including increased endothelial cell monolayer permeability,[9] oxidant stress, and adhesivity for mononuclear phagocytes, as well as induction of mononuclear phagocyte chemotaxis and cytokine/growth factor generation.[11–16]

The most completely characterized cellular receptor for AGEs has been termed receptor for AGE or RAGE, a new member of the immunoglobulin superfamily of cell surface molecules consisting of an extracellular domain of three immunoglobulin-like domains, followed by a single transmembrane spanning domain and a highly charged cytosolic tail.[17] RAGE has been shown to mediate the binding of AGEs to endothelial cells and mononuclear phagocytes, and *in vivo* studies have shown it to be responsible for clearance of AGEs from the intravascular space as well as AGE-induced gene expression.[18] A recently completed bovine tissue survey in normal animals demonstrated RAGE to be expressed in multiple cell types, especially smooth muscle, nerve, and the vasculature.[19] Examination of human atherosclerotic plaques[19,20] or experimentally induced inflammatory lesions in response to local instillation of AGEs[12] showed prominent accumulation of cells strikingly positive for RAGE, as well as increased expression of this receptor in the proximal vasculature. These findings indicated the importance of determining pathophysiologically relevant situations in which RAGE expression was modulated. It should be noted that, in

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addition to RAGE, different AGE binding proteins have been isolated from rat liver.[10]

The current study employing pathological samples from patients with occlusive vascular disease demonstrates that, in vessels involved in the disease process, RAGE expression in endothelial cells of the vasa vasorum, at both the antigen and mRNA level, is enhanced. This was true in patients with diabetes, in which AGEs would be expected to be present in both the intravascular space and vessel wall at higher levels, as well as in nondiabetic patients with extensive peripheral atherosclerotic vascular disease. These data identify a clinically relevant context in which RAGE expression may be important and will serve as the basis for future studies to delineate functional roles of this receptor-ligand system in the pathogenesis of vascular disease.

Materials and Methods

Harvesting and Preparation of Tissues

During operative procedures for therapy of vascular disorders, routinely excised vessel segments were immediately snap-frozen in isopentane/liquid nitrogen and then stored at −80 C. Informed consent was obtained, and the protocol by which tissue was collected was approved by the Internal Review Board of the University of Heidelberg School of Medicine.

All patients were in stages IIb to IV (according to the Fontaine criteria for peripheral occlusive vascular disease) and were compared with normal controls. Control vessels were obtained from patients who had carcinoma of the large bowel, liver transplantation, or removal of a toe due to polydactyly.

Patients were divided in three groups. Group 1 consisted of 19 patients with peripheral occlusive vascular disease and diabetes mellitus (diabetes was defined as preprandial blood glucose of >150 mg/dl or if the patient was regularly taking antidiabetic treatment; ages ranged from 42 to 86 years). Group 2 consisted of 26 patients with peripheral occlusive vascular disease without diabetes mellitus (ages from 51 to 92 years). Group 3 consisted of 11 normal controls without peripheral occlusive vascular disease and without diabetes mellitus (ages from 1 to 79 years).

Preparation and Use of Immunological Reagents

The immunoglobulin (Ig)G fraction from polyclonal antiserum raised in guinea pigs to single-band bovine RAGE (35 kd), the latter purified from lung, was utilized. This antibody has been characterized in detail by Schmidt et al[21] and Brett et al.[19] Its characterization includes Western blotting, enzyme-linked immunosorbent assay, and immunostaining. In each case, any staining observed was blocked by preincubation of antibody with purified bovine RAGE. Observations in the latter system have been confirmed with antibodies generated subsequently to recombinant human RAGE, which were also used by Brett et al.[19] The same anti-RAGE IgG has been used in functional experiments (blocking clearance of AGEs infused into mice) by Schmidt et al[18] and in studies of AGE-induced oxidant stress performed *in vivo* and *in vitro*.[22]

For immunostaining, cryostat sections (4 μ) were cut and fixed in acetone for 10 minutes, incubated with anti-RAGE IgG in a humidified atmosphere overnight at 4 C. Second antibody (rabbit anti-guinea pig Igs, Dako, Glostrup, Denmark) was added for 30 minutes at room temperature, after which third antibody (swine anti-rabbit Igs; Dako) was added for 30 minutes, followed by incubation with DAKOPATTS rabbit PAP (consisting of soluble complexes of rabbit antibody to horseradish peroxidase) for 30 minutes. Between each step of this procedure, sections were washed for 10 minutes in phosphate-buffered saline at room temperature. Peroxidase activity was detected with aminoethylcarbazol as chromogen. In control sections, primary antibody was replaced by phosphate-buffered saline or normal serum. Immunohistochemical sections were evaluated independently by two investigators (U. R. and R. W.) and blindly scored as described in the results.

In situ hybridization was performed by using methodology recently described in detail.[23] On the basis of the human RAGE sequence,[21] a 77-bp insert (bp 70 to 147) was used as an antisense probe to detect expression of RAGE mRNA. This sequence has not been detected in other known genes, on the basis of a computer search of the EMBL data bank. Labeling of the oligonucleotide was performed by using the Boehringer-Mannheim protocol and digoxigenin-UTP. The use of the antisense oligonucleotide, but not the sense oligonucleotide, resulted in positive reactions with cultured human umbilical vein endothelial cells (data not shown).

Cryostat sections of vessel segments were placed on glass slides that had been previously coated with poly-L-lysine (100 μg/ml). Slides were fixed in freshly prepared neutral-buffered paraformaldehyde (4%) and acetylated with acetic anhydride (0 to 25%) in triethanolamine (pH 8.0). Sections were then rinsed in phosphate-buffered saline and dehydrated sequentially in an ascending series of alcohols. Samples

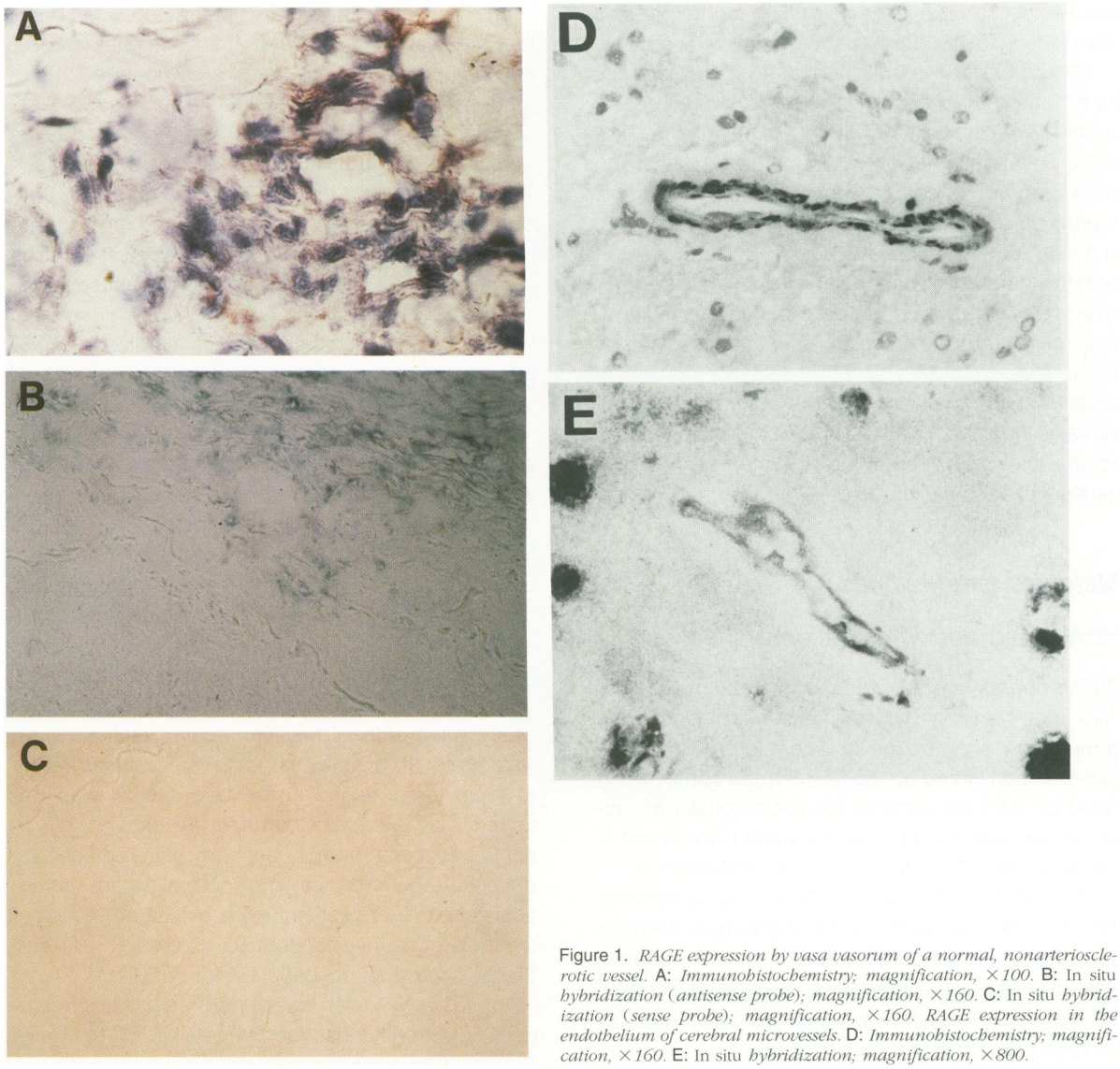

Figure 1. *RAGE expression by vasa vasorum of a normal, nonarteriosclerotic vessel.* A: *Immunohistochemistry; magnification, ×100.* B: In situ *hybridization (antisense probe); magnification, ×160.* C: In situ *hybridization (sense probe); magnification, ×160. RAGE expression in the endothelium of cerebral microvessels.* D: *Immunohistochemistry; magnification, ×160.* E: In situ *hybridization; magnification, ×800.*

were prehybridized (10 minutes at 25 C) in deionized formamide (50%), 4X standard saline citrate (SSC), 1X Denhardt's, salmon sperm DNA (500 μg/ml), yeast tRNA (250 μg/ml), poly (A) RNA (100 μg/ml), dextran sulfate (10%; molecular weight 500,000) and dithiothreitol (10 mmol/L). Sections were then hybridized with rehybridization solution containing digoxigenin-labeled probe (1 ng/μl) for 12 hours at 37 C, followed by washing (25 C) in 1X SSC (10 minutes), 0.5X SSC (30 minutes), and 0.1X SSC (30 minutes). Slices were then rinsed (2 to 3 minutes) in Tris-HCl (100 mmol/L, pH 7.4) and NaCl (150 mmol/L), and color development was performed as described in the manufacturer's instructions (Boehringer-Mannheim, Mannheim, Germany). Results of *in situ* hybridization studies were evaluated independently by two investigators (Y. Z. and R. W.).

Statistical analysis was performed by the Mann-Whitney-Wilcoxon test to determine the *P* value.

Results

Expression of RAGE in Vessels from Normal Individuals and Patients with Occlusive Vascular Disease

Endothelium from vasa vasorum of normal vessels demonstrated variable, low intensity staining for RAGE. Endothelial cells of vasa vasorum from a normal artery demonstrated no or little immunostaining for RAGE antigen (Figures 1A and 2A). Although staining for RAGE was not intense, it was the result of specific binding of the antibody to the antigen, as it

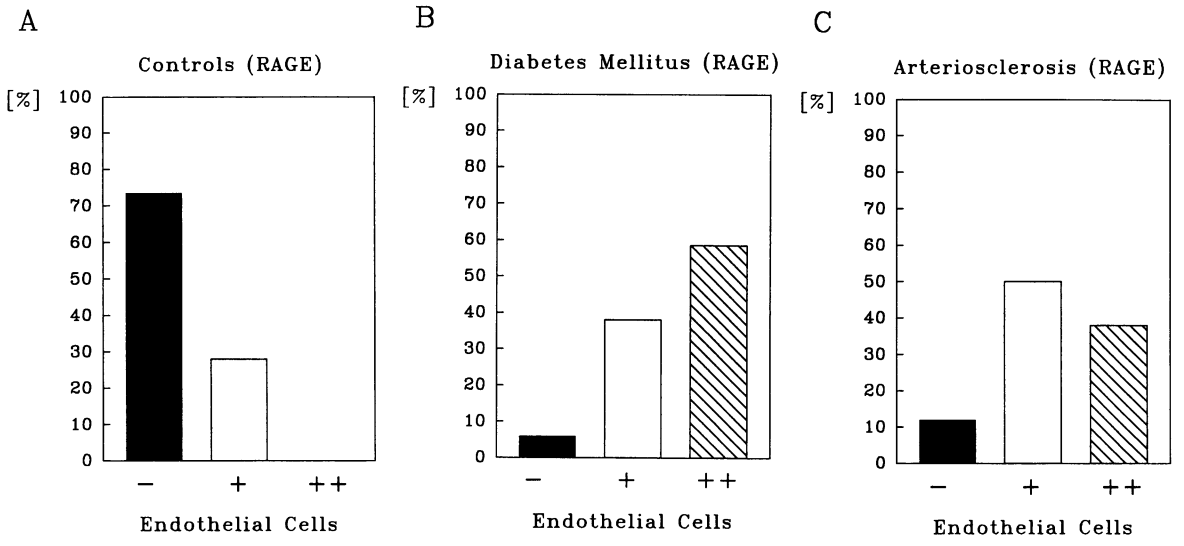

Figure 2. *RAGE expression in vasa vasorum of normal vessels* (**A**, n = *11*), *vessels from patients with diabetes* (**B**, n = *19*), *and vessels from patients with vascular occlusive disease in the absence of diabetes* (**C**, n = *26*). *The intensity of staining (immunohistochemistry) was graded from − to ++ and expressed as percentage of patients investigated. Three to ten sections from each vessel were stained and graded as described in Materials and Methods.*

was not observed with nonimmune IgG (not shown) and preadsorbation of the antibody with purified bovine RAGE abrogated the staining (data not shown, see Materials and Methods). *In situ* hybridization indicated that RAGE mRNA was present, although probably at low levels (Figure 1B). Controls with the sense probe showed no staining (Figure 1C). Levels of RAGE antigen in the endothelium appeared to be greater in cerebral microvessels (Figure 1D) and was correlated with higher levels of RAGE mRNA (Figure 1E). On the basis of examining the endothelium of a range of apparently normal human vessels, RAGE antigen and mRNA appear to be present at low levels that vary throughout the vasculature. These data are consistent with the results of our RAGE survey in normal bovine tissues.[19]

When vessels from patients with diabetes and occlusive arterial disease of other etiologies were examined, virtually all vasa vasorum demonstrated prominent immunostaining for RAGE antigen (Figures 2B, C and 3A), and RAGE mRNA was also present (Figure 3B). The difference in RAGE expression between control and diabetic vessels was evaluated on the basis of the intensity of staining and graded from 0 to 2+. With these criteria, immunostainable RAGE was significantly greater in diabetic compared with normal endothelial cells of the vasa vasorum ($P < 0.0001$; $P < 0.05$ is significant).

When this analysis was extended to vasa vasorum of vessels from patients with hyperlipidemia and patients without an identifiable risk factor for vascular disease, in each case a statistically significant difference in RAGE expression compared with controls was evident ($P < 0.0003$). No difference was found when vessels of patients with arteriosclerosis without diabetes were compared with patients with arteriosclerosis and diabetes ($P < 0.1886$). The intensity of endothelial cell RAGE immunostaining (Figure 2) and the level of mRNA (on the basis of *in situ* hybridization) was similar in patients with occlusive disease regardless of the underlying risk factor for development of the vascular lesions.

In contrast to the apparent induction of RAGE antigen and mRNA in the endothelium in a spectrum of vascular disorders, levels of RAGE appeared unchanged in vascular smooth muscle cells and neurons, two cell types that constitutively express considerable amounts of RAGE according to the bovine survey.[19]

Vascular smooth muscle cells from normal vessels consistently expressed RAGE antigen and mRNA (data not shown). Comparison of the latter results with RAGE expression in vessels from patients with occlusive arterial disease showed no significant difference ($P > 0.05$). Similarly, nerves of all vessels studied expressed RAGE antigen and mRNA (data not shown), and no difference in neuronal RAGE levels was observed between normal and occlusive vascular disease samples ($P > 0.05$).

Discussion

RAGE is a recently described member of the Ig superfamily of cell surface molecules.[21] Its expression

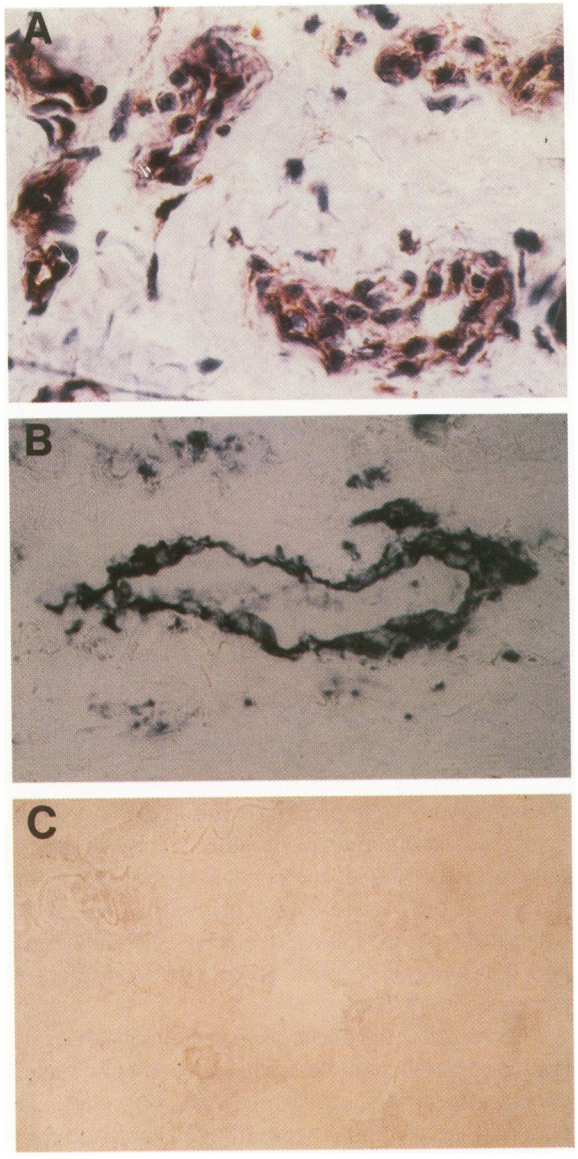

Figure 3. *RAGE expression of vasa vasorum from an arteriosclerotic vessel of a patient with vascular occlusive disease and diabetes mellitus.* **A:** *Immunohistochemistry; magnification, ×100.* **B:** In situ *hybridization (antisense probe); magnification, ×160.* **C:** In situ *hybridization (sense probe); magnification, ×160.*

was first studied in normal bovine tissues, and these results have been extended, in more limited studies, to human and rodent vasculature.[18,19,24] This is the first report of RAGE expression in tissues from individuals with vascular disease. Several pieces of evidence from our study lend support to the hypothesis that RAGE might have diverse functions: the synthesis of RAGE by vascular smooth muscle cells and nerves in very young (1 year), apparently healthy individuals and the lack of a major change in RAGE expression in these cells even at a very advanced age (92 years).

These data suggest that it is unlikely that AGEs are the major determinant of RAGE expression in neurons and smooth muscle, as AGEs would be virtually undetectable in young, normal individuals and have been shown to steadily increase during normal aging.[1,8] This suggests the possibility that non-AGE ligands might be present in the tissue where they would interact with RAGE, potentially regulating cellular activities modulated by other members of the Ig superfamily, such as cell-cell interactions, growth, or cytokine interactions.[11–16] In this context we have recently observed that RAGE promotes neurite extension in neonatal rat cortical neurons, suggesting a role quite distinct from that of a classical scavenger receptor (unpublished observation). However, even the acetylated low density lipoprotein receptor has been shown to mediate adherence of mononuclear phagocytes to diverse surfaces.[25]

Quiescent endothelial cells appear to express variable, but relatively low amounts of RAGE. However, in normal mice, the rapid clearance of AGE albumin infused into the intravascular space was blocked by pretreatment of animals with anti-RAGE IgG, suggesting that RAGE expressed by the endothelium mediates vessel wall uptake of blood-borne AGEs.[18] Consistent with this hypothesis, the induction of cellular oxidant stress by plasma AGEs was blocked by anti-RAGE IgG.[22,25] The prominent up-regulation of RAGE expression in vascular disease is consistent with a potentially important role of RAGE in pathogenetic or repair mechanisms. The observation that levels of RAGE increase not only in diabetes, but also in other types of vascular occlusive disease, also suggests that AGEs are not the principal determinant of RAGE levels. In this context, we have previously observed that cytokines, such as tumor necrosis factor, enhance RAGE expression in cultured endothelial cells (unpublished observation). Such cytokine regulation of RAGE expression is supported by previous work in which tumor necrosis factor increased the binding of AGE albumin to mononuclear phagocytes.[11]

The data indicate that RAGE expression occurs in a range of vascular diseases, and one might conclude that hyperlipidemia is an important factor in RAGE expression. This study was intended as a guide for future work to understand relevant stimuli modulating RAGE expression, and thus we prefer not to draw strong mechanistic conclusions until we have performed studies *in vivo* and *in vitro* using experimental animals to verify such hypotheses (the latter experiments have not been performed at this time). The results indicate the importance of testing the effects of certain cytokines and lipoproteins and modified lipoproteins on RAGE expression. However, it is

possible that there may be common denominators underlying some of these stimuli. For example, accumulations of proteins or lipids that have a delayed turnover (as might be expected to occur in lipid-rich vascular lesions) could be subject to nonenzymatic glycation and formation of AGEs even in euglycemia. For example, it has been found that AGE-modified forms of amyloid-β peptide and paired helical filament tau are present in Alzheimer's disease (in nondiabetic individuals) and an AGE-modified form of β-2-microglobulin is the major component of the amyloid associated with hemodialysis.[26–28] Thus, our current results should stimulate future studies as to the possibility that AGEs, modified lipoproteins (subject to nonenzymatic glycation or other modifications), cytokines, or other factors are pathophysiologically relevant modulators of RAGE expression.

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