Receptor for AGE (RAGE) Mediates Neointimal Formation in Response to Arterial Injury

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Background—Receptor for advanced-glycation end products (RAGE) and its ligands AGEs and S100/calgranulins have been implicated in a range of disorders. However, the role of RAGE/ligand interaction in neointimal hyperplasia after vascular injury remains unclear.

Methods and Results—We examined the expression of RAGE and its ligands after balloon injury of the carotid artery in both Zucker diabetic and nondiabetic rats. Using a soluble portion of the extracellular domain of RAGE, we determined the effects of suppressing RAGE/ligand interaction on vascular smooth muscle cell (VSMC) proliferation and neointimal formation after arterial injury. We demonstrate a significantly increased accumulation of AGE and immunoreactivities of RAGE and S100/calgranulins in response to balloon injury in diabetic compared with nondiabetic rats. Blockade of RAGE/ligand interaction significantly decreased S100-stimulated VSMC proliferation in vitro and brumodeoxyuridine (BrdU)–labeled proliferating VSMC in vivo, and suppressed neointimal formation and increased luminal area in both Zucker diabetic and nondiabetic rats.

Conclusions—These findings indicate that RAGE/ligand interaction plays a key role in neointimal formation after vascular injury irrespective of diabetes status and suggest a novel target to minimize neointimal hyperplasia. (Circulation. 2003; 107:2238-2243.)

Key Words: diabetes mellitus ■ angioplasty ■ restenosis ■ receptors

Restenosis remains the major limitation for the long-term success after percutaneous coronary intervention (PCI), and the restenosis rate is even higher in diabetes.1-4 Studies in affected patients as well as animal models have suggested that the inflammatory and proliferative responses are enhanced in diabetes and may explain the observed increased restenosis rate.5-8 Pathological analyses indicate that neointimal formation is characterized by an inflammatory reaction at the site of injury, migration and proliferation of vascular smooth muscle cells (VSMCs), and the synthesis of excess matrix. Despite the well-defined nature of the lesion, the pathogenic mechanisms leading to exaggerated restenosis remain largely undefined.

One consequence of long-term hyperglycemia is the formation of advanced-glycation end products (AGEs); the accumulation of AGEs in the vessel wall has been implicated in the pathogenesis of diabetic complications.9,10 In diabetic tissues, expression of the receptor for AGEs (RAGE) is enhanced in a manner overlapping with that of its ligands. An important role for RAGE in excessive cellular activation, and enhanced proinflammatory pathways in diabetic tissues was evident by the findings that blockade of RAGE suppressed vascular hyperpermeability in diabetic rats and atherosclerotic lesion development in diabetic apoE-null mice.11,12 In parallel with those observations, indices of endothelial cell activation and macrophage migration/function were suppressed in the presence of RAGE blockade.

Recent observation that AGEs and RAGE together with some inflammatory markers form in many states, such as atherosclerotic lesions, renal failure, and aging,13,14 have suggested that the biology of RAGE and its ligands extends beyond diabetes. RAGE also binds S100/calgranulins, members of a family of proinflammatory cytokines.15 S100/calgranulins binding may lead to ligation of RAGE-bearing cells in the injured/inflamed milieu, thereby providing a mechanism for sustaining cellular perturbation and tissue injury.

We hypothesized that accumulation of AGEs and enhanced expression of RAGE may contribute to the exaggerated neointimal formation after arterial injury. We examined the expression of RAGE and its ligands in the injured vessel wall. In addition, we evaluated RAGE blockade on VSMC prolif-
eration, and neointimal formation in both Zucker diabetic and nondiabetic rats. In the present study, we provide evidence that activation of RAGE is important in the formation and progression of neointimal hyperplasia after arterial injury.

Methods

Cell Culture
Diabetic rat aortic VSMC primary cultures were seeded into 12-well plates (1.2×10^4 cells/well) in DMEM medium with 10% fetal bovine serum for two nights, and the culture medium was replaced by DMEM without fetal bovine serum for 24 hours. The VSMCs were incubated with 2 μmol/L S100 protein (Calbiotech), or 3 U/mL (US Biochemical), and simultaneously by incremental concentration of sRAGE (0, 5, 10, 20, and 40 μg/mL) for 24 hours. Cells were harvested, and cell numbers were counted using Coulter cell counter.

Surgical Procedures
The Zucker obese rats (weight, average 400 g; Charles River Laboratories, Wilmington, Mass) aged 9 to 12 weeks were used as a model of type II diabetes. Zucker lean rats, aged 11 to 14 weeks, as euglycemic controls. All experiments were approved by the Animal Research Committee at the Cleveland Clinic Foundation and conformed to the guidelines of all state, federal, and National Institutes of Health regulation.

Carotid artery injury was induced by balloon deendothelialization. After induction of anesthesia, a midline cervical incision was made to expose the left external carotid artery. The external carotid artery ligated, and the internal carotid artery was temporarily ligated. A 2F Fogarty balloon catheter (Baxter Healthcare Corp) was introduced. The catheter was passed into the aortic arch, and the balloon was distended with saline until a slight resistance was felt on slight traction. After withdrawal into the common carotid artery, the balloon was rotated while pulling back through the common carotid artery. This procedure was repeated 3 times.

At the time of euthanasia, a midline abdominal incision was performed and the distal abdominal aorta exposed. Using an 18-gauge IV catheter introduced at the aortic bifurcation, the aorta was flushed with 50 cc of ringer’s lactate solution at 120 mm Hg followed by in vivo fixation with 200 cc of 5% Histochoice (Amresco) infused over 5 minutes at 120 mm Hg. After 5 minutes of perfusion-fixation, the entire left carotid artery was harvested. Specimens were stored in Histochoice (5%) for at least 24 hours before embedding.

Administration of Soluble RAGE
Four sequential experiments were performed. The first phase examined expression of RAGE, AGEs, and S100/calgranulins after vascular injury. Twenty-four Zucker obese diabetic rats and six Zucker lean nondiabetic rats were subjected to balloon injury and euthanized at 0, 1, 3, 5, 7, 14, and 21 days (2 to 5 animals in each time point). No treatment was administered. The second phase examined the effect of sRAGE treatment on SMC proliferation in vivo: carotid injuries were induced in 11 Zucker obese diabetic rats; murine soluble RAGE or vehicle, murine serum albumin (0.5 mg/day), was administered by intraperitoneal injection beginning one day before and continued until day 3 after injury; and rats received intraperitoneal injection of bromodeoxyuridine (BrdU) 50 mg/kg (Sigma Chemical Co) at 18, 12, and 2 hours before euthanasia. The third phase investigated SAGE on neointimal formation in 15 Zucker diabetic rats: carotid injuries were induced; murine soluble RAGE or vehicle, murine serum albumin (0.5 mg/day), was administered intraperitoneally beginning one day before and continued until day 6 after injury; and rats were euthanized on day 21. The last phase examined sRAGE on neointimal formation in Zucker lean nondiabetic rats: 9 Zucker lean rats were subjected to carotid balloon injury; same treatment was given until day 6; and rats were euthanized at 21 days.

Preparations of Soluble RAGE
Murine sRAGE, which cross-reacts antigenically and functionally with rat sRAGE, was prepared, purified, and devoid of detectable levels of endotoxin as previously described.12

Immunohistochemistry
Immunohistochemistry was performed using polyclonal monospecific antibodies to RAGE, S100/calgranulins, and AGEs (the latter were affinity-purified according to previously described procedures).11,12 Peroxidase-conjugated goat anti-rabbit IgG (Sigma) was used to visualize the sites of primary antibody binding to the antigen. Expression of RAGE, AGEs, and S100/calgranulins was graded according to the following scale by two investigators blinded to the experimental conditions: negative (−), mild staining (+), moderate staining (++), and intense staining (+++).

Assessment of VSMC proliferation was performed using mouse anti-BrdU monoclonal antibody (Dako Co). On application of biotinylated anti mouse IgG secondary antibody, sections were counterstained with hematoxylin. The numbers of BrdU-positive nuclei per section were counted by two observers blinded to the treatment regimen, and the labeling index (positive nuclei/total nuclei) was calculated.

Histological Analysis and Morphometry
The fixed carotid arteries were embedded in paraffin and were obtained in serial sections, 5 mm apart, from the proximal to distal end. Slides were stained with hematoxylin-eosin (H-E) and Van Gieson. Morphometric analyses of the arterial segments were performed by an observer blinded to the study groups, using computerized digital microscopic planimetry software (Image-Pro Plus, Version 4.0 for Windows, Media Cybernetics). The section with the most severe degree of luminal narrowing from 4 to 5 sections of each injured arterial segment was assessed as the “lesion” point. The neointimal and medial boundaries were determined, the cross-section areas subtended by the luminal border, the internal elastic lamina (IEL), and the external elastic lamina (EEL) were measured, and the ratio of intimal and medial area (I/M) was calculated.

Blood Chemistry Assay
Blood samples were collected at 21 days. Serum glucose levels were measured by enzymatic method; cholesterol, HDL cholesterol, and LDL cholesterol were determined by the cholesterol oxidase enzyme assay, triglycerides by the glycerol triphosphate oxidase enzyme assay.

Statistical Analysis
All data were expressed as mean±SD. Statistical analysis was performed with use of SPSS software (Version 7.0 for Windows, SPSS Inc). Continuous variables were compared using unpaired t tests. A value of P≤0.05 was considered to be statistically significant.

Results

Immunoreactivities of RAGE and its Ligands in Response to Balloon Injury
To determine the extent of RAGE/ligand expression after carotid artery injury, Zucker obese diabetic and Zucker lean euglycemic rats were subjected to balloon injury, and euthanized at different time points. Results of expressions of AGEs, RAGE, and S100/calgranulins are shown in Table 1 and Figure 1. Before balloon injury, increased accumulation of AGEs and immunoreactivities of RAGE and S100/calgranulins were evident in the media of carotid arteries retrieved from Zucker obese diabetic rats compared with Zucker lean nondiabetic controls. Subsequent to carotid injury, immunoreactivities of RAGE and S100/calgranulins
and accumulation of AGEs were increased in the neointima and media of injured arteries from both diabetic and euglycemic rats, although to a greater extent in the diabetic rats. At day 21 after balloon injury, immunoreactivities of RAGE, AGE, and S100/calgranulins remained elevated in diabetic rats.

**Effects of sRAGE on VSMC Growth In Vitro**
Quiescent diabetic VSMCs were stimulated by S100 without and with the simultaneous addition of sRAGE. S100 increased VSMC proliferation as measured by cell number at 24 hours in a dose-dependent manner (data not shown). Using a suboptimal dose of S100, we found that sRAGE significantly inhibited VSMC growth in a dose-dependent manner. However, no inhibitory effect of sRAGE was observed in thrombin (3 U/mL)–stimulated VSMC growth (Figure 2).

**Effect of sRAGE on VSMC Proliferation In Vivo**
The number of BrdU-labeled proliferating cells in the media was greatly diminished by sRAGE versus vehicle (30.20±14.01 versus 68.83±19.45 cells per cross section; \(P=0.005\)), as was the BrdU labeling index (8.56±3.38% versus 17.82±4.24%; \(P=0.003\)) (Figure 3). Blockade of RAGE significantly suppressed the number of proliferating VSMCs in the arterial segments compared with vehicle treatment.

**Effect of sRAGE on Neointimal Formation**
Consistent with the hypothesis that RAGE/ligand interaction has a significant role in the proliferative responses after injury, neointimal area in sRAGE-treated diabetic rats was reduced significantly 21 days after balloon injury compared with that observed in vehicle-treated animals (0.08±0.03 versus 0.15±0.04 mm²; \(P=0.002\)). A reduction in the intima/ media ratio was also observed in sRAGE versus control group (0.83±0.35 versus 1.49±0.26; \(P=0.001\)). In concert with a decrease in neointimal hyperplasia, we found greater luminal area in the sRAGE-treated animals (0.24±0.06 versus 0.17±0.02 mm²; \(P=0.04\)). No differences were observed in the medial area between the two groups (0.10±0.01 versus 0.10±0.01 mm²; \(P>0.05\) (Table 2, Figure 4).

To further evaluate if the beneficial effect of sRAGE is diabetes specific, we examined the effect of sRAGE on neointimal formation in Zucker lean nondiabetic rats. Neointimal area was significantly reduced (0.10±0.03 versus 0.16±0.01 mm²; \(P=0.004\)), and the luminal area was markedly increased (0.24±0.04 versus 0.17±0.03 mm²; \(P=0.02\)) in sRAGE-treated group compared with vehicle-treated group (Table 2, Figure 4).

**Figure 1.** Immunohistochemistry for RAGE (A, B, C, D, and E), AGE (F, G, H, I, and J), and S100/calgranulins (K, L, M, N, and O) in the carotid arteries of diabetic and nondiabetic rats (40×). There is increased AGE and RAGE immunoreactivity in parallel with increased S100/calgranulins accumulation in Zucker obese diabetic rats after balloon injury compared with Zucker lean rats. A, F, and K, Negative control. B, G, and L, Uninjured from Zucker lean nondiabetic rats; C, H, and M, Uninjured from Zucker obese diabetic rats. D, I, and N, Uninjured from Zucker lean nondiabetic rats. E, J, and O, Uninjured from Zucker obese diabetic rats. Arrows indicate internal elastic lamina.

**Figure 2.** Effect of sRAGE on S100- and thrombin-stimulated rat VSMC proliferation. Approximately 12,000 cells were plated per well. Cells were rendered quiescent in serum-free media for 24 hours, and then stimulated with S100 (2 μmol/L) or thrombin (3 U/mL) in the presence of sRAGE (0 to 40 μg/mL). Cell number was determined 24 hours later. Data represent the percent increase in the number of cells in treated wells relative to unstimulated control wells. Data represent mean±SD, representative of 5 experiments.
Effect of sRAGE on Serum Glucose and Lipid Profiles in Zucker Diabetic Rats

The serum glucose, total cholesterol, HDL cholesterol, LDL cholesterol, and triglyceride levels were 257 ± 72, 105 ± 20, 34 ± 7, 7 ± 3, and 459 ± 130 mg/dL in sRAGE-treated group, and 253 ± 37, 118 ± 28, 41 ± 13, 7 ± 2, and 440 ± 185 mg/dL in vehicle-treated group (P = NS for all categories). There was also no difference in blood glucose levels in the sRAGE-treated animals compared with controls. sRAGE has previously been shown to have no effect on plasma insulin levels.11

Discussion

One of the most formidable challenges in cardiovascular medicine is reducing the toll of complications in patients after PCI, especially for those with diabetes mellitus, who are particularly prone to restenosis. Our studies have demonstrated that basal immunoreactivities of AGEs, RAGE, and S100/calgranulins were increased in the arterial wall of Zucker diabetic rats versus euglycemic controls. Subsequent to balloon injury, expression of these molecules is further increased, particularly in the diabetic animals. Blockade of RAGE/ligand interaction suppressed VSMC proliferation and neointimal formation after balloon injury.

In the setting of hyperglycemia in diabetes, long-term exposure of free amino groups on polypeptides or lipids to higher levels of glucose eventuates in the formation of advanced-glycation end products.16 AGEs are increased at sites of atherosclerotic lesions, especially in diabetes.17,18 Increased expression of AGEs have also been found in settings like renal failure and amyloidosis, indicating the biology of AGEs extends beyond diabetes. The cellular effects of AGEs are largely mediated by their specific engagement of cell surface receptor RAGE. Studies have demonstrated RAGE expression at a very low levels in a range of cells, including endothelial and smooth muscle cells and mononuclear cells, RAGE expression increases and receptor upregulation is sustained when particular pathological processes intervene.19,20 We found that balloon injury caused the upregulation of RAGE, as well as an increase in

| Table 2. Morphometric Analyses of the Effect of sRAGE |
|-------------------------|-------------------------|-------------------------|
|                         | Zucker Obese Diabetic Rats | Zucker Lean Nondiabetic Rats |
|                         | Placebo (n=8) | sRAGE (n=7) | Placebo (n=5) | sRAGE (n=4) |
| Intimal area, mm²       | 0.15±0.04 | 0.08±0.03† | 0.16±0.01 | 0.10±0.03† |
| Medial area, mm²        | 0.10±0.01 | 0.10±0.01 | 0.10±0.01 | 0.09±0.01 |
| I/M                     | 1.49±0.26 | 0.83±0.35† | 1.51±0.13 | 1.08±0.29* |
| Luminal area, mm²       | 0.17±0.02 | 0.22±0.06* | 0.17±0.03 | 0.24±0.04† |
| IEL area, mm²           | 0.32±0.04 | 0.30±0.03 | 0.33±0.03 | 0.34±0.05 |
| EEL area, mm²           | 0.42±0.05 | 0.40±0.03 | 0.43±0.04 | 0.43±0.06 |

IEL indicates internal elastic laminal; EEL, external elastic lamina.

*P<0.05 vs placebo.
†P<0.01 vs placebo.
the accumulation/expression of its ligands, AGES, and S100/calgranulins.

It is important to note that in the rats subjected to blockade of RAGE within the first 8 days after balloon injury, neointimal hyperplasia was suppressed by over 50%, and the luminal area was significantly increased in both diabetic and nondiabetic rats. Mechanistically, this finding was coupled with inhibition of SMC proliferation in vitro and in vivo.

Our results may suggest an important link between the upregulation of RAGE/ligands and accelerated vascular inflammatory and proliferative responses in Zucker diabetic and nondiabetic rats. AGE-rich injured arteries are populated by high levels of RAGE and are subject to sustained RAGE/ligand interaction, which may result in chronic cellular activation. S100/calgranulins may ligate RAGE-bearing cells in the injured/inflamed environment, thereby providing a mechanism for sustaining cellular perturbation leading to tissue injury, resulting in increased restenosis.

An increasing body of literature has begun to elucidate the potential molecular mechanisms underlying the beneficial effects of inhibition of RAGE/ligand interaction. AGES has been shown to induce significant dose-dependent SMC migration, and RAGE/ligand interaction upregulates the production of various cytokines and growth factors such as TNF-α and PDGF. SMC migration by AGE was significantly inhibited by an antibody against transforming growth factor-β (TGF-β), and TGF-β secreted into the culture medium from AGE-stimulated VSMCs was 7-fold higher than that of control, suggesting a potential role for RAGE/ligand interaction in TGF-β release after vascular injury. In addition, binding of RAGE to its ligand leads to activation of key cell signaling pathways, such as p44/p42 (erk1/erk2), p21, MAP kinases, NF-κB, cdc42/rac, and JAK/Stat, thereby reprogramming cellular properties. Balloon injury activates the MAPK pathway in diabetics, and hyperinsulinemia activation of the MAPK pathway has been shown to be of importance in the exaggerated neointimal hyperplasia after balloon injury in diabetic animals. Blockade of RAGE/ligand interaction decreases MAPK activity in cultured SMCs in a concentration-dependent manner. Inhibition of RAGE/ligand interaction decreased tumor cell proliferation and invasion by suppressing the activation of p44/p42, p38, and SAP/JNK MAP Kinases pathways. Thus, it is possible that in the setting of arterial injury, activation of these, or additional pathways, via RAGE may contribute to enhanced SMC proliferation and migration.

**Limitations**

Our experiments were limited to a rodent model in which molecular pathways leading to exaggerated restenosis parallel, only in part, those observed in human vascular injury. Studies need to be done to determine if successful blockade of restenosis using sRAGE or other RAGE antagonists may be applicable in porcine, nonhuman primate, or human systems. Nevertheless, the present findings support the concept that RAGE contributes importantly to increased restenosis in both diabetic and nondiabetic rats by impacting on key properties of VSMC.

**Conclusion and Clinical Implications**

Our data suggest a central role for RAGE/ligand interaction as a key factor promoting neointimal hyperplasia after balloon injury in both diabetic and nondiabetic rodents. Blockade of RAGE limited to the first 8 days after balloon injury was sufficient to reduce neointimal formation by over 50%. These observations demonstrate a potentially novel target for limiting the development and progression of neointimal hyperplasia after balloon injury.

**References**


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