**OBJECTIVE**—Activation of the receptor for advanced glycation end products (RAGE) in diabetic vasculature is considered to be a key mediator of atherogenesis. This study examines the effects of deletion of RAGE on the development of atherosclerosis in the diabetic apoE\(^{-/-}\) model of accelerated atherosclerosis.

**RESEARCH DESIGN AND METHODS**—ApoE\(^{-/-}\) and RAGE\(^{-/-}/\)apoE\(^{-/-}\) double knockout mice were rendered diabetic with streptozotocin and followed for 20 weeks, at which time plaque accumulation was assessed by en face analysis.

**RESULTS**—Although diabetic apoE\(^{-/-}\) mice showed increased plaque accumulation (14.9 ± 1.7%) and diabetic RAGE\(^{-/-}/\)apoE\(^{-/-}\) mice had significantly reduced atherosclerotic plaque area (4.9 ± 0.4%) to levels not significantly different from control apoE\(^{-/-}\) mice (4.3 ± 0.4%). These beneficial effects on the vasculature were associated with attenuation of leukocyte recruitment; decreased expression of proinflammatory mediators, including the nuclear factor-κB subunit p65, VCAM-1; and MCP-1; and reduced oxidative stress, as reflected by staining for nitrotyrosine and reduced expression of various NADPH oxidase subunits, gp91phox, p47phox, and rac-1. Both RAGE and RAGE ligands, including S100A8/A9, high mobility group box 1 (HMG1), and the advanced glycation end product (AGE) carboxymethyllysine were increased in plaques from diabetic apoE\(^{-/-}\) mice. Furthermore, the accumulation of AGES and other ligands to RAGE was reduced in diabetic RAGE\(^{-/-}/\)apoE\(^{-/-}\) mice.

**CONCLUSIONS**—This study provides evidence for RAGE playing a central role in the development of accelerated atherosclerosis associated with diabetes. These findings emphasize the potential utility of strategies targeting RAGE activation in the prevention and treatment of diabetic macrovascular complications.

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**Receptor for Advanced Glycation End Products (RAGE) Deficiency Attenuates the Development of Atherosclerosis in Diabetes**

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**ORIGINAL ARTICLE**
ROLE OF RAGE IN ATHEROSCLEROSIS

TABLE 1

| Parameters | Control apoE−/− | Diabetic apoE−/− | Control RAGE+/−/apoE−/− | Diabetic RAGE+/−/apoE−/− |
|------------|----------------|-----------------|-------------------------|------------------------|
| n          | 10             | 10              | 10                      | 10                     |
| GHb (%)    | 3.8 ± 0.1      | 15.8 ± 0.7†     | 4.5 ± 0.2‡              | 15.0 ± 0.4§            |
| Body weight (g) | 30.7 ± 0.4 | 24.5 ± 0.6†    | 38.4 ± 1.1‡             | 27.5 ± 0.5§            |
| Plasma glucose (mmol/L) | 11.3 ± 0.9 | 31.2 ± 1.2†    | 9.6 ± 1.2‡              | 31.0 ± 1.9             |
| Total cholesterol (mmol/L) | 14.0 ± 1.1 | 25.6 ± 2.1†    | 12.7 ± 0.6§             | 21.1 ± 1.2§            |
| HDL cholesterol (mmol/L) | 2.6 ± 0.2 | 3.1 ± 0.4      | 3.1 ± 0.2               | 4.0 ± 0.3§             |
| LDL cholesterol (mmol/L) | 10.0 ± 1.0 | 21.6 ± 1.9†    | 8.8 ± 0.4‡              | 16.5 ± 0.8§            |
| Triglycerides (mmol/L) | 1.8 ± 0.3 | 2.1 ± 0.3      | 1.9 ± 0.4               | 1.4 ± 0.2              |

Data are means ± SE. *GHb and weight measurements performed in 24 animals/group. †P < 0.001 vs. control apoE−/− group; ‡P < 0.05 vs. diabetic apoE−/− group; §P < 0.05 vs. diabetic apoE−/− group; †P < 0.001 vs. control apoE−/− group; ‡P < 0.05 vs. diabetic apoE−/− group.

The induction of diabetes in both RAGE+/−/apoE−/− and apoE−/− mice resulted in increased plasma glucose and GHb concentrations, which were comparable between the diabetic groups (Table 1). Plasma concentrations of total and LDL cholesterol levels were also significantly increased in both groups following the induction of diabetes, although this increase was attenuated in RAGE+/−/apoE−/− mice.

RESULTS

Metabolic parameters. The induction of diabetes in both RAGE+/−/apoE−/− and apoE−/− mice led to a significant (approximately fourfold) increase in total atherosclerotic plaque area. However, this diabetes-associated increase in plaque area was completely prevented in diabetic RAGE+/−/apoE−/− mice, with plaque accumulation not significantly different from that seen in control apoE−/− mice (Fig. 1A). The reduction in the plaque accumulation in diabetic RAGE+/−/apoE−/− mice was observed at all three aortic sites: the arch, thoracic, and abdominal aortas. In addition, there was a further reduction in plaque area in nondiabetic RAGE+/−/apoE−/− mice when compared with control apoE−/− mice (Fig. 1A and B).

Plaque morphology and smooth muscle cell recruitment. The induction of diabetes was not associated with plaque accumulation, but, in addition, diabetic plaque was also significantly more complex, characterized by
increased accumulation of foam cells, cholesterol clefts, and leukocyte recruitment similar to that seen in human disease. Furthermore, atherosclerotic plaque in diabetic apoE<sup>−/−</sup> mice involves the proliferation and migration of medial vascular smooth muscle cells into the vessel intima, as demonstrated by increased expression of α-SMA protein when compared with control apoE<sup>−/−</sup> mice (Supplementary Fig. 2). However, the atherosclerotic plaque in diabetic RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice was significantly less complex, with reduced α-SMA expression when compared with diabetic apoE<sup>−/−</sup> mice.

**Vascular accumulation of macrophages and T-cells.** Leukocyte recruitment is a key component of vascular inflammation and atherogenesis. In our study, there was increased CD3 staining in the vessel wall of the diabetic apoE<sup>−/−</sup> mice, consistent with accumulation of T-cells (Fig. 2A and B). T-cell recruitment associated with diabetes was significantly decreased in the RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice to levels not significantly different from control apoE<sup>−/−</sup> mice, with control RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice showing an additional reduction in vascular T-cell recruitment. Similarly, macrophage accumulation, as detected by staining with the F4/80 marker, was significantly increased in diabetic apoE<sup>−/−</sup> mice compared with controls (Fig. 2C and D). Macrophage accumulation was also reduced in the diabetic RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice when compared with diabetic apoE<sup>−/−</sup> mice. Macrophage staining was detected in both the vascular wall and the fibrous cap of the atherosclerotic lesions, particularly in diabetic apoE<sup>−/−</sup> mice.

**Markers of vascular inflammation.** To explore the potential mechanisms responsible for vascular protection in our model, we studied the aortic gene transcription of various proteins that have previously been linked to generation of vascular lesions (9,18,19). In particular, vascular inflammation is thought to be the key initiator of atherosclerosis in both experimental and human disease. Accelerated atherosclerosis in diabetic apoE<sup>−/−</sup> mice was associated with increased aortic expression of the proinflammatory transcription factor NF-κB subunit p65, MCP-1, VCAM-1, tissue factor, and the renin-angiotensin system (RAS)-related C3 botulinum substrate 1 (rac1) (Table 2). Reduction in plaque area in RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice was also associated with a significant reduction in gene expression of these proatherosclerotic mediators to levels similar to those observed in control apoE<sup>−/−</sup> aortas (Table 2). By immunohistochemistry, MCP-1 expression was increased at least twofold in diabetic apoE<sup>−/−</sup> mice particularly within the plaque, specifically foam cells and macrophages, and this increase was not seen in the aortas from diabetic RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice (Supplementary Fig. 3A and B). In addition, Western blot analysis showed that MCP-1 protein expression was increased twofold in aortic extracts from diabetic apoE<sup>−/−</sup> mice and this increased expression was significantly reduced in the aortas from diabetic RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice (Supplementary Fig. 3C). Aortic VCAM-1 protein expression was also assessed by immunohistochemistry. Diabetic apoE<sup>−/−</sup> mice had significantly increased vascular VCAM-1 staining, including within endothelial cells, and this was not seen in control apoE<sup>−/−</sup> mice or diabetic and control RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice (Supplementary Fig. 4).

**Expression of collagens and matrix metalloproteinases.** Gene expression of collagen I, III, IV (α I), and IVα III were increased in the diabetic apoE<sup>−/−</sup> aorta compared with the control apoE<sup>−/−</sup> aorta (Table 2). Collagen III gene expression was significantly decreased in the diabetic RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> aorta when compared with diabetic apoE<sup>−/−</sup> mice. We also assessed collagen protein expression in the vascular wall by immunohistochemistry, specifically assessing the fibrillar type III collagen and the basement membrane type IV collagen. Diabetic apoE<sup>−/−</sup> mice have significantly increased vascular collagen III and collagen IV deposition compared with control apoE<sup>−/−</sup> mice (Supplementary Figs. 5 and 6). The diabetic RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice had significantly less collagen III and IV staining.

We also examined the gene expression of matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9, respectively) that are involved in the inflammatory response and in
collagen degradation (20). There was a threefold induction in the expression of MMP-2 and a twofold induction in the expression of MMP-9 in the diabetic apoE−/− aorta compared with the control apoE−/− aorta. MMP-9 gene expression was significantly attenuated in diabetic RAGE−/− apoE−/− aorta, whereas MMP-2 gene expression was similar to that seen in diabetic apoE−/− aorta.

Accumulation of RAGE ligands. The accumulation of a range of RAGE ligands, including the advanced glycation end product (AGE), CML, HMGB1, and S100A8/A9, in settings such as diabetes are considered to play a role in progressive vascular injury. As expected, diabetic apoE−/− mice had a significant elevation in vascular AGE deposition when compared with the nondiabetic apoE−/− animals, as detected by immunostaining for the well-described AGE, CML (Fig. 3). CML immunostaining was reduced in the aortas of diabetic RAGE−/− apoE−/− mice to levels not significantly different from control animals. A similar pattern was seen with respect to another RAGE ligand, HMGB1 (1), with increased expression in diabetic apoE−/− aorta when compared with the control apoE−/− mice, with normalization in RAGE−/− apoE−/− mice (Fig. 4). We also measured plasma HMGB1 concentrations. Interestingly, plasma HMGB1 levels were higher in diabetic RAGE−/−/apoE−/− mice compared with diabetic apoE−/− mice (369 ± 44 vs. 201 ± 13 ng/ml, P < 0.05). Nondiabetic RAGE−/−/apoE−/− mice tended to have slightly higher circulating HMGB1 levels than nondiabetic apoE−/− mice (356 ± 63 vs. 235 ± 28 ng/ml, P = 0.11).

The diabetic apoE−/− mice had significantly increased positive staining of the proinflammatory cytokine, S100A8/A9, a known RAGE ligand (21) when compared with control animals (Fig. 5A and B), with prominent staining appearing in the aortic lesions, particularly within macrophages, foam cells, and endothelial cells. In the RAGE−/−/apoE−/− mice, the aortic expression of S100A8/A9 protein was significantly decreased when compared with diabetic apoE−/− aortas. The diabetic apoE−/− mice also had a significant elevation in circulating levels of S100A8/A9 when compared with control animals (Fig. 5C). In the
RAGE−/−/apoE−/− mice, S100A8/A9 levels were similar to those seen in control mice.

**Expression of AGE receptors.** As previously described (14,15), the expression of full-length RAGE in apoE−/− mice was significantly increased after the induction of diabetes (Table 2), with focal expression overlying atherosclerotic plaque (data not shown). There was no RAGE expression in RAGE−/−/apoE−/− aorta. The induction of diabetes was also associated with increased expression of AGE clearance receptors, including AGE-R1 (oligosaccharyl transferase-48), AGE-R3 (galectin-3), and the type B macrophage scavenger receptor, CD36. However, in RAGE−/−/apoE−/− mice, the increased gene expression of these other AGE receptors was significantly attenuated (Table 2).

**NADPH oxidase and oxidative stress in the vascular wall.** We determined the potential impact of RAGE on the superoxide-generating NADPH oxidase in the diabetic vasculature. Interestingly, diabetes induced a 5.3-fold increase in expression of NADPH oxidase subunit p47phox and a 3.5-fold increase in gp91phox expression in the apoE−/− aorta, whereas in diabetic RAGE−/−/apoE−/− double knockout mice, this upregulation of NADPH oxidase subunits was attenuated (Table 2). Diabetic apoE−/− mice also had increased expression of nitrotyrosine in their aortas, suggesting increased peroxynitrite-mediated protein oxidation (Fig. 6). Diabetes did not lead to a similar increase in nitrotyrosine staining in the RAGE−/−/apoE−/− mice.

**AT1 receptor expression.** Reflecting an activated vascular RS, which is known to upregulate NADPH-oxidase–dependent reactive oxygen species (ROS) generation (22), diabetic apoE−/− mice had a marked increase in gene expression of the angiotensin II type 1a receptor (AT1a). Aortic AT1a gene expression was significantly reduced in diabetic RAGE−/−/apoE−/− mice when compared with diabetic apoE−/− mice (Table 2).

**DISCUSSION**

Atherosclerotic vascular disease is a major cause of morbidity and mortality in the diabetic population with accelerated plaque accumulation and vascular inflammation. Although a number of mechanisms have been postulated to contribute to the development and progression of atherosclerotic disease in diabetes, in the present study, we provide evidence that RAGE-dependent pathways play a central role in promoting vascular lesions in a rodent model of advanced atherosclerosis, the diabetic apoE−/− mouse. Our results show for the first time that the absence of RAGE suppresses diabetes-accelerated atherosclerosis. Our group has previously shown that the atherosclerotic plaques seen in diabetic apoE−/− mice resemble the complex morphology seen in patients with diabetes, including enhanced accumulation of macrophages, foam cells, and cholesterol clefts within the fatty atheroma and increased expression of inflammatory markers, proinflammatory cytokines, and chemokines (14,15,18). Without normalizing glucose or lipid levels, we show that in the absence of both full-length and sRAGE expression in diabetic RAGE−/−/apoE−/− double knockout mice, the extent of plaque accumulation, vascular inflammation, and oxidative stress was significantly reduced to levels similar to those observed in control apoE−/− mice. In addition, atherosclerosis was further reduced in control RAGE−/−/apoE−/− mice. These data are consistent with previous studies in nondiabetic RAGE−/− mice. RAGE−/− mice demonstrated suppression of neointimal expansion after...
femoral artery denudation (12). Furthermore, a recent study in apoE/RAGE double knockout mice has also demonstrated reduced plaque area, albeit in a nondiabetic context (23).

RAGE is pattern recognition receptor (1) that is able to bind a chemically diverse range of AGEs and other non-AGE ligands, including s100/calgranulins, HMGB1, and β-sheet fibrils. In atherosclerotic vascular disease, the expression of RAGE is focally increased (14,15,21), paralleling an accumulation in RAGE ligands. The major RAGE ligand in the context of diabetes remains to be established. However, the efficacy of AGE-reducing interventions, including alagebrium and aminoguanidine, on diabetes-associated atherosclerosis suggests that AGEs play a pivotal role (14). In this study, we demonstrate increased vascular accumulation of CML along with other RAGE ligands in diabetic apoE<sup>−/−</sup> mice. This increase in vascular CML was markedly attenuated in diabetic RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice, although the hyperglycemia and hyperlipidemia were not corrected. This decrease in vascular AGEs was not due to overexpression of AGE clearance receptors in RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice. In fact, diabetes-associated increases in AGE-R1 (OST-3) and AGE-R3 (galactin-3) were significantly attenuated in diabetic RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice, possibly reflecting reduced AGE accumulation in these animals. Expression of the type B macrophage scavenger receptor CD36 (24) was also attenuated in diabetic RAGE<sup>−/−</sup>/apoE<sup>−/−</sup> mice. Although this change is likely to be secondary to reduced leukocyte recruitment, CD36 has been clearly demonstrated to play a proatherogenic role because the absence of macrophage CD36 protects against vascular lesion formation in mice (25). Thus, one cannot exclude that some of the antiatherosclerotic effects seen with RAGE deletion could be related to changes in the expression of CD36.

Plaque accumulation in the diabetic apoE<sup>−/−</sup> mouse is closely correlated with the generation of ROS, which drive a number of proatherogenic processes, such as monocyte infiltration, smooth muscle cell recruitment, cell adhesion, and increased accumulation of AGEs, which are all observed in the diabetic apoE<sup>−/−</sup> plaque. For example, we
have recently shown that increased oxidative stress in GPx1−/−/apoE−/− mice accelerates diabetes-associated atherosclerosis via upregulation of proinflammatory and profibrotic pathways (26). The role of RAGE as a mediator of oxidative stress in diabetes was also observed in the present study. In our study, diabetes was associated with increases in vascular oxidative stress, as demonstrated by aortic nitrotyrosine staining. This probably occurred partly as a result of increased expression of p47phox and gp91phox, subunits of NADPH oxidase, and rac1, a GTPase involved in NADPH oxidase activation. Expression of the NADPH subunits as well as nitrotyrosine staining were significantly attenuated in diabetic RAGE−/−/apoE−/− mice. Furthermore, aortic gene expression of the AT1a receptor, which is known to enhance ROS generation via NADPH oxidase (27), was increased in diabetic apoE−/− mice yet was significantly reduced in the diabetic RAGE−/−/apoE−/− mice.

RAGE activation is involved not only in the acceleration of atherosclerotic lesion formation but also in promoting proinflammatory pathways considered to play an important role in diabetes-accelerated atherosclerosis. For example, the diabetic plaque is characterized by the increased expression of inflammatory cytokines, chemokines and adhesion molecules that promote leukocyte infiltration and foam cell accumulation. This inflammatory response was clearly attenuated in the diabetic RAGE−/−/apoE−/− mice, as reflected by decreased accumulation of macrophages and T-cells and reduced aortic expression of related chemokines, cytokines, and adhesion molecules. A number of different components contribute to RAGE-induced inflammation, including oxidative stress and AGE accumulation. However, one key mediator appears to be activation of the proinflammatory transcription factor NF-κB, because RAGE has the unique ability to sustain NF-κB activation through de novo synthesis of NF-κB p65-mRNA and to provide a constantly growing pool of transcriptional active NF-κB (28). The downstream effects of this pathway include activation of NADPH oxidase, VCAM-1, and tissue factor and the autoinduction of RAGE expression in a positive feedback loop (29). In our study, the expression of the NF-κB subunit p65 was upregulated in the diabetic apoE−/− aorta. By contrast, diabetes-associated upregulation of NF-κB and subsequent NF-κB-dependent expression of proinflammatory genes were...
attenuated in diabetic RAGE−/−/apoE−/− mice. To complement the finding with respect to NF-κB, we measured gene expression of the NF-κB-dependent protein, MCP-1. MCP-1 gene expression was increased at least sixfold in the diabetic apoE−/− mice, and this increase was significantly attenuated in the RAGE−/−/apoE−/− mice in association with reduced macrophage infiltration.

In the present study, increased gene expression of various collagens was observed in diabetic apoE−/− mice. This result complements previous findings by several groups, including our own by demonstrating increased matrix accumulation as assessed by picrosirius red or increased collagen protein expression as assessed immunohistochemically (9,14). RAGE deletion in these diabetic apoE−/− mice particularly influenced collagen III gene expression, a finding similar to that seen previously with two chemically disparate AGE inhibitors (14). With respect to MMP-9 expression, the increase in mRNA levels in these diabetic apoE−/− mice complements findings by another group that specifically examined MMP-9 expression and activity (9). Furthermore, the decrease in MMP-9 expression seen in the diabetic RAGE−/−/apoE−/− mice is similar to that seen previously in studies using sRAGE (9).

Previously, it has been suggested that the changes observed with respect to matrix and MMP expression in response to inhibition of RAGE may reflect stabilization of the plaque. Nevertheless, the long-term implications of the changes in MMPs and collagens seen in this model remain controversial because this is not considered a model of plaque rupture.

The clear vasculoprotection observed in the diabetic RAGE−/−/apoE−/− mice further support previous experimental studies showing that blockade of RAGE activation, either with neutralizing antibodies or sRAGE (8,9,12), prevents the initiation and progression of atheroma formation in diabetic mice. It is noteworthy that in our study, both full-length and truncated isoforms of RAGE were absent in RAGE−/−/apoE−/− mice. Because RAGE has been reported to not only bind to AGES but also to other ligands such as HMGB1 and certain S100 isoforms, these ligands were examined in this study. S100 A8/9 levels were increased in the plasma and aortic tissues in diabetic apoE−/− mice, and this increase was not observed in the RAGE−/−/apoE−/− double knockout mice, even in the presence of concomitant diabetes. A similar upregulation of HMGB1 in the aorta of diabetic mice was also not seen in vessels from RAGE−/−/apoE−/− mice. However, the circulating levels of HMGB1 were higher in the double knockout mice. This finding is unexplained but may occur because RAGE−/−/apoE−/− mice have no sRAGE to bind HMGB from the circulation, thus enhancing accumulation of this protein within the plasma. These findings extend recent in vitro studies, albeit in a nondiabetic context, describing effects of various RAGE ligands on chemokines, adhesion molecules, and MMPs, including MCP-1, VCAM-1, and MMP-2 (23).

The finding that atherosclerosis was attenuated even in the absence of potentially protective circulating sRAGE strongly supports the postulate that the major mechanism of action of sRAGE is local antagonism of RAGE-dependent signaling. The importance of the full-length RAGE receptor in inflammatory vascular injury, even in the nondiabetic context, is suggested by the finding that even in control RAGE−/−/apoE−/− mice, there was reduced plaque accumulation, as was recently reported by another group (23).

In summary, this study demonstrates that accelerated atherosclerotic plaque accumulation associated with diabetes is prevented in the absence of RAGE. The antiatherosclerotic effects seen in diabetic RAGE−/−/apoE−/− mice were associated with reduced expression of a range of proatherogenic factors, including ROS and inflammatory cytokines, ultimately leading to reduced leukocyte recruitment. Thus, these data provide further support for the development of novel therapies that target RAGE activation in the prevention and treatment of diabetic macrovascular complications.

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