The Receptor RAGE as a Progression Factor Amplifying Arachidonate-Dependent Inflammatory and Proteolytic Response in Human Atherosclerotic Plaques

Role of Glycemic Control

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Background—RAGE (receptor for advanced glycation end products [AGEs]) plays a role in diabetic atherosclerosis. Recently, we have demonstrated enhanced expression of cyclooxygenase-2 and PGE synthase-1 (COX-2/mPGES-1) in human symptomatic plaques, and provided evidence that it is associated with metalloproteinase (MMP)-induced plaque rupture. However, the specific transmembrane signaling pathway(s) influencing plaque COX-2/mPGES-1 expression is unknown. The aim of this study was to characterize RAGE expression in human plaques and to correlate it with the inflammatory infiltration, COX-2/mPGES-1 and MMP expression, and with clinical evidence of diabetes.

Methods and Results—Plaques obtained from 60 patients undergoing carotid endarterectomy were divided into diabetic and nondiabetic according to clinical evidence of type 2 diabetes. Plaques were subjected to analysis of RAGE, NF-κB, COX-2/mPGES-1, MMP-2 and MMP-9, lipid and oxidized LDL (oxLDL) content, and collagen content by immunohistochemistry and Western blot, whereas zymography was used to detect MMP activity. Immunohistochemistry was used to identify CD68 macrophages, CD3 T-lymphocytes, smooth muscle cells (SMCs), and HLA-DR inflammatory cells. Diabetic plaques had more (P<0.0001) macrophages, T-lymphocytes, and HLA-DR cells, more (P<0.0001) immunoreactivity for RAGE, activated NF-κB, COX-2/mPGES-1, and MMPs, increased (P<0.0001) gelatinolytic activity, reduced (P<0.0001) collagen content, and increased (P<0.0001) lipid and oxLDL content. Interestingly, RAGE, COX-2/mPGES-1, and MMP expression was linearly correlated with plasma level of HbA1c.

Conclusions—in conclusion, this study demonstrates in humans that RAGE overexpression is associated with enhanced inflammatory reaction and COX-2/mPGES-1 expression in diabetic plaque macrophages, and this effect may contribute to plaque destabilization by inducing culprit metalloproteinase expression. (Circulation. 2003;108:1070-1077.)

Key Words: diabetes mellitus plaque inflammation prostaglandins metalloproteinases

Diabetes is associated with severe atherosclerosis in humans and represents a leading cause of morbidity and mortality. 1 Chronic perturbation of diabetic vasculature leads to increased number, size, and complexity of atherosclerotic plaques. Furthermore, lesion instability is enhanced in diabetics and mediates increased incidence and severity of clinical events. 1

In the last years, there is increasing evidence that inflammation, particularly in diabetic patients, plays a central role in the cascade of events that result in plaque erosion and fissuring. 2 In fact, several studies have shown that plaque rupture is related to increased inflammation within the plaque rather than plaque morphology or degree of vessel stenosis. 2

Lesional macrophages synthesize matrix metalloproteinases (MMPs), proteolytic enzymes capable of degrading plaque constituents. 3 In particular, 72 kDa (MMP-2) and 92 kDa gelatinase (MMP-9), which are specialized in the digestion of collagen fragments, have been critically associated with acute ischemic events in humans. 4 Interestingly, it has been shown that secretion of these MMPs by macrophages in human atherosclerotic plaques occurs through a prostaglandin (PG) E 2 –dependent mechanism. 4 PGE 2 signaling involves the modulation of cyclooxygenase (COX) and PGE synthase (PGES). 4 Two isoforms of COX and three of PGES have been identified, referred to as COX-1 and COX-2 and cytosolic PGES (cPGES) and type 1/type 2 microsomal...
PGES (mPGES), respectively. Whereas COX-1 and cPGES are constitutively expressed, COX-2 and mPGES-1 are co-regulated in nucleated cells in response to growth factors and cytokines, suggesting that these enzymes are involved in the generation of prostaglandins in inflammatory diseases. Consistent with the hypothesis of COX-2 and mPGES-1 contributing to the clinical instability of plaques, we recently reported enhanced MMP-2 and MMP-9 production by macrophages in symtomatic plaques due to the enhancement in PGE$_2$ synthesis as a result of the induction of the functionally coupled COX-2/mPGES-1. However, the specific transmembrane signaling pathway(s) by which persistent surrounding stimuli such as hyperglycemia may influence MMP generation in human plaque macrophages is still unknown. Previous studies suggest that ligands of RAGE (receptor for advanced glycation end products [AGEs]) such as AGEs are enriched in diabetic vasculature. AGEs deposition occurs in diabetic blood vessel driven by hyperglycemia, oxidant stress, and triggering of proinflammatory mechanisms. Thus, it is time to hypothesize that increased expression of RAGE in a ligand-enriched environment exacerbates proinflammatory mechanisms, thereby accelerating atherosclerotic plaque formation and progression. In this light, it has been recently demonstrated that administration of the extracellular ligand-binding domain of the receptor, soluble (s) RAGE, to apolipoprotein (apo) E–null mice on the diagnosis of hyperglycemia suppressed both accelerated development of atherosclerotic plaques and progression of established lesions toward complexity. On the contrary, still no clear evidences exist in humans about the potential role of RAGE in the accelerated progression of diabetic atherosclerosis and in the evolution of established atherosclerotic plaques toward instability. Notably, it has been recently demonstrated that RAGE may upregulate COX-2 expression in plaque macrophages of diabetic mice. Thus, the possibility that RAGE overexpression might influence the mechanism of COX2/mPGES-1-dependent plaque instability led us to investigate whether RAGE would specifically modulate PGE$_2$-dependent MMP production by macrophages into human atherosclerotic plaques. Our findings extend to humans the previous observations in animal models, and support the premise that upregulation of RAGE is involved not only in lesion formation and progression, but also in sustaining enhanced MMP production by macrophages in carotid plaques of diabetic patients, most likely due to increment in the signaling of the inflammatory PGE$_2$.

**Methods**

**Patients**

We studied 60, not previously examined, surgical inpatients (32 male, 28 female; 69±3 years), enlisted to undergo carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis. Recruitment was completed when 2 balanced groups of 30 patients according to clinical evidence of diabetes were achieved. The first group included 30 patients (16 male, 14 female; 68±3 years) who presented with clinical diagnosis of type 2 diabetes, as defined in accordance with the criteria of the American Diabetes Association (diabetic group). The second group included 30 patients (16 male, 14 female; 70±3 years) without diagnosis of diabetes (nondiabetic group). All the patients had an asymptomatic carotid stenosis. Asymptomatic carotid stenosis was detected on the basis of figure 1.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Stain (×5) for CD68, CD3, HLA-DR, and myeloperoxidase in diabetic or nondiabetic plaques. Similar regions of the plaque are shown. These results are typical of 30 diabetic and 30 nondiabetic plaques.
systematic clinical examination of patients with coronary or peripheral disease. The asymptomatic patients never had an ischemic episode in the territory of the carotid stenosis, but carotid endarterectomy has been shown to be beneficial in these patients, as shown by the Asymptomatic Carotid Atherosclerosis Study (ACAS).\textsuperscript{10} Percentage of carotid diameter reduction, procedural methods, risk factors and concomitant therapy did not differ between the two groups (Table). In particular, by the time of surgery, all patients were taking 100 mg daily of aspirin. The study was approved by local ethics review committees. Written informed consent was obtained from all patients before each examination.

**Immunohistochemistry**

After the surgical procedure, samples were immediately frozen in isopentane and cooled in liquid nitrogen. Serial sections were prepared as previously described.\textsuperscript{4} The specimens were analyzed by an expert pathologist (intraobserver variability 6%) blinded to the patient’s diagnosis. Analysis of immunohistochemistry was performed with a personal computer-based quantitative 24-bit color image analysis system (AlphaEase 5.02, Alpha Innotech Corp).

**Quantification of NF-κB Activity**

Nuclear extracts from plaque specimens were obtained as described by Ohlsson et al.\textsuperscript{11} Next, in order to identify the activated form of the NF-κB p65 subunit, we used a specific antibody that selectively recognizes the activated form of NF-κB.\textsuperscript{12} In addition, we also analyzed the expression of activated p50 subunit by specific Trans-AM NF-κB p50 transcription factor assay kit (Active Motif, Rixensart).\textsuperscript{13}

**Macrophages Extraction from Atherosclerotic Plaques**

Macrophages were selectively extracted from plaques as described by de Vries et al.\textsuperscript{14} Then, immunocytochemistry was performed as illustrated earlier, whereas Western blot and zymography on cell homogenates were performed as described following sections.

**Western Blot**

Proteins were extracted from plaque or plaque-derived macrophages and detected by Western blot as previously described.\textsuperscript{4} Bands were quantified by computer-assisted densitometry (Alpha Ease 5.02) and expressed as densitometric unit (DU).

**Zymography**

Zymography on plaque or plaque-derived macrophages was performed as previously described.\textsuperscript{4} Conditioned medium of human fibrosarcoma cell line HT1080 was used as positive control with known gelatinolytic activity.

**Oil Red O Staining for Lipid Content**

Two parallel sections from each plaque specimen were incubated in 60% isopropanol for 2 minutes and then in Oil Red O (Carlo Erba) solution for 20 minutes and rinsed in water. One of the sections was counterstained with hematoxylin.

**Sirius Red Staining for Collagen Content**

Sirius red polarization microscopy was used to detect interstitial collagen, and performed as previously reported.\textsuperscript{15} The sections were photographed with identical exposure settings for each section.

**Isolation and Culture of Blood Monocytes**

Peripheral blood monocytes were purified and cultured from 5 healthy blood donors as previously described.\textsuperscript{4} AGEs were prepared as described by Basta et al.\textsuperscript{16} Control or stimulated (LPS, 1 μg/mL; AGEs 400 to 800 μg/mL) monocytes (20×10⁷/4 mL of DME) were cultured in the presence or absence of pretreatment (2 hours) with either anti-RAGE or nonimmune IgG (70 μg/mL). The selective COX-2 inhibitor NS-398 (10 μM/L, Sigma) and PGE₂ (10⁻⁷ mol/L, Sigma) were also added to some of the cultures. At the end of the incubation, adherent monocytes were scraped, collected, lysed, and centrifuged to remove dead cells and debris. Then, homogenates were performed as described following sections.

**Results**

**Inflammatory Infiltration**

Immunocytochemistry revealed inflammatory infiltration in all specimens examined, more evident in the shoulder of diabetic plaques (Figure 1). Plaque area occupied by macrophages and T cells was significantly greater ($P<0.0001$) in diabetic than in nondiabetic plaques (Table). Inflammatory cells in the diabetic plaques were always characterized by COX-2/mPGES-1 and MMP expression evaluated by Western blot, while MMP activity analyzed by zymography. The results are representative of three or more experiments using cells from different donors.

**Statistical Analysis**

For clinical data and histological examination, variables were compared by use of the $\chi^2$ test. The significance of difference in biochemical marker expression and inflammatory cell infiltration between diabetic and nondiabetic patients was analyzed by Student’s $t$ test. The strength of the association of plasma HbA1c with RAGE expression in diabetic plaques was assessed by linear regression analysis. Data are expressed as percentage or mean±SD. All calculations were performed using the computer program SPSS 11.0.1.
strong expression of HLA-DR antigen and myeloperoxidase, which contrasted markedly with the low expression of HLA-DR and myeloperoxidase in the nondiabetic plaques (Figure 1).

**Higher RAGE Expression in Diabetic Plaques**

Immunohistochemistry revealed strong RAGE immunoreactivity in atherosclerotic plaques (Figure 2A). Interestingly, by quantitative image analysis, RAGE was more abundant in diabetic lesions (32 ± 5% versus 7 ± 2%, n = 30, mean ± SD; P < 0.0001), corresponding to the content of macrophages (Table). RAGE localized prominently in the shoulder region, in the periphery of the lipid core and in the proximity of vasa vasorum (Figure 2A, box), areas characterized as macrophage-rich. In fact, RAGE staining at high magnification indicated its localization in the activated macrophages (Figure 2A, box).

**RAGE Is Expressed in Higher Amounts in Diabetic Plaques**

Western blot analyses revealed RAGE expression in plaques (Figure 2B), markedly higher in diabetic than in nondiabetic plaques (8241 ± 195 versus 1231 ± 112 DU, mean ± SD, n = 30; P < 0.0001). Notably, RAGE expression in diabetic plaques was strongly dependent on glycemic control (Figure 2C), as also reflected by the statistically significant correlation (R = 0.642, P < 0.0001) between plasma HbA1c and RAGE concentration as quantified by densitometric analysis.

**Enhanced NF-κB Activity in Diabetic Plaques**

NF-κB activation as reflected by the selective analysis of activated form of both p50 (20 ± 3 versus 11 ± 2 pg/µg of nuclear extracts, n = 30; P < 0.0001) (Figure 3A) and p65 (5142 ± 168 versus 1034 ± 104 DU for Western blot analysis, n = 30, P < 0.0001) (Figure 3B) was significantly higher in diabetic plaques as regard to nondiabetic plaques, and showed a strong concordance with RAGE expression, despite a direct relationship that must be confirmed by future in vitro studies. Within the lesion, immunohistochemistry staining for p65a was significantly higher in diabetic plaques (23 ± 4% versus 5 ± 2%, n = 30; P < 0.0001) (Figure 3C) and accumulated always in the CD68+ macrophages in the plaque shoulder.

**Higher COX-2/mPGES-1 Expression in Diabetic Plaques**

COX-2 and mPGES-1 staining was more abundant in diabetic lesions (Figure 4A), as confirmed by quantitative analysis (23 ± 2% versus 4 ± 1%, and 22 ± 3% versus 5 ± 2%, respectively, mean ± SD, n = 30; P < 0.0001). COX-2/mPGES-1 accumulated in the activated macrophages and smooth muscle...
cells (SMCs) of shoulder regions. Western blot confirmed the higher COX-2/mPGES-1 expression in diabetic plaques with respect to nondiabetic plaques (7254±152 versus 1623±108, and 7243±184 versus 1459±111 DU, respectively, mean±SD, n=30; P<0.0001) (Figure 4B).

Plaque Expression of MMPs
Staining for MMP-2 and MMP-9 was significantly more abundant in the diabetic that in the nondiabetic lesions (Figure 5A). By quantitative analysis, levels of MMP-2 and MMP-9 in diabetic plaques significantly exceeded those in nondiabetic plaques (22±3% versus 5±2%, and 24±3% versus 4±1%, n=30; P<0.0001).

Diabetic Plaques Contain Activated MMPs
Increased MMP-2 and MMP-9 immunoreactivity was documented in diabetic plaques by Western blot (6785±142 versus 854±91, and 6857±97 versus 987±88 DU, n=30, mean±SD; P<0.0001) (Figure 5B). In addition, zymography demonstrated that extracts from diabetic plaques contained higher amounts of the activated form of MMP-2 and MMP-9 (2687±108 versus 401±67, and 2981±106 versus 512±77 DU, n=30, mean±SD; P<0.0001) (Figure 5C).

RAGE, COX-2/mPGES-1, and MMPs Are Expressed in Higher Amounts in Macrophages Extracted From Diabetic Plaques
In order to exclude that the higher protein expression observed in diabetic plaques was merely a secondary effect of higher inflammatory infiltration, we repeated immunocytochemistry and Western blot analyses also on macrophages selectively extracted from 5 diabetic and 5 nondiabetic plaques. Only weak positivity for RAGE, COX-2/mPGES-1, and MMPs was observed in macrophages extracted from nondiabetic plaques. In contrast, a 6-fold higher signal (P<0.0001) was demonstrated by Western blot in macrophages isolated from diabetic plaques (5132±111 versus 657±75 DU for RAGE; 6122±132 versus 587±77 DU for COX-2; 5789±115 versus 765±66 DU for mPGES-1; 6574±145 versus 661±48 DU for MMP-2; 5678±103 versus 623±58 DU for MMP-9).

Colocalization of RAGE With COX-2, mPGES-1, and MMPs in Macrophages in Diabetic Plaques
In the first experiment, serial sections of diabetic plaques were incubated with the primary antibodies anti-CD68, anti-RAGE, anti-COX-2, anti-mPGES-1, anti-MMP-2, and anti-MMP-9. Within the lesion, all proteins accumulated in the shoulder as well as in the periphery of the lipid core. In two other experiments, immunofluorescence double labeling associated the expression of RAGE with COX-2, mPGES-1, and MMPs in CD68+ macrophages both in plaque sections and in plaque-derived macrophages (Figure 6). Thus, these analyses confirmed the concomitant presence of RAGE, COX-2/mPGES-1, and MMPs in macrophages at the vulnerable region of diabetic plaque.

Plaque Extracellular Components
Sirius Red polarization showed considerably lower content of interstitial collagen in the tissue sections of diabetic patients compared with nondiabetic patients (11±2% versus 17±3%, n=30, mean±SD; P<0.0001). In contrast, the plaque content of lipid and oxLDL was significantly higher in diabetic plaques than in nondiabetic plaques (25±9% versus 6±3%, and 22±7% versus 7±2%, respectively, n=30, mean±SD; P<0.0001).

RAGE Activation Induces PGE2-Dependent MMPs in Monocytes In Vitro
To determine if monocyte MMP production is regulated through RAGE-dependent pathway involving the concomitant induction of functionally coupled COX-2 and mPGES-1, we initially examined the effect of AGEs on MMP production (Figure 7). AGEs caused a dose-dependent enhancement in COX-2, mPGES-1, MMP-2, and MMP-9 levels over that detected in control monocytes. MMP induction by AGEs was significantly inhibited by anti-human RAGE IgG and NS-398; however, the inhibition of MMPs was reversed by the addition of PGE2. Thus, MMP production in monocytes/macrophages appears secondary to the induction of COX-2/mPGES-1 and the subsequent generation of PGE2 by engagement of RAGE by AGEs.
Discussion

In the present report, we provide evidence for the functional involvement of RAGE in PGE$_2$-dependent MMP overexpression in diabetic atherosclerotic plaques in humans. In particular, the present findings are the first, to the best of our knowledge, to (1) clearly identify differences for RAGE in diabetic versus nondiabetic human atherosclerotic lesions, (2) recognize higher COX-2/mPGES-1 as well as PGE$_2$-dependent MMP expression in diabetic versus nondiabetic human plaques, and (3) relate the presence of RAGE in human plaque macrophages to an unstable phenotype and to an increased risk of future acute ischemic events precipitated by COX-2/mPGES-1–dependent rupture of atherosclerotic plaque.

Concomitantly higher expression of RAGE, NF-κB, COX-2, mPGES-1, MMP-2, and MMP-9 was found in specimens obtained from the asymptomatic carotid lesions of patients with type 2 diabetes compared with specimens obtained from nondiabetic patients.

In this study, macrophages were significantly more abundant in diabetic plaques, always outnumbered the lymphocytes, and represented the major source of RAGE. In addition, the site of inflammatory infiltration was also always characterized by strong expression of RAGE antigen on
HLA-DR+ inflammatory cells, which contrasted markedly with the low expression of these proteins elsewhere in the fibrous cap. Thus, these data suggest the presence of an active inflammatory reaction in diabetic plaques. In fact, in agreement with the difference in RAGE staining pattern, the histological milieu of the lesions appears different with regard to cellularity and presence of foam cells, but not in the degree of vessel stenosis, suggesting that diabetic and non-diabetic lesions are only different as regard to inflammatory burden and that differences in plaque behavior stem from differences in the presence of stimuli (ie, persistent hyperglycemia) for selective expression of RAGE, capable of disrupting plaque stability via COX-2/mPGES-1 induction.

Our previous studies have reported enhanced COX-2/mPGES-1 expression in symptomatic atherosclerotic lesions. However, these studies did not provide any evidence about specific enzyme expression in subgroups of high-risk asymptomatic plaques such as those found in diabetic patients. Furthermore, they did not provide any information about the specific transmembrane signaling pathway transducing environmental stimuli in COX-2/mPGES-1 overexpression ultimately leading in turn to PGE₂-dependent atherosclerotic plaque rupture.

Interestingly, macrophages of the shoulder region contain most of the RAGE and COX-2/mPGES-1 within the lesion. This finding may have functional importance, because RAGE-dependent PGE₂ can regulate the expression of different genes in different cell types. In particular, macrophages, not present in normal arterial tissue, produce an array of metalloproteinases, including the PGE₂-dependent MMP-2 and MMP-9, considered two of the most important in the process of atherosclerotic plaque rupture. Now, the finding that RAGE predominantly localizes with lesional macrophages complete our previous observations in human carotid atherosclerotic plaques, where macrophages also represented the majority of COX2/mPGES-1-expressing cells. Furthermore, our description of MMP-2 and MMP-9 in plaque regions RAGE-positive and found to be macrophage-enriched suggests that such regulation of MMP expression by RAGE may operate in vivo in diabetic patients. Thus, we can speculate that increased PGE₂ generation in plaque macrophage as consequence of RAGE overexpression may enhance the synthesis of MMPs in the same cell, possibly representing a crucial step in the pathophysiology of diabetic plaque instability.

Furthermore, because ligand-RAGE interaction results in upregulation of RAGE and NF-κB activated by RAGE may further amplify RAGE expression in inflammatory cells by a direct, positive control on gene promoter, RAGE overexpression in plaque macrophages may establish a positive, self-stimulatory autocrine and paracrine feedback loop, amplifying and sustaining the inflammatory response leading to progressive plaque destabilization. Finally, our observation that diabetic lesions displayed augmented expression of oxLDL, probably as consequence of RAGE-dependent increased lipid peroxidation, supports the concept that RAGE-driven inflammatory response within the diabetic blood vessel may contribute to accelerated progression of diabetic plaques.

Figure 6. Confocal microscopy showed staining for RAGE (green staining) on plaque-derived macrophages, concomitant (red staining) with CD68, COX-2, MMP-2, and MMP-9 expression. These results are typical of 30 diabetic and 30 nondiabetic plaques.

Figure 7. RAGE-dependent MMP production in monocytes. Purified monocytes were cultured in the presence/absence of pretreatment (2 hours) with anti-RAGE IgG, and then AGes, LPS, NS-398, and PGE₂ were added to some cultures. Cultures were harvested at 48 hours for Western blot analysis.
atherosclerosis also by amplifying and sustaining other glucose-independent proatherogenic mechanisms.

Notably, the intriguing and novel proatherogenic mechanism of RAGE in human diabetes is supported in this study not only by the observation that RAGE expression is higher in diabetic plaques with respect to nondiabetic plaques, but also by the information that it is strongly correlated with the intensity of glycemic control as reflected by HbA1c. In this light, the peculiar RAGE expression in the proximity of vasa vasorum, areas designed for blood-vessel interchange, is quite relevant. On the other hand, the observed enhanced myeloperoxidase staining pattern in plaque macrophages found positive for RAGE suggests that the direct generation of AGE by macrophage myeloperoxidase may be operative and potentially relevant in the setting of those clinical conditions, such as hypercholesterolemia, characterized by high infiltration of activated macrophages in atherosclerotic plaques.

In conclusion, this study demonstrates the high prevalence of RAGE in diabetic atherosclerotic lesions, and provides evidence that expression of this receptor by activated macrophages is associated with COX-2/mPGES-1-dependent increase in the biosynthesis of MMP potentially promoting plaque rupture. Because recent clinical data have raised the possibility that intensive glycemic control may reduce ischemic events in subjects with type 2 diabetes, these findings are potentially important from a fundamental standpoint because they identify a potential mechanism by which hyperglycemia may influence the evolution of atherosclerotic lesions. From a practical standpoint, these findings raise the interesting possibility that modification of the RAGE-COX-2/mPGES-1 signaling, for example by selective RAGE antagonists, might provide a novel form of therapy for plaque stabilization of diabetic patients with atherosclerotic disease and prevention of acute ischemic syndromes.

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