Chronic Vascular Inflammation in Patients With Type 2 Diabetes

Endothelial biopsy and RT-PCR analysis

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OBJECTIVE — Chronic vascular inflammation may play a role in the development of macrovascular complications in diabetic patients. In this study, we examine the association of endothelial expression of two inflammatory mediators, receptor for advanced glycation end product (RAGE) and monocyte chemoattractant protein-1 (MCP-1), with type 2 diabetes using novel endothelial biopsy and RT-PCR techniques.

RESEARCH DESIGN AND METHODS — Endothelial samples are obtained from the aorta of 12 patients with type 2 diabetes and 23 control subjects who underwent cardiac catheterization for chest pain syndrome or heart transplant follow-up. Endothelial cells are purified using magnetic beads with adsorbed CD146 antibody and subjected to RT-PCR analysis of RAGE and MCP-1 transcripts. The association of RAGE and MCP-1 expression with type 2 diabetes is assessed with χ^2 test and confirmed with in vitro experiments on human aorta endothelial cells.

RESULTS — RT-PCR reveals gene expression patterns in patient-derived endothelial cells. Strong associations are observed between induction of RAGE mRNA and diabetes ($P < 0.01$) and between induction of RAGE and MCP-1 transcripts ($P < 0.05$). Treatment of cultured human aortic endothelial cells with S100b induces the expression of MCP-1 and RAGE transcripts.

CONCLUSIONS — Endothelial cells can be harvested during cardiac catheterization and can be characterized with respect to molecular phenotypes under the influence of both genetic and environmental factors. Induction of RAGE and MCP-1 transcripts in patients with diabetes supports a role of chronic vascular inflammation in macrovascular complications.

Diabetes Care 28:379–384, 2005

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Received for publication 30 June 2004 and accepted in revised form 26 October 2004.

A.M.S. is a member of the Scientific Advisory Board of, has been a paid consultant for, and has received research support from TransTech Pharma.

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Abbreviations: AGE, advanced glycation end product; GAPDH, glyceraldehydes-3-phosphate; HAEC, human aortic endothelial cell; LCA-1, leukocyte common antigen-1; MCP-1, monocyte chemoattractant protein-1; RAGE, receptor for AGE; vWF, von Willebrand factor.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Experimental data have shown that hyperglycemia may induce a chronic inflammatory state in the vessel wall, thus accelerating the development of macrovascular complications (1–4). Hyperglycemia and oxidant stress promote nonenzymatic glycoxidation of proteins and lipids. The final products of these reactions, termed advanced glycation end products (AGEs), have both direct and indirect effects. Direct activities of AGEs include the capacity to alter vessel wall architecture through formation of intermolecular crosslinks and trapping of plasma components, reducing elasticity. AGE-mediated generation of low levels of reactive oxygen species can result in quenching of the endogenous vasorelaxant nitric oxide. By interacting with the receptor for AGE (RAGE), AGEs magnify their biologic effects considerably by recruiting cellular elements. RAGE is a member of the immunoglobulin superfamily of cell surface molecules with a ligand repertoire, including AGEs (5,6), S100/calgranulins (7), amyloid-β peptide (8), and amphoterin (9). Engagement of RAGE triggers signaling cascades in which activation of NADPH oxidase recruits multiple downstream pathways, including $p21^{ras}$, the mitogen-activated protein kinases, nuclear translocation of nuclear factor-kB, phosphatidylinositol 3-kinase, cdc42/rac, and the Jak/stat pathway (7,10,11). Two general outcomes of RAGE-mediated cellular activation include an elicitation of a proinflammatory phenotype, resulting from expression of mediators (cytokines) and effectors (metalloproteinases and tissue factor), and upregulation of the receptor itself. The latter has the potential to create a positive feedback loop in which ligand-induced expression of RAGE places more receptor on the cell surface to potentiate subsequent rounds of RAGE-induced cellular activation.

One of the key proinflammatory mediators whose expression is stimulated by the RAGE signaling pathway is monocyte chemoattractant protein-1 (MCP-1; JE is

the murine counterpart) (12). MCP-1 is a member of the low–molecular mass, chemotactic cytokine family that is involved in the recruitment of leukocytes to inflammatory sites and in the constitutive trafficking of lymphocytes and antigen-presenting cells through secondary lymphoid organs (13). The involvement of MCP-1 in atherogenesis has been suggested by its strong chemotactic effect on monocytes both in vitro and in vivo (14), the upregulation of MCP-1 in endothelial and smooth muscle cells after exposure to minimally modified LDL (15), the presence of abnormal MCP-1 in macrophage-rich areas of atherosclerotic lesions (16), and protection from the development of vascular lesions in genetically manipulated atherosclerosis-prone mice deficient in JE/MCP-1 (17,18). If, indeed, the expression of MCP-1 on endothelial cells is enhanced by activation of RAGE in vivo, MCP-1, thus formed, would enhance the adhesion of monocytes to the vessel walls.

Despite the extensive animal studies, the extent of chronic vascular inflammation in diabetic patients remains largely unknown for the lack of a reliable and noninvasive method to assess vascular inflammation. Catheter and guide wire-based endothelial biopsy offers a minimally invasive method to obtain a few endothelial cells from patients (19). By examining gene expression of RAGE and MCP-1 in these patient-derived endothelial cells, it is possible to confirm the presence of vascular inflammation attributable to hyperglycemia. This molecular information may help identify therapeutic targets to slow down the progress of macrovascular complications and serve as an indicator for therapeutic efficacy in clinical trials and future clinical practice. In this pilot case control study, we report the use of endothelial biopsy and RT-PCR to demonstrate elevated levels of RAGE and MCP-1 transcripts in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

— Guide wires were collected from 86 patients, randomly selected from ~3,100 patients who underwent cardiac catheterization at our institution from March 2000 to March 2001. Endothelial samples were successfully obtained from 35 patients (average age, 63 ± 2 years). Indications for cardiac catheterization included angina or acute myocardial infarction in 21 patients and annual follow-up for heart transplant in 14 patients. Twelve of these 35 patients had type 2 diabetes; the other 23 nondiabetic patients served as control subjects. The study was approved by the institutional review board, and all patients gave routine surgical consent that included permission for the use of tissues/organs removed during the procedure for subsequent research/educational purposes.

Endothelial cell isolation from guide wires

Benson or Rosen guide wires, used to guide the catheter to the artic arch, were collected immediately after use. Tips of the wires were removed (the distal 10 cm) and washed in endothelial cell dissociation solution (19). Cells eluted from the wire tips were purified with magnetic beads (2×10^5; Dynal, Lake Success, NY) coated with anti-CD146 antibody (Chemicon, Temecula, CA), a mouse monoclonal antibody specific for endothelial cells (20). Immediately after purification with magnetic beads, cells were resuspended in lysis buffer and frozen in −80°C. The lysis buffer contains 50 mmol/l Tris-HCl (pH 8.3 at 37°C), 40 mmol/l KCl, 6 mmol/l $MgCl_2$, 10 mmol/l dithioerythritol, 0.5 units/μl RNase inhibitor, and 0.2% NP40.

RT-PCR

After adding 20 μmol/l dNTP and 20 nmol/l oligo dT24 to patient-derived endothelial cells, the cells were heated at 72°C for 3 min to disrupt cells membranes and denature proteins. Reverse transcription was subsequently carried out using 10 units of Moloney murine leukemia virus and avian myeloblastosis virus reverse transcriptase mix (1:1) (Roche, Indianapolis, IN) at 37°C for 15 min. After adding a poly-A end with terminal transferase, the cDNAs were amplified with 25-cycle PCR amplification using a primer containing oligo dT24 and polycloning sites (5′-ATATGGATCCAAGCTTCGAATTCGTTTTTTTTTTTTTTTTTTTTTTT-3′). To allow analysis of gene expression, the amplified cDNAs were reamplified for another 25 cycles with the same primer, diluted appropriately to normalize the level of glyceraldehydes-3-phosphate dehydrogenase product (GAPDH) and used as the template for the following specific PCRs. Twenty-five cycles of PCRs were performed by using a battery of cell markers, including GAPDH, thrombomodulin, von Willebrand factor (vWF), Tie-2, smooth muscle α-actin, and leukocyte common antigen-1 (LCA-1). β-Actin was use as an internal control to assure that the possible variation in GAPDH level did not skew the ratio of gene expression in the samples (21). Only samples that were positive for all three endothelial markers (thrombomodulin, vWF, and Tie-2) and negative for α-actin and LCA-1 were used for the subsequent studies. The expression of RAGE and MCP-1 was assessed with RT-PCR in patient-derived and cultured endothelial cells. All primers were derived from the most 3′ 500 bp of the cDNA sequences, and when possible, the primers were selected from different exons to avoid amplification of genomic DNA. The sequences of these primers are listed in Table 1.

Statistical analysis

The association between RAGE and MCP-1 expression with diabetes was assessed with χ^2 test with Yates' correction. The patients were also stratified by their indications for cardiac catheterization, presence of severe coronary artery disease, age, and sex. These confounding factors were analyzed with the same statistical test.

Confirmation of AGE-induced RAGE and MCP-1 expression in cultured endothelial cells.

Human aortic endothelial cells (HAECs) were obtained from Clonetics (Walkersville, MD) and grown in endothelial cell basal medium/microvascular medium. To test the hypothesis that ligand engagement of RAGE induces the expression of MCP-1 transcript in endothelium, HAECs were incubated with 5 μg/ml, 10 μg/ml, and 20 μg/ml S100b (human and bovine brain S100b at a 1:1 ratio; Calbiochem, San Diego, CA) for 45 min and 1.5, 3, 6, and 12 h. To ascertain that S100b-induced MCP-1 expression was mediated by RAGE, HAECs were pretreated with $F(ab')_2$ fragments of anti-RAGE IgG (60 μg/ml) (22) for 12 h before the addition of S100b (10 μg/ml). The endothelials were stimulated with 50 ng/ml lipopolysaccharide for control. Fifty cells from the treated or untreated samples were processed for RT-PCR to detect transcripts for MCP-1 and RAGE.

Table 1—*Sequences of primers used in RT-PCR*

Gene	GenBank accession number	Forward primer sequence	Reverse primer sequence	PCR product (bp)
GAPDH	M17851	5′GGTGGTGAAGCAGGCGTCGGA3′	5′GGTCTACATGGCAACTGTGAG3′	403
β-Actin	NM001101	5′CAATGTGGCCGA GGACTTTGA3′	5′CTGGTCTCAAGTCAGTGTACA3′	370
TM	X05495	5′AGGCTAGGTACACAGCTCTAG3′	5′GTACAAGTGATGTCATAAGCAAG3′	240
Tie-2	L06139	5′GACATTTGGGAGACATGTGAC3′	5′AGGCAAGACATTTATTCACTC3′	156
vWF	X04385	5′GGCTGCAGTATGTCAAGGTGG3′	5′GGAGCATTTGCACTCCATGGC3′	233
LCA-1 (CD45)	Y00062	5′GCATGCTCGATTATTCCCTGTAC3′	5′TAACATGGGAACTATGCATATGC3′	220
α-Actin	J05192	5′CCATGAAGATCAAGATCATTG3′	5′CACATAGGTAACGCGTCAGAG3′	273
RAGE	M91211	5′AGAGGAGAGGAAGGCCCCAGA3′	5′GGCAAGGTGGGGTTATACAGG3′	231
MCP-1	X14768	5′GCCTTAAGTAATGTTAATTCTTAT3′	5′GGTGTAATAGTTACAAAATATTCA3′	239

TM, thrombomodulin.

RESULTS — Endothelial cells suitable for RT-PCR–based gene expression analysis were obtained from patients with guide wires used in endovascular procedures. The expression of three endothelial markers, thrombomodulin, Tie-2, and vWF, was successfully detected in 35 samples. The lack of expression of smooth muscle α-actin and LCA-1 argues against the contamination of smooth muscle cells or leukocytes and eliminates the possibility of genomic amplification. An example of the expression of these cell markers in representative cell samples is shown in Fig. 1. The success rate of endothelial biopsy was 41% (35 of 86), consistent with our previous report (19). Delayed transfer of wire tips or inadvertent wash of the wire tip in saline accounted for most of the failures, whereas laboratory error in PCR caused the others.

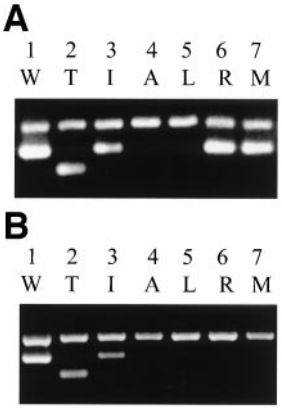

Figure 1—*Expression of cell markers, RAGE, and MCP-1 in patient-derived endothelial cells. Two examples of RT-PCR results of patient-derived endothelial cells are shown. The patient in* A *has type 2 diabetes; the patient in* B *does not. β-Actin is used as the internal control, which appears as the upper bands in each lane. Three endothelial markers, vWF (W), thrombomodulin (T), and Tie-2 (I), are the lower bands in* lanes 1–3. *No smooth muscle α-actin (*A*) or LCA-1 (L) is detected in* lanes 4 *and* 5. *Patient* A *expresses both RAGE (R) and MCP-1(M), whereas patient* B *shows no detectable RAGE or MCP-1 (*lanes 6 *and* 7*, respectively).*

Ten of 12 patients with type 2 diabetes showed RAGE transcripts in their aortic endothelium in contrast to 4 of 23 patients in the control group (Table 2), indicating a strong association between RAGE expression and diabetes ($\chi^2 = 14.21$; $P < 0.01$). Transcripts for MCP-1 were detected in 16 patients, 10 of whom were RAGE positive (Table 3). Fifteen of 19 MCP-1–negative patients were also negative for RAGE. The association between induction of RAGE and MCP-1 mRNA was statistically significant by χ^2 test with Yates' correction ($\chi^2 = 6.17$; $P < 0.05$). We did not find any association of RAGE or MCP-1 expression with severe coronary artery disease or acute myocardial infarction. The degree of atherosclerotic disease in the aorta was not documented, and thus the association of RAGE expression with the severity of atherosclerosis was not determined in this study. Age and sex did not show any effect on RAGE or MCP-1 expression in this study. No association was observed between RAGE expression and heart transplantation. It should be noted that in the heart transplant patients, the endothelial cells were obtained from the host side instead of the donor heart. Endothelial expression of RAGE in the donor heart is not assessed. The strong association of two proinflammatory cytokines, RAGE and MCP-1, with diabetes in this small sample suggests the presence of chronic vascular inflammation in diabetic patients.

Cell culture experiments suggested that the activation of RAGE could mediate the elevated expression of MCP-1. After the cultured HAECs were incubated with S100b, a known RAGE ligand (7), the induction of MCP-1 mRNA was detected after 45 min, and transcripts were evident up to the longest time point, 6 h (Fig. 2*A*). After 3 h of exposure to S100b, expression of RAGE was also increased (Fig. 2*B*). Although no PCR products for MCP-1 and RAGE could be detected in the control sample with this specific combination of template dilution and PCR cycles, low levels of mRNA for MCP-1 and RAGE were detected with higher template concentrations and longer cycles (data not shown). When the cells were pretreated with $F(ab')_2$ fragments of anti-RAGE IgG to block low-level RAGE present on quiescent endothelial cultures, both MCP-1 and RAGE induction were suppressed (Fig. 2*C* and *D*). This result suggests that low level of RAGE present on endothelial cells, not detected under standard condition in Fig. 2*A*, interacts with S100b to induce MCP-1 expression early and enhance RAGE expression later. The exact mechanism is unclear, and other RAGE-independent mechanisms could also be involved. The induction of MCP-1 expression could be detected after treatment with an S100b concentration as low as 5 μg/ml.

CONCLUSIONS — This study demonstrates the feasibility of analyzing endothelial gene expression in patients using a minimally invasive endovascular tech-

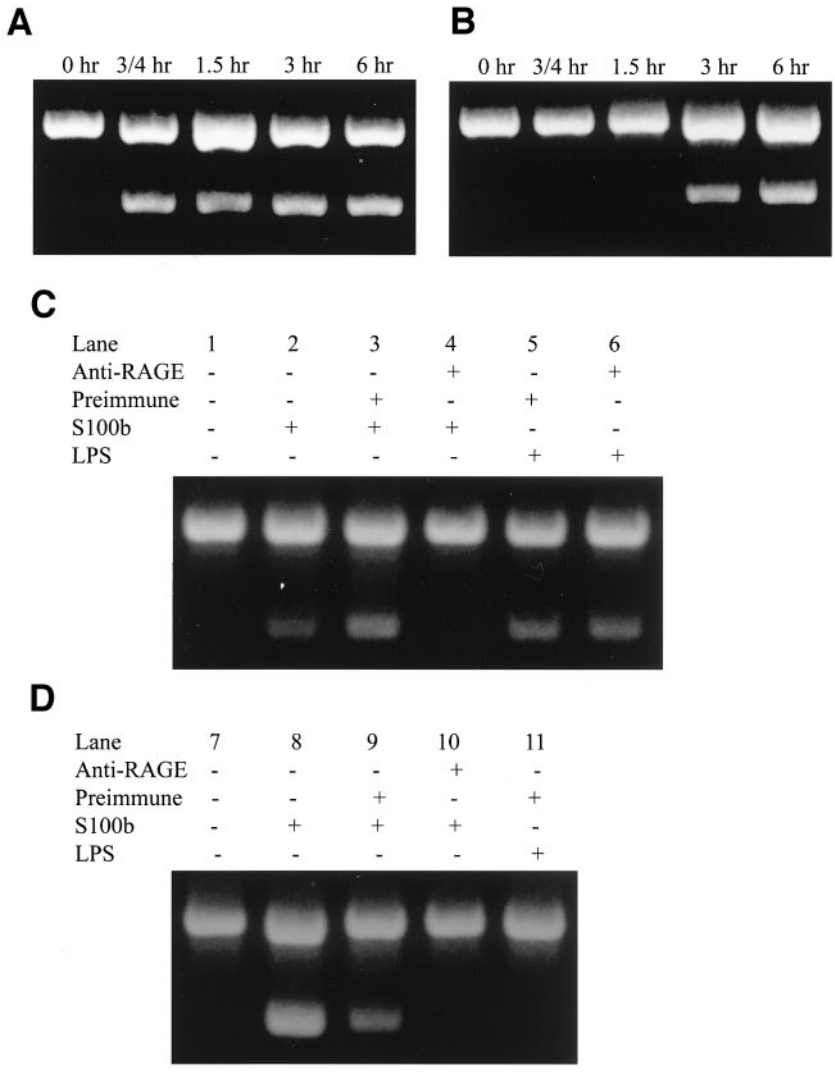

Figure 2—*Stimulation of MCP-1, RAGE, and RGE-1 expression in HAECs.* A*: MCP-1 expression is induced after 45 min of exposure to 10 μg/ml S100 proteins.* B*: It takes 3 h for significant RAGE induction to occur.* C*: MCP-1 induction is suppressed by F(ab′)$_2$ fragments prepared from anti-RAGE IgG but not by preimmune serum* (lanes 3 *and* 4)*. In the presence of anti-RAGE F(ab′)$_2$, LPS is still able to induce the expression of MCP-1* (lanes 5 *and* 6). D*: Similar suppression of RAGE induction is also seen with anti-RAGE F(ab′)$_2$ but not with preimmune serum. The slightly lower level of expression of RAGE after preimmune serum treatment may be secondary to the dilution effect of the preimmune serum, which is half as concentrated as anti-RAGE F(ab′)$_2$ in terms of IgG content. The top bands are GAPDH, and the lower bands are MCP-1* (A *and* C) *and RAGE* (B *and* D).

nique and RT-PCR. Coordinately increased expression of RAGE and MCP-1 mRNAs, two genes implied in atherogenesis in rodent models and cell culture, is detected in the endothelium of a small group of diabetic patients with symptoms and signs of cardiac ischemia significant enough to warrant cardiac catheterization, suggesting a role for chronic vascular inflammation in the development of macrovascular complications in diabetic patients. Consistent with the results from patient-derived endothelial cells, the cell culture experiments with HAECs confirm the induction of RAGE and MCP-1 by S100b.

Atherosclerosis is a multifactorial disease, involving hemodynamic, metabolic, and lifestyle determinants that cannot be easily mimicked in experimental animals. Therefore, disease mechanisms evaluated in animal models cannot be automatically extrapolated to patients. The observation of elevated RAGE expression in the endothelium of diabetic patients and its association with an inflammatory mediator, MCP-1, suggests that RAGE-dependent mechanisms may stimulate a proinflammatory cascade in humans. This preliminary result justifies further prospective study with targeted endothelial biopsy and direct correlation with the presence of vascular lesions to characterize the role of RAGE in chronic vascular inflammation and atherogenesis.

The association of elevated RAGE and MCP-1 expression provides a plausible link between hyperglycemia and the early development of atherosclerosis. AGE-RAGE interaction has the capacity to upregulate RAGE, resulting in chronic vascular inflammation through a positive feedback loop. Elevated levels of MCP-1 attract monocytes to the vessel wall, an important initial step in atherogenesis. Expression of other proinflammatory factors, also elicited by RAGE-induced cellular activation, enhances monocyte migration into the vessel wall and their differentiation into macrophages. These macrophages will, in turn, accumulate lipids and become foam cells, a hallmark of early atherosclerosis. MCP-1 is likely to be a downstream effect of RAGE activation, as demonstrated by RAGE-dependent induction of MCP-1 in cultured endothelial cells. Although MCP-1 can be induced by tissue necrosis factor-α and interleukin-1, which can be stimulated by AGE-RAGE interaction, the short time course to MCP-1 induction after S100/calgranulin exposure suggests a possible direct or parallel activation of MCP-1 by RAGE.

The endothelial biopsy technique can be easily adapted to target endothelium in various vascular beds. For example, the catheter can be advanced to a diseased vessel segment, and a road map of the vascular lesion can be obtained with contrast injection. A new wire can then be passed through the catheter and used to scrape the vascular lesion under fluoroscopic guidance. Because the wire is shielded from the circulation except for over the vascular lesion, a highly selective endothelial biopsy can be achieved.

Several improvements made in this study facilitated the analysis of gene expression in patient-derived endothelial cells. Purification of endothelial cells with magnetic beads negated the tedious task of picking individual endothelial cells. The use of a small number of cells as the

RT-PCR templates improved the sensitivity and reliability of the study over single-cell RT-PCR and conferred better representation of the endothelium in vivo. The performance of RT-PCR in the same PCR tube minimized loss of mRNA. The short reverse transcription time generated first-strand cDNA of 300–700 bp, which could be reliably amplified by subsequent cycles of PCR, avoiding discrimination against large messages. With the above attention to detail, it is possible to use RT-PCR to identify patients who express high levels of RAGE and MCP-1. This RT-PCR–based gene expression analysis is clearly superior to immunocytochemistry, as the amplified cDNAs can be used to examine unlimited number of genes in the same sample.

This study has several limitations. First, the small sample size and the random selection of patients and control subjects limit the generalizability of the conclusions. Second, the success rate of the biopsy can be improved further with the use of multiple wires and several passes. This modification will enable the biopsy of smaller vessels with smaller wires. Finally, we can expect further improvement of the sensitivity and quantification of the RT-PCR analysis with the use of real-time PCR.

Using similar combination of catheters and guide wires, endothelial cells can also be obtained from radial artery and antecubital vein (23). This less invasive technique makes it possible to assess the expression of inflammatory genes in the endothelium over time and to correlate it with clinical development of vascular diseases. The reduction of endothelial inflammation can also be used to gauge the effect of drug therapy and behavioral modifications. These findings could potentially be used to advantage in the monitoring of potential therapeutic agents specifically targeted to the reduction of vascular inflammation.

Acknowledgments— This study is supported by a generous donation of the Weitzman family for the study of the mechanisms of vascular diseases.

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