Blockade of Endogenous Cytokines Mitigates Neointimal Formation in Obese Zucker Rats

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Background—It is well known that diabetes mellitus is a major risk factor for cardiovascular diseases such as atherosclerosis and restenosis after angioplasty. It has become clear that advanced glycation end products (AGE) and their receptor (RAGE) are implicated in vascular diseases, especially in diabetes mellitus. Nevertheless, the mechanisms by which diabetes mellitus is often associated with vascular diseases remain unclear.

Methods and Results—To study the role of endogenous cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 in the development of vascular diseases and in the expression of RAGE, we used semapimod, a pharmacological inhibitor of cytokine production, and examined its effect on neointimal formation in the femoral artery of obese Zucker (OZ) rats. We also used an adenovirus construct expressing a dominant negative mutant of the receptor for TNF-α (AdTNFRαC) to block the action of endogenous TNF-α. Semapimod significantly suppressed neointimal formation and RAGE expression in OZ rats compared with untreated OZ rats. This inhibitory effect of semapimod on neointimal formation was overcome by infection of an adenovirus expressing RAGE into the femoral artery of OZ rats. Furthermore, AdTNFRαC infection significantly suppressed neointimal formation and RAGE expression in the femoral artery of OZ rats.

Conclusions—These results suggest that endogenous cytokines, especially TNF-α, were implicated in neointimal formation in OZ rats and that RAGE was a mediator of the effect of these cytokines on neointimal formation. (Circulation. 2005;111:1398-1406.)

Key Words: diabetes mellitus ■ angioplasty ■ gene therapy

It is well established that diabetes mellitus (DM) is a major risk factor for cardiovascular diseases. Most diabetic patients have type II DM, which is characterized by obesity and insulin resistance. It has been shown that vascular function is impaired in DM. Endothelium-dependent vasorelaxation in response to acetylcholine was impaired in obese Zucker (OZ) rats, an animal model of insulin resistance. Neointimal formation after balloon injury of the carotid artery was enhanced in OZ rats compared with control lean Zucker (LZ) rats. Furthermore, it has been demonstrated that neointimal proliferation in coronary arteries after stent implantation was accelerated in diabetic patients. Nevertheless, the mechanisms by which DM is often associated with vascular dysfunction are not clearly understood.

Recently, the role of advanced glycation end products (AGE) and their receptor (RAGE) in the pathogenesis of vascular diseases has become clear. Expression of AGE/RAGE was augmented in the neointima of OZ rats compared with LZ rats after balloon injury of the carotid artery. Blockade of RAGE function, with the use of a RAGE mutant that encodes only the extracellular domain of RAGE (soluble RAGE [sRAGE]) and sequesters its ligands, resulted in amelioration of the extent of neointimal formation after endothelial denudation in diabetic animals as well as in nondiabetic animals. Furthermore, progression of atherosclerosis was inhibited in diabetic mice when they were treated with sRAGE. Still, little is known about the mechanisms by which expression of AGE/RAGE is enhanced in diabetic animals.

Although the precise mechanisms of insulin resistance remain unclear, it has been demonstrated that cytokines released from the adipose tissue, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6, induce insulin resistance. Because these cytokines are known to induce vascular dysfunction, it is possible that vascular diseases often progress in the diabetic state because of the action of these cytokines. To test this hypothesis, we used semapimod (formerly known as CNI-1493), a tetravalent guanylhydrazone compound that inhibits the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6. Semapimod, reportedly, does not suppress the production of transforming growth factor-β or the expression of major...
histocompatibility complex class II antigens. Furthermore, it does not affect total cellular protein synthesis, suggesting the specificity of its effects. Phase II trials are ongoing to examine the efficacy of this drug in patients with Crohn’s disease and endoscopic retrograde cholangiopancreatography-induced pancreatitis. We administered this drug to OZ rats and examined its effect on neointimal formation after placement of a polyethylene cuff around the femoral artery. We also constructed an adenovirus encoding a deletion mutant of the receptor for TNF-α (TNFR), which lacks the carboxyl terminal signal transduction domain and blocks TNF-α signaling, and examined its effect on neointimal formation.

Methods

Reagents
Semapimod was kindly supplied by Cytokine PharmaSciences, Inc (King of Prussia, Pa). Anti-RAGE, -S100/calgranulins, –proliferating cell nuclear antigen (PCNA), -TNFR, -p65, –extracellular signal-regulated kinase (ERK), and –vascular cell adhesion molecule-1 (VCAM-1) antibodies were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, Calif). Anti-ED1 antibody was purchased from Serotec (Kidlington, UK).

Animals
Male OZ rats and LZ rats matched for age were purchased from Charles River Laboratories (Wilmington, Mass). They were fed a standard chow and had free access to water. LZ and OZ rats were divided into 2 groups: an untreated group and a group treated with semapimod. The rats in the treated group received 5 mg/kg per day of semapimod intraperitoneally for 2 weeks before and after the cuff placement. The untreated rats received the same amount of normal saline. All animal studies were performed in accordance with the guidelines for animal care of Tokyo University.

Preparation of Protein Extracts and Enzyme-Linked Immunosorbent Assay
The concentrations of TNF-α, IL-1β, and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (BioSource International, Inc). The rat visceral fat, thoracic aorta, heart, and lungs were homogenized on ice in a Triton X-100 cell lysis buffer. After centrifugation for 20 minutes at 4°C, the supernatant was used for the assay. Serum concentration of C-reactive protein (CRP) was also measured with an ELISA kit (Alpha Diagnostic International, Inc).

Construction of Adenoviruses
Total RNA was extracted from the thoracic aortas of OZ rats with the use of TRIZOL reagent (GIBCO-BRL) according to the instructions provided by the manufacturer. Rat RAGE (RAGEwt), sRAGE corresponding to amino acids 1 to 336, and the carboxyl terminal–truncated TNFR (TNFRΔ) were isolated and subjected to PCR. The PCR primers used were as follows: rat RAGE sense, 5’-GAATTCCTCCCCAATGTTCA-3’; rat RAGE antisense, 5’-GCCGACACCGGAAAGT-3’; and rat GAPDH antisense, 5’-TTCCCCGTATGACCAGCT-3’. PCR-amplified products were subcloned into the pcDNA3 vector (Invitrogen, Carlsbad, Calif). The entire DNA sequence was determined by cycle sequence with the use of a CEQ8000 DNA sequencer (Beckman Coulter, Fullerton, Calif). Adenoviruses expressing RAGEwt (AdRAGEwt), sRAGE (AdSRAge), or TNFRΔ (AdTNFRΔ) were constructed as previously described. A recombinant adenovirus that expresses green fluorescence protein (AdGFP) was obtained from Quantum Biotechnologies (Montreal, Quebec, Canada).

Cuff Placement and Gene Transfer
A cuff was placed around the femoral artery as previously reported with slight modifications. Rats were anesthetized with pentobarbital injected intraperitoneally. The femoral artery was isolated, and a polyethylene tube (inner diameter, 0.58 mm; outer diameter, 0.965 mm; length, 5 mm) was placed around the artery. In some experiments, a virus suspension (1.0×10⁹ plaque-forming units) was injected into the femoral artery from its muscular branch immediately before the cuff placement as previously described. The vessels were incubated with the virus suspension for 30 minutes. The femoral artery was harvested 2 weeks after the cuff placement.

Histological Analysis and Immunohistochemistry
Histological analysis and immunohistochemistry were performed as previously described. Primary antibodies were used at a dilution of 1:100. Expression of RAGE, S100/calgranulins, p65, VCAM-1, and ED1 was evaluated by investigators blinded to the experimental protocol.

RNA Extraction and Real-Time PCR
RNA extraction and real-time PCR analysis were performed as previously described with the use of an SYBR Green dye. The primers used were as follows: rat RAGE sense, 5’-GAAATTCCTCCCCAATGTTCA-3’; rat RAGE antisense, 5’-GCCGACACCGGAAAGT-3’; rat GAPDH sense, 5’-GTATGACCTCTACCCACCGCAAGT-3’; and rat GAPDH antisense, 5’-TTCCCCGTATGACCAGCT-3’. To confirm that no significant amounts of primer dimers were formed, dissociation curves were analyzed.

Western Blot Analysis
Western blot analysis was performed as previously described.

Statistical Analysis
Values are mean±SEM. Statistical analyses were performed with ANOVA followed by the Student-Newman-Keuls test. Differences with a value of P<0.05 were considered statistically significant.

Results

Physical and Metabolic Features of OZ and LZ Rats
Body weight and systolic blood pressure measured by the tail-cuff method were significantly higher in 16-week-old OZ rats than in LZ rats matched for age. Serum glucose, triglycerides, and total cholesterol were significantly higher in OZ rats than in LZ rats. Semaipimod did not have any significant effect on these parameters in either LZ or in OZ rats (Table 1). There was no significant difference in heart rate among the 4 groups.

Serum and Tissue Levels of Adipocytokines
The concentrations of TNF-α, IL-1β, and IL-6 in serum and visceral fat were significantly higher in OZ rats than in LZ rats, and this increase was significantly suppressed by treatment with semapimod (Table 2). TNF-α and IL-1β levels in the thoracic aorta were significantly higher in OZ rats than in LZ rats, and this increment was also significantly suppressed by treatment with semapimod. In contrast, the concentrations of TNF-α, IL-1β, and IL-6 in the heart and lungs and those of IL-6 in the aorta did not differ significantly among the 4 groups. Semaipimod did not have any significant effect on these cytokine levels in LZ rats. We also measured serum
CRP concentration (Table 3). Serum CRP level was significantly higher in OZ rats than in LZ rats, and semapimod significantly suppressed CRP in OZ rats but not in LZ rats.

**Semapimod Inhibits Neointimal Formation in OZ Rats**

Neointimal formation was observed in the femoral artery 2 weeks after placement of the polyethylene cuff around the artery. The extent of neointimal formation was significantly higher in OZ rats than in LZ rats, as assessed by the ratio of the intimal to medial area (I/M ratio). Semaipimod significantly suppressed neointimal formation in OZ rats compared with untreated OZ rats (Figure 1A and 1C). In accordance with the results, the number of PCNA-positive proliferating cells in the neointima was significantly higher in OZ rats than in LZ rats, as assessed by the ratio of the number of PCNA-positive cells in the neointima (Figure 1B and 1D) in OZ rats compared with untreated LZ rats. Because semapimod did not significantly affect metabolic parameters, cytokine levels, or neointimal formation in LZ rats, we focused on the effects of semapimod in OZ rats in the following experiments.

It has been shown that RAGE is implicated in neointimal formation in diabetic animals. We therefore examined the expression of RAGE and its ligand S100/calgranulins in the wall of the sham-operated femoral artery and cuff-injured femoral artery (Figure 2). Expression of RAGE and S100/calgranulins was barely detected in the artery of sham-operated LZ and OZ rats (Figure 2A through 2C and 2G through 2I). In contrast, expression of both RAGE and S100/calgranulins was observed in the cuff-injured arteries of LZ and OZ rats. Immunoreactivity of RAGE and S100/calgranulins was remarkably upregulated in the neointima, especially in the endothelial layer, of cuff-injured OZ rats compared with LZ rats (Figure 2D, 2E, 2J, and 2K). Inter-

### Table 1. Physical and Metabolic Characteristics of the 4 Groups of Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LZ</th>
<th>LZ/Sem</th>
<th>OZ</th>
<th>OZ/Sem</th>
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<tr>
<td>Body weight, g</td>
<td>383.3±9.9</td>
<td>367.9±13.7</td>
<td>616.0±11.7*</td>
<td>605.0±9.9*</td>
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<tr>
<td>Heart rate, bpm</td>
<td>400.1±18.3</td>
<td>401.8±9.9</td>
<td>421.3±10.6</td>
<td>417.2±8.6</td>
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<td>Systolic blood pressure, mm Hg</td>
<td>138.5±1.6</td>
<td>138.1±1.5</td>
<td>167.7±1.9*</td>
<td>159.9±6.1*</td>
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<tr>
<td>Blood glucose, mg/dL</td>
<td>104.8±2.5</td>
<td>92.3±12.5</td>
<td>217.2±6.1*</td>
<td>223.3±12.1*</td>
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<tr>
<td>Triglyceride, mg/dL</td>
<td>95.7±18.0</td>
<td>94.4±4.2</td>
<td>383.7±57.0*</td>
<td>335.3±68.4*</td>
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<tr>
<td>Total cholesterol, mg/dL</td>
<td>87.8±9.9</td>
<td>88.0±2.2</td>
<td>200.0±18.2*</td>
<td>201.5±53.7*</td>
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</table>

Data are mean±SEM. LZ/Sem indicates semapimod-treated LZ rats; OZ/Sem, semapimod-treated OZ rats.

*P<0.05 vs LZ rats (n=6).

### Table 2. Cytokine Levels in Serum and Various Tissues

<table>
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<tr>
<th>Cytokine</th>
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<th>LZ/Sem</th>
<th>OZ</th>
<th>OZ/Sem</th>
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<td>TNF-α</td>
<td>Serum, pg/mL</td>
<td>19.8±4.0</td>
<td>21.7±3.8</td>
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<td>Visceral fat, pg/100 μg protein</td>
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<td>84.5±8.8</td>
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<td>Aorta, pg/100 μg protein</td>
<td>43.1±4.0</td>
<td>41.6±5.2</td>
<td>90.8±7.6*</td>
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<td></td>
<td>Heart, pg/100 μg protein</td>
<td>208.4±29.6</td>
<td>236.4±23.3</td>
<td>295.6±41.6</td>
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<tr>
<td></td>
<td>Lung, pg/100 μg protein</td>
<td>47.8±4.1</td>
<td>38.9±6.1</td>
<td>46.4±8.9</td>
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<tr>
<td>IL-1β</td>
<td>Serum, pg/mL</td>
<td>14.3±0.5</td>
<td>14.5±0.6</td>
<td>19.4±1.6*</td>
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<td></td>
<td>Visceral fat, pg/100 μg protein</td>
<td>188.3±27.8</td>
<td>195.2±14.6</td>
<td>483.0±98.8*</td>
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<td></td>
<td>Aorta, pg/100 μg protein</td>
<td>181.9±28.3</td>
<td>200.1±40.2</td>
<td>463.9±78.4*</td>
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<tr>
<td></td>
<td>Heart, pg/100 μg protein</td>
<td>478.1±45.7</td>
<td>475.6±27.8</td>
<td>470.0±42.8</td>
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<td>Lung, pg/100 μg protein</td>
<td>345.7±31.1</td>
<td>360.5±37.4</td>
<td>461.0±94.3</td>
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<td>IL-6</td>
<td>Serum, pg/mL</td>
<td>15.0±0.9</td>
<td>17.3±2.3</td>
<td>23.3±1.6*</td>
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<td>Visceral fat, pg/100 μg protein</td>
<td>35.3±4.7</td>
<td>33.5±3.1</td>
<td>89.3±1.7*</td>
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<td></td>
<td>Aorta, pg/100 μg protein</td>
<td>54.7±11.2</td>
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<td>57.6±13.9</td>
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<td></td>
<td>Heart, pg/100 μg protein</td>
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<td>478.3±25.1</td>
<td>494.6±72.9</td>
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<tr>
<td></td>
<td>Lung, pg/100 μg protein</td>
<td>38.7±3.1</td>
<td>41.0±2.1</td>
<td>39.3±8.0</td>
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</tbody>
</table>

Data are mean±SEM. LZ/Sem indicates semapimod-treated LZ rats; OZ/Sem, semapimod-treated OZ rats.

*P<0.05 vs LZ rats; †P<0.05 vs OZ rats (n=8).
estingly, staining of RAGE and S100/calgranulins was decreased in OZ rats administered semapimod compared with untreated OZ rats (Figure 2F and 2L).

**Effect of Adenovirus-Mediated Overexpression of RAGEwt and sRAGE on Neointimal Formation**

To examine whether RAGE plays a central role in neointimal formation and whether semapimod inhibits neointimal formation via suppression of RAGE expression, we infected the cuff-injured femoral artery with adenovirus expressing RAGEwt (AdRAGEwt) and sRAGE (AdsRAGE). Expression of RAGEwt and sRAGE was confirmed by Western blot analysis (Figure 3A). We first injected the viral suspension of AdRAGEwt into the femoral artery to confirm that the adenoviral construct was effectively expressed in the femoral artery. RAGE was predominantly expressed in the endothelial layer of the femoral artery 2 weeks after AdRAGEwt infection, whereas no remarkable staining was observed when AdGFP was injected as the negative control (Figure 3B). We then infected the cuff-injured femoral artery of OZ rats with AdsRAGE and examined its effect on neointimal formation. AdsRAGE infection significantly inhibited neointimal formation in OZ rats compared with AdGFP infection, confirming that RAGE plays a critical role in neointimal formation in OZ rats (Figure 3C, 3D, and 3G). We next infected the cuff-injured femoral artery of OZ rats administered semapimod with AdRAGEwt. The inhibitory effect of semapimod on neointimal formation was overcome by AdRAGEwt, and neointimal formation was significantly more marked in the AdRAGEwt-infected femoral artery than in the AdGFP-infected artery of OZ rats administered semapimod (Figure 3E, 3F, and 3H). These results suggested that semapimod inhibited neointimal formation, at least partly, by suppressing RAGE expression.

Expression of a Dominant Negative Mutant of TNFR Inhibits Neointimal Formation and RAGE Expression

It has been reported that TNF-α stimulates RAGE expression in human skin microvascular endothelial cells (HMVECs).16

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**Table 3. Serum CRP Levels in the 4 Groups of Rats**

<table>
<thead>
<tr>
<th></th>
<th>LZ</th>
<th>LZ/Sem</th>
<th>OZ</th>
<th>OZ/Sem</th>
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<tbody>
<tr>
<td>CRP, μg/mL</td>
<td>338.5±51.9</td>
<td>349.7±70.1</td>
<td>633.2±111.1*</td>
<td>372.8±64.1†</td>
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</table>

Data are mean±SEM. LZ/Sem indicates semapimod-treated LZ rats; OZ/Sem, semapimod-treated OZ rats.

*P<0.05 vs LZ rats; †P<0.05 vs OZ rats (n=6).
To examine more specifically the role of endogenous cytokines, especially TNF-α, in semapimod-mediated inhibition of RAGE expression, we constructed an adenovirus that expresses a dominant negative mutant of TNFR that lacks the intracellular signal transduction domain (AdTNFRΔC). We first tested whether this construct worked as a dominant negative mutant of TNF-α signaling. We infected cultured vascular smooth muscle cells with AdTNFRΔC and examined whether TNF-α-induced phosphorylation of ERK was inhibited by AdTNFRΔC infection. Expression of TNFRΔC was confirmed by Western blot analysis (Figure 4A). AdTNFRΔC remarkably inhibited TNF-α-induced ERK phosphorylation (Figure 4B). We then infected the cuff-injured femoral artery of OZ rats with AdTNFRΔC and examined its effect on neointimal formation. We also examined whether AdTNFRΔC inhibited the expression of RAGE and S100/calgranulins. Infection of AdTNFRΔC significantly suppressed neointimal formation compared with AdGFP infection. AdTNFRΔC infection remarkably suppressed the immunoreactivity of RAGE and S100/calgranulins compared with AdGFP infection (Figure 4C and 4D).

We also performed real-time PCR to quantitatively analyze the expression of RAGE in the femoral artery. Expression of RAGE mRNA was significantly enhanced in the cuff-injured femoral artery of OZ rats compared with LZ rats. Treatment of OZ rats with semapimod significantly inhibited the expression of RAGE mRNA compared with untreated OZ rats. AdTNFRΔC infection also significantly suppressed RAGE mRNA expression in the cuff-injured femoral artery of OZ rats compared with that observed in AdGFP infection (Figure 5).

**Expression of p65 Subunit of Nuclear Factor-κB in the Neointima Is Mediated by TNF-α and RAGE**

It is well known that nuclear factor-κB (NF-κB) is activated by the RAGE-mediated pathway as well as by TNF-α.17,18 It is also reported that NF-κB is implicated in TNF-α induction of RAGE expression.16 To examine whether NF-κB is activated in the neointima, we examined the expression of the p65 subunit of NF-κB in the neointima of LZ and OZ rats by immunohistochemical analysis. p65 was found to express in the neointima of LZ rats (Figure 6). Immunoreactivity of p65 was remarkably enhanced in the neointima of OZ rats, and semapimod remarkably suppressed p65 staining in the neointima. Interestingly, AdGFP infection remarkably augmented the immunoreactivity of p65 in the neointima of OZ rats compared with AdGFP infection. Furthermore, infection with AdsRAGE or AdTNFRΔC substantially inhibited p65 staining in the neointima of OZ rats compared with AdGFP infection. These results suggested that TNF-α activated the NF-κB-dependent pathway in the neointima, at least in part, via stimulation of RAGE expression.

**Expression of VCAM-1 and Macrophage Infiltration in the Neointima Are Mediated by TNF-α and RAGE**

It has been shown that macrophages are critically implicated in neointimal formation.19,20 To further examine the mechanisms by which endogenous TNF-α and RAGE stimulate neointimal formation in OZ rats, we analyzed the expression of VCAM-1, which mediates adhesion of monocytes to the endothelial cells and infiltration of monocytes/macrophages in the vessel wall.21,22 Immunoreactivity of VCAM-1 was remarkably enhanced in the neointima of OZ rats compared with LZ rats (Figure 7). S100/calgranulins remarkably inhibited VCAM-1 staining in the neointima. AdGFP infection substantially augmented VCAM-1 staining in the neointima of OZ rats compared with AdGFP infection. Furthermore, infection with AdsRAGE or AdTNFRΔC remarkably suppressed VCAM-1 staining in the
neointima of OZ rats compared with AdGFP infection. Consistent with these results, macrophage infiltration in the neointima significantly increased in OZ rats compared with LZ rats, and semapimod significantly inhibited macrophage infiltration. AdRAGEwt infection significantly stimulated macrophage infiltration in the neointima of OZ rats administered semapimod compared with AdGFP infection. Furthermore, infection with AdsRAGE or AdTNFRΔC significantly suppressed macrophage infiltration in OZ rats compared with AdGFP infection. These results suggested that endogenous TNF-α was implicated in neointimal formation in OZ rats, at least partly, via stimulation of VCAM-1 expression and recruitment of macrophages in the neointima. These results also suggested that TNF-α induced VCAM-1 expression and macrophage infiltration, at least in part, via stimulation of RAGE expression.

**Discussion**

Although it has become apparent that RAGE and its ligands, AGE and S100/calgranulins, are critically implicated in the development of neointimal formation after balloon injury as well as in atherosclerosis in animal models of DM, the mechanisms by which expression of RAGE is augmented in DM remain unclear. One explanation was that AGE per se stimulated RAGE expression. On the other hand, it was demonstrated in the same report that TNF-α stimulated RAGE expression in HMVECs. Because production of cytokines such as TNF-α and IL-6 was reportedly enhanced in adipose tissue in a diabetic state, we hypothesized that these cytokines would be implicated in the enhanced RAGE expression in the vessel wall and in neointimal formation in a diabetic state. To test this hypothesis, we utilized 2 strategies to block the actions of these endogenous cytokines. One was semapimod, a pharmacological inhibitor of proinflammatory cytokine production. The other was an adenovirus construct that expresses a dominant negative mutant of TNFR and blocks the signaling of TNF-α.

Although semapimod was originally reported to inhibit the production of cytokines such as TNF-α in monocytes/macrophages at the level of translation, it effectively suppressed production of TNF-α and IL-β in visceral adipose tissue and the thoracic aorta of OZ rats. It also significantly suppressed the production of IL-6 in visceral adipose tissue of OZ rats. Thus, this compound seemed to be suitable to suppress the production of endogenous cytokines in the adipose tissue and blood vessels of diabetic animals. Neointimal formation was significantly augmented in the cuff-injured femoral artery of OZ rats compared with that observed in LZ rats. Semapimod significantly inhibited neointimal formation in OZ rats. Staining of RAGE and S100/calgranulins was remarkably enhanced in the cuff-injured femoral artery of OZ rats compared with LZ rats. Semapimod remarkably inhibited the immunoreactivity of RAGE and S100/calgranulins in OZ rats. Furthermore, this inhibitory effect of semapimod on neointimal formation in OZ rats was overcome by forced expression of RAGE with the use of AdRAGEwt. These results suggested that semapimod inhibited neointimal formation, at least partly, by suppressing the expression of RAGE. These results also suggested that endogenous cytokines such as TNF-α were involved in the expression of RAGE.

To examine more specifically the role of endogenous cytokines in neointimal formation and RAGE expression, we used an adenovirus construct that expresses a dominant negative mutant of TNFR. Neointimal formation in the cuff-injured femoral artery of OZ rats was significantly suppressed by AdTNFRΔC infection compared with AdGFP infection. AdTNFRΔC infection also inhibited the immunoreactivity of RAGE and S100/calgranulins in OZ rats compared with AdGFP infection. Furthermore, AdTNFRΔC infection significantly suppressed the expression of RAGE mRNA in the cuff-injured femoral artery of OZ rats compared with AdGFP infection as assessed by real-time PCR. These results strongly suggested that endogenous TNF-α was implicated in RAGE expression and neointimal formation in the cuff-injured femoral artery of OZ rats. Our results also
suggested that visceral fat and/or blood vessel itself might be
the origin of TNF-α that enhanced vascular inflammation.

RAGE and S100/calgranulins were expressed predominantly in the endothelial layer, as assessed by immunohistochemical analysis, in our model. Previous reports showed broad staining of RAGE and S100/calgranulins in the neointima after endothelial denudation.5,6 The difference in their localization probably resulted from the fact that the endothelium was not mechanically denuded in our model. Interestingly, although expression of RAGE and S100/calgranulins was largely restricted to the endothelial layer, a significant amount of neointimal formation (proliferation of vascular myocytes) was observed. Furthermore, although RAGE was predominantly expressed in the endothelial layer after injection of AdRAGEwt into the femoral artery in our model, it effectively induced neointimal formation. These results suggested that activation of RAGE in the endothelium was sufficient to initiate the cascades of vascular inflammation to induce neointimal formation. It was reported that the activation of AGE/RAGE stimulated the expression of VCAM-1 in cultured vascular endothelial cells.25 Because it is well known that VCAM-1 stimulates the recruitment of monocytes/macrophages in the vessel wall12,22 and that macrophages play critical roles in neointimal formation,19,20 it was possible that RAGE expressed in the endothelium was implicated in neointimal formation via stimulation of VCAM-1 expression and macrophage infiltration. To test this hypothesis, we examined the expression of VCAM-1 in the neointima of OZ rats. Expression of VCAM-1 was remarkably increased in the neointima of OZ rats, and infection with AdsRAGE or AdTNFRαC substantially suppressed VCAM-1 expression. In accordance with these results, macrophage infiltration was significantly inhibited by infection with AdsRAGE or AdTNFRαC. Thus, our results suggested the possibility that RAGE, whose expression was induced by TNF-α in the endothelium, enhanced neointimal formation via stimulation of VCAM-1 expression and macrophage infiltration.

Serum CRP concentration was significantly higher in OZ rats than in LZ rats, and semapimod significantly suppressed CRP in OZ rats. These results were compatible with previous

Figure 5. Quantitative analysis of RAGE mRNA expression in the cuff-injured femoral artery. A, Statistical analysis of RAGE expression in the femoral artery. Total RNA was extracted from the femoral artery of LZ, OZ, semapimod-treated OZ, AdGFP-infected OZ, and AdTNFRαC-infected OZ rats 2 weeks after the cuff placement. Real-time PCR analysis was performed to evaluate the amount of RAGE and GAPDH mRNA (n=6). B, Typical photograph showing ethidium bromide staining of the PCR-amplified products.

Figure 6. Expression of the p65 subunit of NF-κB in the neointima of LZ and OZ rats. Expression of p65 in the neointima of LZ rats, OZ rats, and OZ rats administered semapimod (Sem) was examined histologically. Expression of p65 in the neointima was also analyzed in OZ rats administered semapimod that had been infected with AdGFP or AdRAGEwt and in OZ rats infected with AdGFP, AdsRAGE, or AdTNFRαC. Arrowheads indicate the internal elastic lamina.
reports that serum CRP levels correlated well with the indicators of obesity, such as body mass index, in humans.\(^{26,27}\) Although the mechanisms by which semapimod suppressed serum CRP remain unclear, it is possible that semapimod inhibited CRP by suppressing the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 because it has been reported that these cytokines stimulate CRP production in the liver and vascular smooth muscle cells.\(^{28,29}\) Future studies will be required to clarify the mechanisms by which serum CRP levels increase in obesity.

In summary, endogenous cytokines, especially TNF-α, seemed to be involved in the enhancement of RAGE and S100/calgranulins expression and in neointimal formation in OZ rats. Blocking the action of these cytokines may be a promising strategy to modulate vascular diseases such as atherosclerosis and restenosis after angioplasty, which are often observed in a diabetic state.

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