Remnant Lipoproteins Induce Proatherothrombogenic Molecules in Endothelial Cells Through a Redox-Sensitive Mechanism

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Background—Triglyceride-rich lipoproteins (TGLs) are atherogenic. However, their cellular mechanisms remain largely unexplained. This study examined the effects of isolated remnant-like lipoprotein particles (RLPs) on the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and tissue factor (TF), proatherothrombogenic molecules, in cultured human endothelial cells.

Methods and Results—RLPs were isolated from plasma of hypertriglyceridemic patients by use of the immunoaffinity gel mixture of anti–apoA-1 and anti–apoB-100 monoclonal antibodies. The incubation of cells with RLPs significantly upregulated mRNA and protein expression of these molecules. Total TGLs (d < 1.006) and LDL had fewer or minimal effects on expression of these molecules compared with RLPs. RLPs increased intracellular oxidant levels, as assessed with an oxidant-sensitive probe. Combined incubation with α-tocopherol or N-acetylcysteine, both antioxidants, suppressed RLP-induced increase in expression of these molecules. In patients with higher plasma levels of RLPs, plasma levels of soluble forms of ICAM-1 and VCAM-1 were significantly higher than in patients with lower RLP levels. Treatment with α-tocopherol for 1 month decreased levels of the soluble adhesion molecules concomitantly with an increase in resistance of RLPs to oxidative modification in patients with high RLP levels.

Conclusions—RLPs upregulated endothelial expression of ICAM-1, VCAM-1, and TF, proatherothrombogenic molecules, partly through a redox-sensitive mechanism. RLPs may have an important role in atherothrombotic complications in hypertriglyceridemic patients. (Circulation. 2000;102:670-676.)

Key Words: lipoproteins ■ endothelium ■ cell adhesion molecules ■ antioxidants ■ atherosclerosis

There is increasing evidence that triglycerides and triglyceride-rich lipoproteins (TGLs) carry a risk for coronary artery disease (CAD).1,2 However, cellular mechanisms for the relation of hypertriglyceridemia with atherosclerotic development remain largely unexplained, in contrast to hypercholesterolemia, in which oxidatively modified LDLs contribute importantly to atherogenesis.3 We recently showed that remnant-like lipoprotein particles (RLPs), isolated by an immunoseparation method, have a causative role in endothelial vasomotor dysfunction in human coronary arteries and that RLPs directly induced endothelial dysfunction in the isolated rabbit aorta.4,5 Thus, RLPs may have a direct role in endothelial dysfunction, an early event leading to atherosclerotic development, in hypertriglyceridemic patients. Indeed, we recently demonstrated that high RLP levels predict coronary events in patients with CAD independently of traditional coronary risk factors.6 Most individuals spend ≥12 hours daily in a postprandial state; thus, postprandial RLPs may play a more important role in atherogenesis in hypertriglyceridemic patients than fasting RLPs.

Altered or activated endothelial functions play an important role in atherogenesis through a variety of endothelium-derived proatherothrombogenic molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and tissue factor (TF).7–9 This study was thus aimed at examining the effects of isolated postprandial RLPs on endothelial production of these molecules, considering a possible relevance of RLPs in atherothrombogenesis.

Methods

Lipoprotein Preparations

EDTA-plasma (1 mg/mL EDTA) was obtained at 5 hours after the test meal from 18 hypertriglyceridemic subjects who had fasting RLP levels of >5.1 mg cholesterol/dL (75th percentile of the distribution of fasting RLP levels in 250 consecutive hospitalized patients in our hospital). The postprandial RLP levels were 12.2 ± 2 mg cholesterol/dL. The energy content of the test meal was 490 kcal/m² body surface area (86.8% from fat, 8.7% from carbohydrate, and 4.5% from protein; the ratio of polyunsaturated fatty acids to saturated fatty acids was 0.51). The subjects had no serious diseases.
and had not taken cardiovascular medications, pharmacological doses of antioxidants, or estrogen for ≥7 days. Patients with familial hypertriglyceridemia were not included in the study subjects.

RLPs were routinely prepared with columns (1.5×30 cm) packed with 50 mL of immunoaffinity gel containing anti–apoA-1 and anti–apoB-100 monoclonal antibodies (Japan Immunoresearch Laboratories).4–6,10 This unique anti–apoB-100 antibody has been shown to recognize apoB-100 in LDL and most VLDLs but not in apoE-enriched VLDL.10 The plasma samples (5 mL) from each subject were applied to the columns, and the unbound fraction containing apoB-enriched lipoproteins and albumin was eluted with PBS (mmol/L: NaCl 138, KCl 2.7, NaH₂PO₄ 8.1,KH₂PO₄ 1.1; pH 7.4) at a rate of 30 mL/h for 6 hours at 4°C. The bound fraction was subsequently eluted at a rate of 30 mL/h for 3 hours with 3 mol/L NaSCN containing 1 mg/mL BSA (fatty acid–free BSA) and was immediately dialyzed against EDTA/saline (pH 7.4). The unbound and bound fractions were ultracentrifuged (d<1.006) to isolate RLPs and bound TGLs, respectively, and they were concentrated by the membrane filtration method. According to analyses4–8 with SDS-PAGE, elution profiles with high-performance liquid chromatography (HPLC), agarose gel electrophoretograms, electron photomicrographs, and compositions of lipids and apolipoproteins, the unbound fraction isolated by this method from plasma 5 hours after the meal consisted mainly of VLDL remnants and small amounts of chylomicron remnants (amount ratio of apoB-48 relative to apoB-100 was 0.13±0.01, determined by densitometric analysis on SDS-PAGE). Total TGLs and LDLs were isolated from the same EDTA-plasma as used for RLP isolation by ultracentrifugation (d<1.006) for total TGLs, 1.019<d<1.063 for LDLs). The prepared lipoproteins were extensively dialyzed for 24 hours at 4°C against PBS containing EDTA (50 μmol/L) and then sterilized by filtration (filter pore size, 0.22 μm; Millipore). Total cholesterol and triglycerides were measured by enzymatic methods.4 Final concentrations of the prepared lipoproteins were 4.5 to 6.5 mg cholesterol/mL.

In Vivo Treatment With α-Tocopherol

A consecutive series of 74 patients who underwent cardiac catheterization for atypical chest pain in Kumamoto University Hospital were studied for a possible relation of RLP levels with plasma levels of soluble forms of ICAM-1 and VCAM-1. Of these patients, 12 who had higher plasma levels of fasting RLPs were studied for a possible relation of RLP levels with plasma levels of soluble forms of ICAM-1 (sICAM-1) and VCAM-1, respectively (R&D Systems).11 Standard curves assayed for measurements of expression of mRNA and protein and lipid peroxides.

Cell Culture

Primary cultures of human umbilical vein endothelial cells (HUVECs) were obtained as previously described.12 Confluent HUVECs at passage 2 were used in this study. After serum starvation for 8 hours, the medium was replaced with serum-free medium 199, and the cells were then incubated with one of the lipoprotein preparations in the presence or absence of α-tocopherol or η-aminocysteine (NAC) for 5 hours at 37°C in the dark. The treated cells were assayed for measurements of expression of mRNA and protein and lipid peroxides.

Cell-Surface ELISA for Adhesion Molecules on HUVECs

After treatment in 96-well microplates, the cells were rinsed and incubated with 0.5% periodic acid at 4°C for 20 minutes for inactivation of endogenous peroxidase. Then, the cells were rinsed and treated with mouse anti-human ICAM-1 (Dako) or VCAM-1 monoclonal antibodies (Coulter-Immunotech) at 4°C for 2 hours. The plates were subsequently washed with PBS and then treated with peroxidase-conjugated goat anti-mouse IgG at 4°C for 1 hour. The plates were then washed and incubated with 0.1 mL/well of 3,3',5,5'-tetramethylbenzidine substrate (Dako) at 4°C for 30 minutes. The reaction was stopped by addition of 50 μL of 1N hydrochloric acid and 3N sulfuric acid mixture. The plates were read on an ELISA reader (M-Emax, Wako) at OD 450 nm after blanking on rows stained only with second-step antibody.13

Assays of Soluble Forms of Adhesion Molecules in Culture Medium

After the treatment of HUVECs in 12-well plates for 12, 24, and 36 hours, the conditioned medium was collected and then centrifuged at 20 000 g for 10 minutes to remove cell debris. The levels of the soluble forms of the adhesion molecules in the culture medium were measured with the same ELISA kits as described above.

Determination of TF Protein

After treatment in 6-well plates, the cells were detached by addition of cold PBS containing 1% Triton X-100 and stirred for 12 hours at 4°C. The suspension was centrifuged to separate cell debris, and TF antigen levels in the cell extracts were measured by ELISA (Imubind TF ELISA Kit, Loxo GmbH).14

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from the treated cells by the guanidine thiocyanate method.15 Northern blot analysis was then performed by loading of 20 μg of RNA in each lane of 1% agarose-formaldehyde gels, electrophoretic separation, transfer to nylon membranes (Schleicher & Schuell), and ultraviolet cross-linking. Complementary cDNA probes were 32P-labeled by the random primer method to a specific activity of ~5×10⁶ cpm/μg DNA. The cDNA probes in this study included the following: (1) a 1.8-kbp cDNA probe for ICAM-1 and a 700-bp cDNA probe for VCAM-1 (a kind gift from Dr Ron Cob, Tanabe Research Laboratories, San Diego, Calif); (2) a 641-bp cDNA probe for TF (a kind gift from Dr Lindsey A. Miles, The Scripps Research Institute, La Jolla, Calif); and (3) a 1-kb cDNA probe for GAPDH. The membranes were hybridized with the ICAM-1 probe, or VCAM-1, or TF probe. The membranes were hybridized with the GAPDH probe to normalize the amount of ICAM-1, VCAM-1, and TF mRNA. The intensity of hybridization signals was determined by use of an FLA 2000 (Fujiﬁlm).

Electrophoretic Mobility Shift Assay

Nuclear extraction from the treated cells and electrophoretic mobility shift assay were performed as described previously.16 The sequence of the probe for nuclear factor (NF)-κB used in this study was 5'-CCAGAGGGGGACTTTCGCCAGAGG-3'.

Measurements of Intracellular Oxidant Levels, Lipid Peroxide Levels in the Medium, and Susceptibility of RLPs to Oxidative Modification

Intracellular levels of reactive oxygen species were measured by flow cytometric analysis using an oxidant-sensitive fluorescence probe, 2',7'-dichlorofluorescein diacetate (DCFH-DA, Eastman Kodak) as previously described.17 After the treatment, the cells were incubated with phenol red-free medium 199 containing 5 μmol/L DCFH-DA for 30 minutes at 37°C in the dark. Then, the cells were washed 3 times with cold PBS (4°C) and detached by addition of cold PBS containing 0.05% EDTA. The suspended cells were washed and immediately analyzed with a fluorescence-activated cell
sorter (FACScan, Becton Dickinson). For each analysis, 10,000 events were recorded.

Lipid peroxide levels in the culture medium were measured by colorimetric assay (Bioxytech, OXIS International, Inc).

The susceptibility of RLPs to oxidative modification was determined by measuring Cu²⁺-induced formation of conjugated dienes. The conjugated diene formation in RLPs (0.1 mg triglyceride/mL) was monitored by the change in absorbance at 234 nm in a spectrophotometer.

Reagents
All reagents for cell culture were from Gibco BRL. NAC, α-tocopherol, and other chemicals were purchased from Sigma Chemical Co. Poly (dI-dC) was from Pharmacia Biotech Inc. [α-32P]CTP and [γ-32P]ATP were from Amersham Corp. Antibody raised against p50, p65, RelB, and c-Rel were from Santa Cruz Biotechnology Inc.

Statistical Analysis
All values were expressed as mean±SEM otherwise indicated. The difference between 2 mean values was analyzed with unpaired Student's t test. The mean values in >3 groups were compared by 1-way ANOVA, then the difference between 2 mean values was analyzed with Fisher’s protected least significant difference test. The effects of treatments on the plasma levels of soluble adhesion molecules in patients were compared by 2-way ANOVA with repeated measures followed by post hoc testing with the Scheffe test. A value of P<0.05 was considered statistically significant.

Results

Effects of RLPs on mRNA and Protein Expression of ICAM-1, VCAM-1, and TF and Levels of sICAM-1 and sVCAM-1 in Culture Medium
The incubation of HUVECs with RLPs increased mRNA levels of ICAM-1, VCAM-1, and TF, as shown in Figure 1. Furthermore, RLPs increased cell surface expression of ICAM-1 and VCAM-1 protein and TF protein levels in cell homogenates, as shown in Figure 2. The effects were dose-dependent, and similar relative values were obtained if triglycerides were used as lipoprotein concentrations (data not shown). Bound TGLs, total TGLs, and LDL had fewer or minimal effects on mRNA and protein expression of...
ICAM-1, VCAM-1, and TF compared with RLPs, as shown in Figures 1 and 2. Combined incubation with α-tocopherol or NAC suppressed the RLP-induced mRNA and protein expression of these molecules, as shown in Figures 3 and 4. The inhibitory effects of these antioxidants were dose-dependent, i.e., 50 to 150 μmol/L of α-tocopherol suppressed the increase in the cell surface expression of ICAM-1 by 50% to 80%, and 0.1 to 10 mmol/L of NAC suppressed it by 50% to 70%. In human coronary artery endothelial cells (Applied Cell Biology Research Institute), RLPs also caused an increase in cell surface protein expression of adhesion molecules, which was suppressed by α-tocopherol and NAC (data not shown). RLPs isolated from the patients after treatment with α-tocopherol for 4 weeks had significantly less effect on mRNA and protein expression of ICAM-1 and VCAM-1 than RLPs from patients before treatment (mRNA, 43.3±6.4% and 35.0±5.4% of the respective pretreatment values; protein, 34.8±6.4% and 48.9±9.2% of the respective pretreatment values, n=6).

RLPs (0.2 mg cholesterol/mL) increased the levels of sICAM-1 and sVCAM-1 in the culture medium in a time-dependent manner, but the combined incubation with α-tocopherol (20 μmol/L) and NAC (10 mmol/L) suppressed the RLP-induced increase in these levels (sICAM-1 and sVCAM-1: 24 hours after incubation with PBS, 1.4±0.1 and 4.8±0.1 ng/mL; with RLPs alone, 2.6±0.2* and 6.0±0.2* ng/mL; with RLPs+α-tocopherol, 1.6±0.1# and 5.2±0.1# ng/mL; with RLPs+NAC, 1.5±0.1# and 5.4±0.1# ng/mL, respectively; n=6 in each experiment; *P<0.01 versus PBS, #P<0.05 versus RLPs alone).

Endotoxin levels, measured by the Limulus assay, were very low in all of the lipoprotein preparations, namely, <10 pg/0.1 mg cholesterol of lipoprotein, at which level there was...
no significant effect on the protein expression of the adhesion molecules.

Quantification of Lipid Peroxides in Cells and in Culture Medium

In flow cytometric analysis, 2',7'-dichlorofluorescein fluorescence intensity was increased in the cells after 1 hour of treatment with RLPs (0.1 mg cholesterol/mL) compared with the time-control cells (mean peak flow intensities were 19.2±1.9 [control] versus 65.5±2.9 [RLPs], n=6, P<0.001) (Figure 5). The increase in the fluorescence intensity was suppressed by combined incubation with α-tocopherol (100 μmol/L) (mean peak flow intensities were 65.5±2.9 [RLPs] versus 39.6±1.3 [RLPs+α-tocopherol], n=6, P<0.001) (Figure 5). Incubation of HUVECs with RLPs (0.1 mg cholesterol/mL) increased lipid peroxide levels in the culture medium (125.9±2.6 nmol · L⁻¹ · 6 h⁻¹ before the incubation versus 385.1±59.1 nmol · L⁻¹ · 6 h⁻¹ after the incubation, n=6, P<0.001).

Effects of RLPs on DNA-Binding Activity of NF-κB

Incubation of the cells with RLPs for 90 minutes increased DNA-binding activity of NF-κB, as shown in Figure 6A. Combined incubation with α-tocopherol attenuated the NF-κB activation by RLPs (Figure 6B).

Effects of In Vivo Treatment With α-Tocopherol on Plasma Levels of Adhesion Molecules

Plasma levels of sICAM-1 and sVCAM-1 were higher in patients with higher RLP levels (>5.1 mg cholesterol/dL, 75th percentile of the RLP distribution, n=12) than those with lower RLP levels (<2.4 mg cholesterol/dL, 25th percentile, n=12) (sICAM-1, 406±34 ng/mL in patients with higher RLP levels versus 231±19 ng/mL in patients with lower RLP levels, P<0.001; sVCAM-1, 659±39 ng/mL in patients with higher RLP levels versus 462±29 ng/mL in patients with lower RLP levels, P<0.001). Treatment with α-tocopherol for 4 weeks significantly suppressed plasma levels of both adhesion molecules in patients with higher RLP levels (sICAM-1, 406±34 ng/mL before treatment versus 326±20 ng/mL after treatment, n=6, P<0.001; sVCAM-1, 659±39 ng/mL in patients with higher RLP levels versus 462±29 ng/mL in patients with lower RLP levels, P<0.001). Treatment with α-tocopherol for 4 weeks significantly suppressed plasma levels of both adhesion molecules in patients with higher RLP levels (sICAM-1, 406±34 ng/mL before treatment versus 326±20 ng/mL after treatment, n=6, P<0.001; sVCAM-1, 659±39 ng/mL in patients with higher RLP levels versus 462±29 ng/mL in patients with lower RLP levels, P<0.001). Treatment with placebo had no effect (sICAM-1, 379±32 ng/mL before treatment versus 361±43 ng/mL after treatment, n=6, P=NS; sVCAM-1, 615±39 ng/mL before treatment versus 613±33 ng/mL after treatment, n=6, P=NS). The lag time of the oxidation of RLPs isolated from patients under treatment with α-tocopherol was longer than that of RLPs before treatment (438±98 versus 253±21 minutes, respectively.)
Discussion
The present study showed that RLPs upregulated endothelial expression of ICAM-1 and VCAM-1, which are responsible for monocyte recruitment into the arterial walls,7,8 and TF, which is essential for thrombotic events,9 at the same range of RLP concentrations as in peripheral plasma in patients with CAD (0.05 to 0.5 mg cholesterol/mL). Thus, high plasma levels of RLPs may have an important role in development of atherosclerosis and thrombotic events through endothelial upregulation of these proatherothrombogenic molecules. Furthermore, the present study showed that RLPs increased the endothelial release of sICAM-1 and sVCAM-1 into the culture medium and that plasma levels of both sICAM-1 and sVCAM-1 were increased in patients with higher RLP levels compared with those with the lower levels. Although the tissue sources of plasma sICAM-1 and sVCAM-1 are multiple, the plasma levels might be derived partly from the activated vascular endothelium.9,11,13,19 Thus, these data may support potentially stimulatory effects of RLPs on endothelial expression of adhesion molecules and their release in vivo in patients with high RLP levels. The present study further showed that RLPs increased intracellular oxidant levels in the cultured cells and that α-tocopherol and NAC suppressed RLP-induced increases in mRNA and protein expression of ICAM-1, VCAM-1, and TF. These results suggest that the induction of these molecules by RLPs was at least partly mediated by redox-sensitive mechanisms.

Several mechanisms for the RLP-induced increase in oxidative stress in endothelial cells can be considered. During incubation of endothelial cells with RLPs, endothelium-derived reactive oxygen species may initiate and propagate a chain of free radical reactions, especially in polyunsaturated fatty acids in RLPs, leading to production of highly reactive intermediates that could, in turn, be transferred from RLPs to endothelial cells.20,21 This sequence of events may cause an increase in oxidative stress in the cultured endothelial cells. This mechanism is supported by the present results showing the increase in intracellular oxidant levels and in lipid peroxide levels in the culture medium containing RLPs after the incubation of the endothelial cells with RLPs. In patients with high levels of remnant lipoproteins, remnants can flow into the subendothelial space, where the same interaction between remnants and endothelial cells as observed in the present in vitro study could occur and result in the endothelial upregulation of these redox-sensitive molecules. Indeed, the present study further showed that administration of α-tocopherol, an antioxidant, decreased plasma levels of both sICAM-1 and sVCAM-1 in patients with high RLP levels concomitantly with the increase in resistance of RLPs to Cu2+-induced oxidation. RLPs isolated from the patients under treatment with α-tocopherol also had fewer effects on mRNA and protein expression of adhesion molecules in the cultured cells.

The present study did not examine the transcriptional activity of these genes and the downstream signals leading to the upregulation of these proatherothrombogenic genes by oxidant stress. However, these genes are known to have oxidative stress–responsive elements, such as NF-κB, in their promoter/enhancer regions.22,23 The present study showed that RLPs activated the DNA-binding activity of NF-κB in endothelial cells, which was inhibited by coinubcation with α-tocopherol. Thus, it is possible that RLPs may cause an increase in oxidative stress, leading to transcriptional activation of these genes in endothelial cells, possibly through a mechanism mediated by the activation of oxidative stress–responsive elements. It remains largely undefined why the cellular effects were greater in RLPs than in total TGLs. We previously reported that RLPs induced endothelial dysfunction in an apolipoprotein receptor–independent manner.4 It is possible that smaller size and different lipid structure in RLPs may be partly responsible for the greater cellular effects of RLPs than of their precursors, ie, unmetabolized VLDLs and chylomicrons. Although a pathophysiological role and clinical usefulness of the RLP fraction in atherogenesis have been demonstrated,4–6,24 it is not yet established that RLPs are completely identical with remnant lipoproteins because of their heterogeneous characteristics in size and composition.

A previous in vitro experiment showed that RLPs were taken up by macrophages and caused foam cell formation.24 Furthermore, RLPs caused endothelial vasomotor dysfunction, as shown in our previous reports.4,5 Very recently, we showed that high RLP levels predict coronary events in patients with CAD independently of traditional coronary risk factors.6 Thus, the previous data and the present results suggest that these proatherothrombogenic properties of RLPs may play an important role in the cardiovascular events in hypertriglyceridemic patients.

In conclusion, RLPs induced ICAM-1, VCAM-1, and TF in cultured endothelial cells through redox-sensitive mechanisms. Thus, remnant lipoproteins may have a direct and causative role in atherothrombotic development in hypertriglyceridemic patients.

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