Preventive Effects of Heregulin-β1 on Macrophage Foam Cell Formation and Atherosclerosis

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Rationale: Human heregulins, neuregulin-I type I polypeptides that activate proliferation, differentiation, and survival of glial cells, neurons, and myocytes, are expressed in macrophage foam cells within human coronary atherosclerotic lesions. Macrophage foam cell formation, characterized by cholesterol ester accumulation, is modulated by scavenger receptor class A (SR-A), acyl-coenzyme A:cholesterol acyltransferase (ACAT)1, and ATP-binding cassette transporter (ABC)A1.

Objective: The present study clarified the roles of heregulins in macrophage foam cell formation and atherosclerosis.

Methods and Results: Plasma heregulin-β1 levels were significantly decreased in 31 patients with acute coronary syndrome and 33 patients with effort angina pectoris compared with 34 patients with mild hypertension and 40 healthy volunteers (1.3±0.3, 2.0±0.4 versus 7.6±1.4, 8.2±1.2 ng/mL; P<0.01). Among all patients with acute coronary syndrome and effort angina pectoris, plasma heregulin-β1 levels were further decreased in accordance with the severity of coronary artery lesions. Expression of heregulin-β1 was observed at trace levels in intracoronary atherothrombosis obtained by aspiration thrombectomy from acute coronary syndrome patients. Heregulin-β1, but not heregulin-α, significantly reduced acetylated low-density lipoprotein–induced cholesterol ester accumulation in primary cultured human monocyte-derived macrophages by reducing SR-A and ACAT1 expression and by increasing ABCA1 expression at both mRNA and protein levels. Heregulin-β1 significantly decreased endocytic uptake of [125I]acetylated low-density lipoprotein and ACAT activity, and increased cholesterol efflux to apolipoprotein (Apo)A-I from human macrophages. Chronic infusion of heregulin-β1 into ApoE−/− mice significantly suppressed the development of atherosclerotic lesions.

Conclusions: This study provided the first evidence that heregulin-β1 inhibits atherogenesis and suppresses macrophage foam cell formation via SR-A and ACAT1 downregulation and ABCA1 upregulation. (Circ Res. 2009;105:00-00.)

Key Words: atherosclerosis ■ ischemic heart disease ■ LDL cholesterol ■ macrophages ■ vascular biology

Heregulins, also called neuregulin-I type I, are a group of polypeptide growth factors that behave as natural ligands for the epidermal growth factor receptor family such as erbB3 and erbB4.1 Heregulins promote proliferation, differentiation, and survival of cells in many types of tissue, including breast epithelial cells, glial cells, neurons, and cardiomyocytes during normal development and healing after injury.2 Heregulins also induce neoangiogenesis when expressed in breast cancer cells and promote the expression of angiogenic factors, such as vascular endothelial growth factor or cysteine-rich protein 61.3,4 Human monocytic THP-1 cells express high levels of heregulins and their receptors erbB3 and erbB4.5 Heregulins bind with low affinity to erbB3 and with high affinity to erbB4.6 There are 4 different heregulin isoforms (α, β1, β2, and β3), which are produced through alternative splicing.7 The β isoform is 10 to 100 times more biologically active than the α isoform.8 Intravenous administration of heregulin into rats rescued ischemia-induced brain damage and cardiac dysfunction.9,10 Recent studies demonstrated the expression of heregulin in atherosclerotic lesions in human coronary and carotid arteries.11,12

The development of atherosclerosis is promoted by cholesterol ester (CE) accumulation in macrophages. The intracellular free cholesterol level is increased by the uptake of oxidized low-density lipoprotein (LDL) via...
scavenger receptors and is decreased by efflux of free cholesterol mediated by ATP-binding cassette transporter (ABC)A1, ABCG1, and scavenger receptor class B type I (SR-BI). As excessive accumulation of free cholesterol is toxic to cells, free cholesterol must be either removed through efflux to extracellular acceptors, such as apolipoprotein (Apo)A-I and high-density lipoprotein (HDL), or esterified to CE by acyl-CoA:cholesterol acyltransferase (ACAT). ACAT promotes CE accumulation in macrophages, thereby contributing to foam cell formation, a key event in the early phase of atherosclerosis. However, the roles of heregulin-β₁ in macrophage foam cell formation and atherosclerosis remain unclear.

The present study was performed to examine the effects of heregulin-β₁ on foam cell formation and its molecular and cellular mechanisms in primary cultured human monocyte-derived macrophages. Furthermore, the effects of chronic heregulin-β₁ infusion into ApoE⁻/⁻ mice on the development of atherosclerosis were examined. Most importantly, we clarified the relationships between plasma and tissue levels of heregulin-β₁ and human coronary artery disease (CAD), especially, acute coronary syndrome (ACS).

**Methods**

**Sample Collection of Human Blood, Intracoronary Atherothrombosis, and Coronary Atherosclerotic Lesions**

Blood was collected from a total of 138 subjects: 31 patients with ACS (8 unstable angina pectoris, 23 ST-elevation acute myocardial infarction) hospitalized for emergent coronary catheterization within 4 hours after onset (26 men, 5 women; aged 49 to 95); 34 patients with mild hypertension (26 men, 5 women; aged 50 to 86); 33 patients with stable effort angina pectoris (22 men, 11 women; aged 24 to 81) who were taking no medications. Human coronary atherothrombosis samples were collected from ACS patients and ACS (3 men; aged 71 to 77) at autopsy. This study was approved by the Ethics Committee of Showa University.

**Heregulin-β₁ Measurement**

Heregulin-β₁ concentrations in human plasma from 138 subjects and culture media of human monocytes (4×10⁶ cells/2 mL per 6-cm dish) were measured by ELISA kits (Human NRG1-β₁/HRG1-β₁, R&D Systems).

**Immunohistochemistry**

Immunohistochemical analyses were carried out to identify the expression of heregulins in human coronary atherosclerotic lesions and intracoronary atherothrombosis from ACS patients. Sections were stained with anti-human heregulin-β₁ epidermal growth factor domain antibody (R&D) and antimonocice heregulins (α and β) extracellular domain antibody (clone 7D5, Sigma).

**Cell Culture**

Human peripheral mononuclear cells were isolated from the blood of healthy volunteers as described previously. Monocytes purified using anti-CD14 antibody-conjugated magnetic microbeads (Miltenyi Biotec) were seeded onto 6-cm dishes (4×10⁶ cells/2 mL per dish) for immunoblotting analysis, real-time RT-PCR, and ACAT activity assays, and 3.5-cm dishes (1.3×10⁶ cells/1 mL per dish) for assays of cholesterol esterification, cholesterol efflux, and endocytosis. Cells were incubated at 37°C in 5% CO₂ for 7 days in RPMI-1640 medium supplemented with 10% pooled human serum, streptomycin, penicillin G, and the indicated concentrations of recombinant human heregulin-α or -β₁ (purity >98%, Sigma). The medium in each dish was replaced with fresh medium containing heregulin-α or -β₁ every 3 days.

**Immunoblotting Analysis**

Cells were extracted with 80 μL of 10% sodium dodecyl sulfate. In a standard experiment, aliquots of 25 μg of protein were separated by 10% SDS-PAGE and subjected to immunoblotting with the following antibodies: anti-ACAT1 (Dartmouth Medical School), anti– SR-A (R&D), anti–scavenger receptor class A (SR-A) (R&D), anti–scavenger receptor class B type I (SR-BI) (Novus), anti–apolipoprotein (Apo) (Abgent), anti–human epidermal growth factor receptor domain (R&D), anti–erbB3 (Abgent), anti–erbB4 (Abgent), or anti– β-actin (Sigma).

**Quantitative Real-Time RT-PCR**

Total RNA and first-strand cDNA were prepared from samples. Quantification of mRNA was conducted by real-time RT-PCR using SYBR Premix Ex Taq II (Takara Bio) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Amplification was carried out in 50 μL of reaction mixture at 95°C for 10 seconds followed by 40 cycles at 95°C for 5 seconds and 60°C for 31 seconds, and then at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. The mRNA levels were determined after normalization of RNA concentration with that encoding human GAPDH, and values are expressed as fold changes over control. The primers were as follows: SR-A, 5'-ACCAGGGATTTCAGGTCACA (forward) and 5'-CGACGCCATCGCAACTTTTGATGTAATG (reverse); ACAT1, 5'-TTOGGGAATATCAACAGAGGCC (forward) and 5'-TGGTGAAGACGCAC-ACGCAC (reverse); ABCA1, 5'-ATTCCGCTCTGAGATGACCA (forward) and 5'-TTTTAAGCCCCAGATAAGCCA (reverse); and GAPDH, 5'-GACACGTCAAAGGCTGAGAAC (forward) and 5'-TGGTGAAGGTCGACA (reverse).

**ACAT Enzyme Activity**

Cell extracts (125 μg) were incubated at 37°C for 30 minutes with 20 μL of 250 μmol/L [14C]oleoyl-CoA followed by determination of cholesterol [14C]oleate.
Assay for Cholesterol Esterification

Human macrophages differentiated by 7-day culture with the indicated concentrations of heregulin-α and -β1 were incubated for 18 hours with the indicated concentrations of acetylated LDL (AcLDL) in RPMI-1640 medium containing 10% human sera. [3H]Cholesterol-labeled macrophages were incubated for 2 hours with RPMI-1640 medium containing 0.1% BSA, and then efflux was initiated by adding RPMI-1640 medium containing 0.1% BSA with 15 μg/mL ApoA-I or HDL. After a 16-hour incubation, the medium was collected and centrifuged at 15 000 rpm for 10 minutes to remove cell debris. Cells were washed twice with PBS and lysed with 0.3 mL of 1 N NaOH. The radioactivity of [3H]cholesterol in the medium and in the cell lysate was measured by liquid scintillation counting. Cholesterol efflux is expressed as the percentage of the medium [3H]cholesterol radioactivity of the total [3H]cholesterol radioactivity measured from the medium and the cells.

Cellular Assay for Endocytic Uptake of [125I]AcLDL

Human macrophages differentiated by 7-day culture with the indicated concentrations of heregulin-β1 were incubated for 18 hours with [125I]AcLDL. Trichloroacetic acid–soluble radioactivity in the supernatant and cell-associated radioactivity were determined as described previously.

Assays for Cholesterol Efflux

Human macrophages differentiated by 7-day culture with the indicated concentrations of heregulin-β1 were incubated for 24 hours with 10 μg/mL AcLDL prelabeled with 74 kBq/mL [3H]cholesterol in RPMI-1640 medium containing 10% human sera. [3H]Cholesterol-labeled macrophages were incubated for 2 hours with RPMI-1640 medium containing 0.1% BSA, and then efflux was initiated by adding RPMI-1640 medium containing 0.1% BSA with 15 μg/mL ApoA-I or HDL. After a 16-hour incubation, the medium was collected and centrifuged at 15 000 rpm for 10 minutes to remove cell debris. Cells were washed twice with PBS and lysed with 0.3 mL of 1 N NaOH. The radioactivity of [3H]cholesterol in the medium and in the cell lysate was measured by liquid scintillation counting. Cholesterol efflux is expressed as the percentage of the medium [3H]cholesterol radioactivity of the total [3H]cholesterol radioactivity measured from the medium and the cells.

In Vivo Examination for Atherosclerosis

A total of 88 ApoE−/− mice were fed a standard chow diet. Two different experiments were performed as follows. Experiment 1: Heregulin-β1 (8 ng/mL per kilogram body weight per hour; based on Figure 1 in the online data supplement, available at http://circres.ahajournals.org) or saline (vehicle) was infused into 12-week-old ApoE−/− mice for 4 weeks by osmotic minipumps (Alzet). Experiment 2: Anti-hergulin-β1 neutralizing antibody (Acris, 5 μg/mL per kilogram body weight per hour) or vehicle was infused into 13-week-old ApoE−/− mice for 4 weeks by osmotic minipumps. The entire aorta was stained with oil red O, and cross-sections of the aortic sinus were stained with oil red O or anti-hergulins antibody (clone 7D5). Macrophage infiltration was visualized with anti-mouse MOMA-2 antibody (Chemicon International) staining. Lesion area of the aorta was quantified using Scion Image software.

Statistical Analysis

All values are expressed as means±SEM. Data were compared by 2-tailed paired Student’s t test between 2 groups and by 1-way ANOVA, followed by Bonferroni’s post hoc test when >2 groups were involved. Differences were considered statistically significant at P<0.05.

Results

Expression of Heregulin-α and -β1, ErbB3, and ErbB4 in Human Monocyte-Macrophages and Heregulin-β1 Secretion From These Cells

Figure 1 shows the protein expression of heregulin-α, heregulin-β1, and their receptors erbB3 and erbB4 during differentiation from human monocytes into mature macrophages. The expression levels of heregulin-α and heregulin-β1 increased significantly on day 7 in primary culture (Figure 1A and 1B). Expression of erbB3 and erbB4 increased significantly on day 3 and reached a plateau on days 5 and 7 (Figure 1C and 1D). The concentration of endogenous heregulin-β1 in culture media increased significantly on day 3 but did not increase further on days 5 and 7 (Figure 1E), which was negligible compared with exogenous heregulin-β1 added.

Effects of Heregulin-α and -β1 on AcLDL-Induced CE Accumulation in Human Monocyte-Derived Macrophages

Figure 2 shows the effects of heregulin-α and -β1 on foam cell formation as assessed by AcLDL-induced CE accumulation in macrophages. In the absence of heregulins, coincubation with AcLDL at 5 and 10 μg/mL significantly increased CE accumulation in macrophages (Figure 2A). However, at 10 ng/mL, heregulin-β1 but not heregulin-α significantly reduced AcLDL-induced CE accumulation. As shown in Figure 2B, 10 μg/mL AcLDL-induced CE accumulation in macrophages was significantly decreased by heregulin-β1 in a concentration-dependent manner, with a maximal reduction of 40% observed at 10 ng/mL. Heregulin-α at all concentrations tested had no statistically significant effects on AcLDL-induced CE accumulation in macrophages.

Dose-Dependent Effects of Heregulin-α and -β1 on SR-A, CD36, ACAT1, ABCA1, ABCG1, and SR-BI Expression in Human Monocyte-Derived Macrophages

The concentration-dependent effects of heregulin-α and -β1 on SR-A, CD36, ACAT1, ABCA1, ABCG1, and SR-BI protein expression in monocyte-derived macrophages were assessed on day 7. Heregulin-α had no statistically significant effects on SR-A, CD36, ACAT1, ABCG1, or SR-BI expression at any concentrations tested (Figures 3 and 4). In contrast, heregulin-β1 significantly decreased SR-A and ACAT1 expression in a concentration-dependent manner (Figure 3). Heregulin-β1 significantly increased ABCA1 protein expression to a greater extent than heregulin-α at 1 ng/mL (Figure 4). However, heregulin-β1 had no statistically significant effects on protein expression of CD36 (Figure 3), ABCG1, or SR-BI (Figure 4).

Time-Dependent Effects of Heregulin-α and -β1 on SR-A, ACAT1, and ABCA1 Expression in Human Monocyte-Macrophages

As shown above, significant changes in SR-A, ACAT1, and ABCA1 protein expression on day 7 were induced by heregulin-β1 at the appropriate concentrations (1 ng/mL for ABCA1, 10 ng/mL for others). Therefore, we evaluated the time-dependent effects of heregulin-α and -β1 at the above concentrations on expression of these proteins as compared with a macrophage differentiation marker CD68 during differentiation from monocytes into mature macrophages (Figure 5). Expression of SR-A, ACAT1, and ABCA1, as well as CD68, increased with monocyctic differentiation into macrophages in the absence of heregulin-α and -β1 (as a control).
Heregulin-α did not significantly affect SR-A or ACAT1 expression during monocytic differentiation into macrophages but significantly increased ABCA1 expression on day 7. On the same day, heregulin-β1 significantly reduced SR-A and ACAT1 expression, and increased ABCA1 expression to a greater extent than heregulin-α.

**Effects of Heregulin-β1 on SR-A, ACAT1, and ABCA1 mRNA Levels in Human Monocyte-Derived Macrophages**

The effects of heregulin-β1 on SR-A, ACAT1, and ABCA1 mRNA levels in monocyte-derived macrophages on day 7 are shown in Figure 6. Heregulin-β1 (10 ng/mL) significantly decreased SR-A and ACAT1 mRNA levels. Heregulin-β1 (1 ng/mL) significantly increased ABCA1 mRNA level.

Changes in these mRNA levels by heregulin-β1 were consistent with the protein expression.

**Effects of Heregulin-β1 on Endocytosis of AcLDL, ACAT Enzyme Activity, and Cholesterol Efflux in Human Monocyte-Derived Macrophages**

We assessed the effects of heregulin-β1 on endocytic uptake of [125I]AcLDL by macrophages (SR-A activity), ACAT activity, and cholesterol efflux from macrophages to ApoA-I (ABCA1 function) and to HDL (ABCG1 and SR-BI function). Heregulin-β1 at 10 ng/mL significantly decreased both cell association and endocytic degradation of [125I]AcLDL (Figure 6D and 6E) and ACAT activity (Figure 6F). Heregulin-β1 at 1 ng/mL significantly increased cholesterol
efflux to ApoA-I (Figure 6G) but did not induce significant changes in cholesterol efflux to HDL (data not shown).

**Effects of Heregulin-β1 or Anti-Heregulin-β1 Neutralizing Antibody on Atherosclerotic Lesion Development in ApoE<sup>−/−</sup> Mice**

After the 4-week infusion, plasma heregulin-β1 levels in ApoE<sup>−/−</sup> mice infused with heregulin-β1 were significantly higher than those with vehicle (2.1±0.2 versus 1.0±0.1 ng/mL; P<0.05). The 2 groups exhibited no significant differences in body weight, systolic blood pressure, and plasma levels of total cholesterol, triglyceride, and glucose (Online Table I). Histochemical analyses revealed remarkable atherosclerotic lesions in the aorta from vehicle-infused ApoE<sup>−/−</sup> mice (Figure 7A and 7C). However, chronic heregulin-β1 infusion significantly reduced the surface area of aortic atherosclerotic lesions (Figure 7I) as compared with vehicle-infused mice, which was accompanied by marked reductions in plaque size (Figure 7J), macrophage infiltration (Figure 7K), and endogenous heregulins expression (Figure 7L) in the aortic sinus lesions. In addition, chronic infusion of anti–heregulin-β1 neutralizing antibody resulted in the acceleration of aortic atherosclerotic lesions (Figure 7U and 7V) and macrophage infiltration (Figure 7W) without affecting systolic blood pressure, total cholesterol, triglyceride, or glucose levels (Online Table I). Plasma heregulin-β1 levels were significantly reduced in ApoE<sup>−/−</sup> mice infused with anti–heregulin-β1 antibody compared with those with vehicle (0.02±0.01 versus 0.75±0.10 ng/mL, P<0.0001). In ApoE<sup>−/−</sup> mice infused with anti–heregulin-β1 antibody, the least amount of endogenous heregulins was observed in the intima of aortic wall, which was in accordance with the region infiltrated by macrophages (Figure 7T).

**Plasma Heregulin-β1 Levels in Patients With Coronary Heart Diseases**

Plasma levels of heregulin-β1 were significantly lower in patients with angiographically proven CAD, such as EAP and ACS, than in those with mild hypertension and healthy volunteers (Figure 8A). ACS patients had the lowest levels of
plasma heregulin-\(\beta_1\). Among patients with ACS and EAP, plasma heregulin-\(\beta_1\) levels decreased in accordance with the severity of coronary artery lesions, with significantly lower levels of heregulin-\(\beta_1\) observed when patients with 3-vessel disease were compared with those with 1-vessel (Figure 8B).

**Expression of Heregulin-\(\beta_1\) in Coronary Atherosclerotic Plaque Progression and Rupture and Atherothrombosis Formation in ACS Patients**

Expression of heregulins was not observed in nondiseased coronary arteries (Figure 8F) but became high levels in macrophage foam cells in accordance with the progression of coronary atherosclerotic plaques from ACS patients (Figure 8G and 8H). However, expression of heregulins and heregulin-\(\beta_1\) disappeared in the rupture site of advanced plaques (Figure 8I and 8J), which was extremely lower compared with these expression in nonruptured advanced plaques (Figure 8D and 8E). Furthermore, expression of heregulin-\(\beta_1\) was observed at trace levels within intracoronary atherothrombosis (formed after rupture) in ACS patients (Figure 8K).

**Discussion**

The original findings of the present study are novel atheroprotective effects of heregulin-\(\beta_1\). We provide the first evidence that heregulin-\(\beta_1\) prevents foam cell formation in primary cultured human monocyte-derived macrophages and the development of atherosclerosis in ApoE\(^{−/−}\) mice. The molecular mechanisms involve significant decreases by heregulin-\(\beta_1\) of endocytic uptake of AcLDL and ACAT activity caused by downregulation of SR-A and ACAT1 and increased cholesterol efflux to ApoA-I attributable to upregulation of ABCA1 in human macrophages. We extend the data that neutralization of heregulin-\(\beta_1\) accelerates the development of atherosclerotic lesions in ApoE\(^{−/−}\) mice. To our knowledge, we have demonstrated for the first time the relationships of the reduced levels of heregulin-\(\beta_1\) in plasma and tissues with the presence and severity of CAD in humans.

Several animal studies have shown that suppression of SR-A contributes to the prevention of atherosclerotic lesion progression by inhibiting vascular inflammation other than by suppressing macrophage foam cell formation.\(^{20}\) Our previous studies have shown that ACAT1 is involved in macrophage foam cell formation and atherosclerotic lesion development,\(^{17,18,21}\) and ACAT1 specific inhibition suppresses both the phenomena.\(^{22,23}\) Cholesterol efflux by ApoA-I via ABCA1 plays a key role in preventing atherosclerosis.\(^{14}\) In the present study, the most likely mechanism underlying a significant suppression of macrophage foam cell formation...
and atherosclerotic lesion development may be harmonized upregulation of ABCA1 and downregulation of SR-A and ACAT1 by heregulin-β1.

Heregulin stimulates vascular endothelial growth factor expression in vascular smooth muscle cells (VSMCs) and facilitates the migration and proliferation of human umbilical vein endothelial cells, leading to angiogenesis in vitro and in vivo. The expression levels of heregulin, erbB3, and erbB4 increase during differentiation of THP-1 monocytes into macrophages. Expression of heregulin is stimulated by lipopolysaccharide in human monocytes/macrophages, which is declined within 24 hours. Heregulin prevents proinflammatory responses by attenuating cyclooxygenase-2 mRNA expression in monocytic U937 cells. Heregulin has antioxidant properties, and activates endothelial nitric oxide synthase in rat cardiomyocytes. In ischemic rat brain tissues, heregulin exerts the antinflammatory and antipoptotic effects by suppression of interleukin-1β, monocyte chemotactic protein-1, intercellular adhesion molecule-1, lectin-like oxidized LDL receptor-1, and caspase-3. A recent study showed that endogenous heregulin-β1 and erbB4 are upregulated following balloon injury of rat carotid arteries. Upregulation of heregulin-β1 is regarded as an immediate protective response to inhibit VSMC proliferation and extracellular matrix production after vascular injury. Infusion of exogenous heregulin-β1 into rats attenuates balloon injury–induced neointimal formation by inhibiting VSMC proliferation. In the present study, the increasing intimal levels of heregulin in the aorta of ApoE−/− mice may reflect a protective response to atherosclerosis, comparable to the attempt of macrophages to take up atherogenic oxidized LDL from the extracellular intima.

We acknowledge that the concentrations of heregulin-β1 (∼2 ng/mL) required to prevent atherosclerosis in vivo in ApoE−/− mice may be relatively low compared with the higher concentration (1 to 10 ng/mL) used in the in vitro studies with human macrophages. There are some possible explanations. First, upregulation of heregulin-β1 and erbB receptors in the atherosclerotic lesion could make macrophages more responsive to heregulin-β1 and require less exogenous heregulin-β1 to see an in vivo effect. Second, heregulin-β1 is known to suppress VSMC proliferation and also reduce lesional inflammation in vivo, which may synergistically suppress atherosclerotic plaque formation. Finally, mouse macrophages may be more sensitive to heregulin-β1 than human macrophages, requiring lower concentrations of heregulin-β1. These factors may account for the differences in the effective concentrations of heregulin-β1 in vivo versus in vitro.

Clinical data from the present study suggest important roles of heregulin-β1 in atherosclerotic cardiovascular diseases. Panutsopulos et al showed that heregulin is expressed at high levels in macrophage foam cells, but not in VSMCs, fibroblasts, or extracellular matrix, within human coronary atherosclerotic lesions. The expression of heregulin was absent in endothelial cells in both normal and atherosclerotic human coronary arteries. They showed that the levels of heregulin expression increased as the stage of atherosclerotic lesions progressed in 26 subjects. This finding is compatible in part with our results. Macrophages infiltrated into the arterial wall may produce heregulin to counteract the ongoing atherosclerosis. However, our study showed that heregulin levels were decreased in extremely advanced coronary plaques in ACS. Furthermore, heregulin (Online Figure II) and heregulin-β1 were expressed at trace levels in atherothrombosis immediately formed after plaque rupture in ACS. It is unknown whether decreased levels of heregulin-β1 in plasma and atherothrombosis may contribute to, or be the result of, coronary events in ACS. We speculate that the expression of heregulin-β1 in macrophages within extremely advanced atherosclerotic plaques may be reduced by its secretion because of upregulation of ADAM-17 (a disintegrin and metalloproteinase 17), which is required for cleavage of heregulin-β1. Moreover, heregulin-β1 may be exhausted by the activation of coagulation system during thrombus formation after rupture. Further analyses are required to clarify the relationship between decreased levels of heregulin-β1 expression and coronary plaque destabilization and rupture (Figure 8C through 8E).
It is not clearly understood where plasma heregulin-β₁ originates or what causes its marked decrease in CAD patients. Because heregulin-β₁ levels in plasma of normal controls were much higher than those secreted into media from macrophages (Figure 1E), it is unlikely that arterial macrophages are the main source. Therefore, we speculate that a great extent of systemic atherosclerosis occurred in parallel with ACS and severe CAD results in downregulation of hepatic macrophages and/or nonmacrophage heregulin-β₁ production in other tissues. Systemic, rather than arterial, production of heregulin-β₁ may be the crucial factor in the modulation of atherosclerosis. Plasma levels of heregulin-β₁ range from 0.86 to 33.6 ng/mL in normal controls, which are compatible with the concentrations of heregulin-β₁ used in in vitro study. Future studies are needed to clarify whether the levels of heregulin-β₁ in arterial wall achieved by circulating heregulin-β₁ could reach high enough to show the significant effects on macrophages. The 2 main findings (the antiatherogenic action of systemic heregulin-β₁ and its effects on macrophages) are novel enough even when it is openly stated that there is at present insufficient evidence for a causal link between the 2.

Both erbB3 and erbB4 binding with heregulin-β₁ induce the heterodimerization with erbB2, which leads to phosphorylation of intrinsic tyrosine kinase followed by various intracellular signaling pathways. The activation of these pathways stimulates distinct transcriptional programs in the nucleus, affecting glial, neuronal, and myocyte proliferation. However, heregulin-β₁ inhibits skin endothelial cell and VSMC proliferation. This discrepancy may be attributed to the difference in cell type. Clement et al have shown that heregulin-β₁ inhibits platelet-derived growth factor–stimulated VSMC proliferation by suppressing extracellular signal-regulated kinase 1/2 phosphorylation. In the present study, the intracellular signaling mechanisms by which heregulin-β₁ modulates SR-A, ACAT1, and ABCA1 expression in macrophages remain to be addressed. Further studies are required to determine the signaling molecules and transcription factors concerned.

In conclusion, the results of the present study suggest that heregulin-β₁ has atheroprotective effects by suppressing macrophage foam cell formation. The molecular mechanisms involve the reduced endocytosis of AcLDL and reduced CE formation caused by downregulation of SR-A.
Figure 7. Atherosclerotic lesions of the aorta in ApoE−/− mice. The 17-week-old ApoE−/− mice were infused with saline (vehicle) or heregulin-β1 (8 ng/mL per kilogram body weight per hour) by osmotic minipumps for 4 weeks. Otherwise, the 13-week-old ApoE−/− mice were infused with vehicle or anti–heregulin-β1 neutralizing antibody (5 μg/mL per kilogram body weight per hour) in the same manner. The excised aorta was opened longitudinally, followed by oil red O staining (A, B, M, and N). Atherosclerotic lesions in cross-sections of the aortic sinus were stained with oil red O (C, D, O, and P), anti–MOMA-2 antibody (E, F, Q, and R), or anti-herengulin antibody (G, H, S, and T). Hematoxylin was used for nuclear staining. I and U, The surface area of aortic atherosclerotic lesions. C through H and O through T, The percentage of intimal atherosclerotic lesions of the aortic wall.
and ACAT1, respectively, and increased ApoA-I–mediated cholesterol efflux attributable to ABCA1 upregulation in macrophages. Clinically, the results presented here provide insights into the potential use of heregulin-β, as a biomarker for CAD and an extended therapeutic window for combating atherosclerosis and restenosis after coronary angioplasty.

**Figure 8.** Heregulin-β1 levels in plasma, coronary atherosclerotic plaque, and atherothrombosis in ACS patients. Plasma heregulin-β1 levels were measured in 64 patients with CAD including 31 ACS and 33 EAP, 34 patients with mild hypertension (HT), and 40 healthy volunteers (A and B). 1-VD indicates 1-vessel disease; 2-VD, 2-vessel disease; 3-VD, 3-vessel disease. Coronary artery from an ACS patient involves the ruptured (arrow) and nonruptured atherosclerotic plaques stained with hematoxylin/eosin (C) and anti-heregulins (D) or anti–heregulin-β1 (E) antibody. Nondiseased area (F), early (G), and advanced (H) atherosclerotic plaques, and plaque rupture site (I and J) in coronary arteries from ACS patients were stained with anti-heregulins (F through I) or anti–heregulin-β1 (J) antibody. Intracoronary atherothrombi from ACS patients were stained with anti–heregulin-β1 antibody (K). Hematoxylin was used for nuclear staining. Scale bars: 1 mm (C through E); 100 μm (F through K).

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Disclosures

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Online Figure 1. Relationship between dose of heregulin-β1 infusion into ApoE−/− mice and their plasma heregulin-β1 levels after a 4-week infusion. Each point represents the mean ± SEM from 3 mice. *P < 0.05 vs 0 ng/mL/kg per hour of heregulin-β1. Thereby we chose 8 ng/mL/kg per hour of heregulin-β1 that significantly increased plasma heregulin-β1 levels in experiment 1.
Systolic blood pressure (SBP) was measured by an indirect tail-cuff equipment (Muromachi Model MK-2000) in conscious mice. Plasma levels of total cholesterol, triglyceride, and glucose were measured by enzymatic methods using an autoanalyzer (Hitachi 7020). Data are expressed as means ± SEM.
Online Figure 2. Expression of heregulins, erbB3, and erbB4 in atherothrombosis in acute coronary syndrome (ACS). Intracoronary atherothrombi obtained by aspiration thrombectomy from ACS patients were stained with anti-heregulins (A), anti-erbB3 (B), or anti-erbB4 (C) antibody. Hematoxylin was used for nuclear staining. Scale bars = 100 µm. ErbB4 and erbB3 were expressed in macrophage foam cells at high and low levels, respectively. However, expression of heregulins was observed at trace levels in all cell types within atherothrombosis.