Vascular smooth muscle cells migrate, proliferate, and transform to foam cells during the atherosclerotic process. We have reported that smooth muscle cells derived from the intima of atherosclerotic lesions express the proto-oncogene c-fms and a scavenger receptor, which are not normally expressed in normal medial smooth muscle cells. In the present study, we demonstrated that heparin binding epidermal growth factor-like growth factor (HB-EGF) induced the expression of c-fms and the scavenger receptor in normal human medial smooth muscle cells to the level observed in the intima. The expression of c-fms was partially inhibited by a protein kinase C inhibitor, suggesting that HB-EGF induces c-fms via pathways that are both dependent on and independent of protein kinase C. By contrast, most of the scavenger receptor induction by HB-EGF was suppressed by protein kinase C inhibitors. These results indicate that two characteristic genes of monocyte-derived macrophages were induced by HB-EGF via different mechanisms. The alteration of gene expression in response to HB-EGF may play an important role in the phenotypic change of smooth muscle cells to macrophage-like foam cells during the atherosclerotic process.

Heparin binding epidermal growth factor-like growth factor (HB-EGF), a member of the epidermal growth factor (EGF) family, was originally identified as a 20–22-kDa glycoprotein secreted by human monocytes, macrophages, and a macrophage-like cell line (U-937) (1–4). Recently, secretion of HB-EGF from vascular smooth muscle cells (SMCs) (5) and the high expression of HB-EGF in atherosclerotic lesions (6) was reported, suggesting significant involvement in the progression of atherosclerosis. It has been suggested that HB-EGF binding to the EGF receptor through an interaction with heparan sulfate proteoglycan on the cell surface (7). Rapid phosphorylation of the EGF receptor at tyrosine initiates a cascade of metabolic and gene regulatory events, including migration and proliferation of smooth muscle cells.

Foam cell formation is the most characteristic event of early atherosclerosis. Foam cells are derived from both monocyte-derived macrophages and smooth muscle cells. Peripheral monocytes enter the subendothelial space and differentiate to tissue macrophages that actively take up lipoprotein cholesterol through receptor-mediated endocytosis, including the scavenger receptor (8–10). Smooth muscle cells migrate from the media to the intima of the arterial wall, where they proliferate and transform into foam cells. Various cytokines and growth factors secreted by vascular cell components are presumed to play important roles in the phenotypic change of monocyte-derived macrophages and smooth muscle cells (10). However, the precise mechanism of phenotypic change in cells (especially smooth muscle cells) in atherosclerotic lesions is not fully understood. As a characteristic change of gene expression between two phenotypes of vascular smooth muscle cells derived from the intima and media, we demonstrated the expression of macrophage colony-stimulating factor (M-CSF) receptor encoded by proto-oncogene c-fms on smooth muscle cells derived from the intima of atherosclerotic lesions (11). Furthermore, we have reported that PDGF-BB homodimer transiently induces gene expression of c-fms in normal vascular smooth muscle cells (12); adding EGF or FGF makes the gene expression similar to that in intimal smooth muscle cells (13). Our observations suggest that the factors involved in the atherosclerotic process cause changes in the regulation of c-fms gene expression in normal vascular smooth muscle cells by inducing phenotypic change to macrophage-like cells. In the present study, we found that HB-EGF independently raises the stable expression of c-fms in normal vascular smooth muscle cells to that in intimal smooth muscle cells.

**EXPERIMENTAL PROCEDURES**

Cells—Human aortic medial smooth muscle cells were explanted by the method described by Fischer-Dzoga et al. (14). Cells were passaged three times by exposure to trypsin and seeded in 10 ml of Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc) containing 10% fetal bovine serum in 100-mm dishes; Cells from the fourth to sixth passages were used for experiments. Subconfluent cells were cultured with serum-free medium for 24 h before the experiments, and medial smooth muscle cells were cultured with DMEM containing 1% FCS in the presence of HB-EGF at 37 °C. The protein concentration was determined using the BCA assay (Pierce). HB-EGF was donated by Dr. Judith A. Abraham (Scios Nova Inc., Mountain View, CA).

**Quantitative Analysis of M-CSF Receptor mRNA**—Human smooth muscle cells were treated with HB-EGF for various periods (1–48 h) or with various concentrations (1–5 ng/ml) of HB-EGF for 8 h in DMEM containing 1% FCS after incubation with serum-free medium for 24 h.
Induction of c-fms in SMCs by HB-EGF

After incubation with HB-EGF, total RNA was isolated using the acid-guanidinium phenol-chloroform method (15). A competitive polymerase chain reaction method was used to measure M-CSF receptor (c-fms) mRNA levels as described previously (11).

RNAse Protection Assay—A 1.2-kilobase probe fragment (2048–3194) of the human c-fms cDNA was subcloned into pBluescript II SK− (Stratagene Corp., La Jolla, CA), and this plasmid was digested with BglII. In the case of the scavenger receptor, a 290-base pair cDNA fragment was prepared for cloning the polymerase chain reaction product with primers human scavenger receptor 1 (5′-TGCTCTAATGACAGCTTTGGC-3′) and human scavenger receptor 2 (5′-CCATTGGTGTCATGTGGTCC-3′) into EcoRV-digested pBluescript II, and this plasmid was linearized by digesting with XhoI. The linearized DNA was transcribed and labeled with [32P]UTP (specific activity, 800 Ci/mmol; Amersham Corp.) using a Riboprobe Gemini system according to the method described in the instructions (Promega Corp., Madison, WI). A resultant 354-base pair antisense RNA probe for c-fms and a 324-base pair antisense RNA probe for the scavenger receptor were used for the ribonuclease protection assay as described in the instructions (Ambion Inc., Austin, TX). Total RNA (25 μg) was hybridized with approximately 20,000 cpm of the RNA probe overnight at 42 °C, and the mixture was digested with RNase A and RNase T1. Protected fragments were separated on 5% acrylamide/8 M urea gels. We performed the run-on assay according to the method described previously (16). Five μg of plasmids containing either human c-fms DNA or human β-actin cDNA were linearized, and the linearized plasmids were blotted onto a Hybond-N membrane. The membrane was hybridized with 32P-labeled nuclear RNAs (104 cpm) isolated from nuclei after transcription and exposed to Kodak XAR-5 film at −80 °C.

Western Blot Analysis—Subconfluent cells were solubilized in 50 mM Tris-malate buffer containing 3% SDS, 8 M 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride at pH 6.0 after incubation for 24 h with fresh medium containing 5 ng/ml HB-EGF. The cell lysates containing 150 μg of protein were subjected to 7% SDS-polyacrylamide gel electrophoresis under reducing conditions. Proteins electrotransferred were transferred to nitrocellulose paper, and the paper was incubated with anti-c-fms monoclonal antibody (Oncogene Science Inc., New York, NY). After the nitrocellulose sheet was incubated with [32P]UTP (specific activity, 800 Ci/mmol; Amersham), autoradiography was performed. Gels were calibrated based on the following molecular mass standards: 200 kDa for myosin, 116 kDa for β-galactosidase, 94 kDa for phosphorylase b, 68 kDa for bovine serum albumin, and 43 kDa for ovalbumin.

Uptake of Acetylated LDL—Vascular medial smooth muscle cells were cultured with 5 ng/ml HB-EGF in the presence or absence of 50 μM staurosporine and 10 μM calphostin C (proteinkinase C inhibitors) and 100 μM staurosporine and 10 μM calphostin C (proteinkinase C inhibitors) into 5% acrylamide/8 M urea gels. We performed the run-on assay according to the method described previously (16). Five μg of plasmids containing either human c-fms DNA or human β-actin cDNA were linearized, and the linearized plasmids were blotted onto a Hybond-N membrane. The membrane was hybridized with 32P-labeled nuclear RNAs (104 cpm) isolated from nuclei after transcription and exposed to Kodak XAR-5 film at −80 °C.

RESULTS

c-fms mRNA Analysis—The mRNA of c-fms was not detected in human aortic smooth muscle cells using a RNase protection assay with a riboprobe of c-fms. In the presence of 5 ng/ml HB-EGF, mRNA of c-fms was obviously detected in human smooth muscle cells both in the presence and absence of 1% FCS, whereas it was not detected in the presence of 10 ng/ml PDGF-BB (Fig. 1A and B). This expression was less than that of human monocyte-derived macrophages.

To estimate the mRNA level of c-fms, we used a sensitive competitive polymerase chain reaction method. In assays of human aortic smooth muscle cells in the presence of 5 ng/ml HB-EGF, the mRNA levels were estimated at 7 × 10−2 pg/μl genomic DNA. This indicates that the transcription of c-fms in human aortic smooth muscle cells was increased 3,500-fold in the presence of 5 ng/ml HB-EGF or 10 ng/ml human PDGF in DMEM containing 1% FCS for 8 h after a 24 h preincubation in serum-free medium. Human monocyte-derived macrophages (HMM) were cultured as described (11). B, cells were cultured with 5 ng/ml human HB-EGF in DMEM in the presence (HB-EGF) or absence (HB-EGF only) of 1% FCS for 8 h after a 24-h preincubation in serum-free medium. Therefore, 25 μg of total RNA was applied to a ribonuclease protection assay with the RNA probe for human c-fms. The protection assay was performed three times, and the representative result is shown. C, a competitive polymerase chain reaction method was used to measure M-CSF receptor (c-fms) mRNA levels. One μg of total RNAs isolated from the cells was reverse transcribed with random hexamer primers. Aliquots of the cDNA products were co-amplified with indicated amounts of the control genomic DNA. It is possible to quantitate the amount of cDNA present by titrating an unknown amount of cDNA (lower band) against a dilution series containing known amounts of the corresponding genomic DNA (upper band). The same amounts of genomic DNA and cDNA fragments were obtained when the concentration of the genomic template was 7 × 10−1 pg/μl in the assay for the estimation of c-fms mRNA level in human aortic smooth muscle cells in the presence of 5 ng/ml HB-EGF. D, the cell nuclei were incubated with reaction buffer for 30 min at 30 °C. Then, the reaction mixture was treated with DNase I (20 μg/ml) and proteinase K (100 μg/ml) and extracted with phenol-chloroform (1:1). Five μg of plasmids containing either human c-fms cDNA or human β-actin cDNA were blotted onto a Hybond-N membrane, and the membrane was hybridized with α-32P-labeled nuclear RNAs.
expression were estimated by a competitive polymerase chain reaction method and expressed as genomic c-fms concentrations. Rabbit intimal SMCs were isolated from intima of balloon-injured aorta (11), and human monocyte-derived macrophages and human and rabbit aortic medial SMCs were cultured as described (11). Total RNA was isolated from cells, and levels of mRNA expression were estimated by a competitive polymerase chain reaction method and expressed as genomic c-fms concentrations. Total RNA was isolated from cells, and levels of mRNA expression were estimated by a competitive polymerase chain reaction method and expressed as genomic c-fms concentrations. We have reported that the c-fms expression by PDGF-BB alone is not mediated through protein kinase C activation and the progression factors such as EGF and FGF stimulate smooth muscle cells. M-CSF receptor encoded by c-fms, which generally coincides with monocyte-derived macrophage lineages. Among growth factors, we previously demonstrated that a combination of PDGF-BB homodimer and either EGF or FGF induced c-fms expression in vascular smooth muscle cells, which do not normally express this gene, to the level equivalent to that of rabbit intimal smooth muscle cells isolated from atherosclerotic lesions (11). However, neither EGF nor FGF alone induces c-fms expression, and PDGF-BB alone induces only transient and low expression. The present study demonstrated that HB-EGF independently induced the stable expression of c-fms in medial smooth muscle cells to the expression level of intimal smooth muscle cells.

We have reported that the c-fms expression by PDGF-BB alone is not mediated through protein kinase C activation and then augmented by EGF and FGF through certain pathways, including protein kinase C activation. PDGF-BB initially renders cells competent to respond to progression factors (18, 19), and the progression factors such as EGF and FGF stimulate the signal induced by PDGF-BB. The potency of HB-EGF in c-fms expression is approximately 10-fold greater than that of PDGF-BB and rather close to that of combinations of PDGF-BB and FGF.

**DISCUSSION**

Numerous cytokines and growth factors are secreted by vascular cell components, including endothelial cells, monocyte-derived macrophages, smooth muscle cells, and lymphocytes, and are involved in atherosclerotic processes such as transformation, migration, and proliferation of vascular smooth muscle cells, monocyte differentiation, and foam cell formation (9, 10). We have focused on the mechanisms for the phenotypic change from vascular smooth muscle cells to phagocytic cells that is characterized by expression of the scavenger receptor gene and SMC expression of scavenger receptor mRNA in the presence of 5 ng/ml HB-EGF (Fig. 5B). Staurosporine (50 nM) and calphostin C (10 nM), protein kinase C inhibitors, suppressed c-fms expression in the presence of HB-EGF to 40–50% of that in controls (Table I and Fig. 3).

**c-fms Western Blot Analysis**—Expression of M-CSF receptor was demonstrated in human medial smooth muscle cells treated with 3 ng/ml HB-EGF for 24 h. As shown in Fig. 4, a 165-kDa protein of M-CSF receptor was detected in human medial smooth muscle cells treated with HB-EGF as well as in human monocyte-derived macrophages, whereas no specific band for c-fms was detected in nontreated medial smooth muscle cells.

**Scavenger Receptor Activity**—Using the method of RNase protection assay with a riboprobe of human scavenger receptor, we detected mRNA of the scavenger receptor. HB-EGF induced mRNA of the scavenger receptor in human medial smooth muscle cells in a dose-dependent manner (Fig. 5A). Staurosporine (50 nM) and calphostin C (10 nM) completely inhibited the expression of scavenger receptor mRNA in the presence of 5 ng/ml HB-EGF (Fig. 5B).

To confirm these findings, we estimated the accumulation of cellular cholesterol ester in human medial smooth muscle cells incubated with 100 μg/ml acetylated LDL. As shown in Fig. 6, cholesterol ester accumulated in vascular smooth muscle cells in response to HB-EGF in the presence of acetylated LDL (Fig. 6). The accumulation of cholesterol ester in response to HB-EGF was inhibited by the protein kinase C inhibitors staurosporine (50 nM) and calphostin C (10 nM).

**DISCUSSION**

Numerous cytokines and growth factors are secreted by vascular cell components, including endothelial cells, monocyte-derived macrophages, smooth muscle cells, and lymphocytes, and are involved in atherosclerotic processes such as transformation, migration, and proliferation of vascular smooth muscle cells, monocyte differentiation, and foam cell formation (9, 10). We have focused on the mechanisms for the phenotypic change from vascular smooth muscle cells to phagocytic cells that is characterized by expression of the scavenger receptor gene and

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**Table I**

**mRNA expression of c-fms in vascular smooth muscle cells**

<table>
<thead>
<tr>
<th>Cell</th>
<th>mRNA level</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human aortic medial SMC in the presence of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ng/ml PDGF-BB</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>10 ng/ml PDGF-BB + 3 ng/ml EGF</td>
<td>8.0</td>
<td>10.0</td>
</tr>
<tr>
<td>5 ng/ml HB-EGF</td>
<td>7.0</td>
<td>8.75</td>
</tr>
<tr>
<td>5 ng/ml HB-EGF + 10 nm calphostin C</td>
<td>2.8</td>
<td>3.5</td>
</tr>
<tr>
<td>5 ng/ml HB-EGF + 50 nm staurosporine</td>
<td>3.5</td>
<td>3.98</td>
</tr>
<tr>
<td>Human aortic medial SMC</td>
<td>0.002</td>
<td>2.5 × 10⁻³</td>
</tr>
<tr>
<td>Human monocyte-derived macrophage</td>
<td>17.5</td>
<td>21.9</td>
</tr>
<tr>
<td>Rabbit aortic medial SMC</td>
<td>0.001</td>
<td>1.25 × 10⁻³</td>
</tr>
<tr>
<td>Rabbit aortic intimal SMC</td>
<td>10.0</td>
<td>12.5</td>
</tr>
</tbody>
</table>

**Fig. 2.** Time- and dose-related expression of c-fms mRNA in response to HB-EGF in human aortic smooth muscle cells. The subconfluent cells were cultured with 5 ng/ml HB-EGF in DMEM for the indicated hours (A) and with the specified amounts of HB-EGF for 8 h (B) in DMEM containing 1% FCS at 37 °C after a 24-h preincubation in serum-free medium. Each value was estimated by a competitive polymerase chain reaction method. One μg of total RNAs isolated from the cells was reverse transcribed with random hexamer primers. Aliquots of the cDNA products were co-amplified with indicated amounts of the control genomic DNA. mRNA levels were estimated by titrating an unknown amount of cDNA against a dilution series containing known amounts of the corresponding genomic DNA.
and either EGF or FGF with regard to both magnitude and stability of c-fms expression (13). It has been suggested that HB-EGF has functions of both competent and progression factors in cell growth (1, 7). HB-EGF may switch on high and stable c-fms expression by consecutively stimulating the signals of both protein kinase C-dependent and -independent pathways.

Treatment of aortic smooth muscle cells with staurosporine and calphostin C, which are protein kinase C inhibitors, suppressed c-fms expression in the presence of HB-EGF to 40–50% of that in the absence of protein kinase C inhibitors. This result indicates that the effect of HB-EGF cannot be attributed to protein kinase C activation only; therefore, HB-EGF induces c-fms expression through other pathways as well. The induction of c-fms expression by different growth factors in normal medial smooth muscle cells may serve as a good model for studying complex signaling cascades initiated by growth factors and cytokines in the arterial wall.

Recently, we demonstrated gene expression of the scavenger receptor as well as c-fms in vascular smooth muscle cells isolated from atherosclerotic lesions (11). We proposed that this gene expression may be related to the phenotypic conversion of vascular smooth muscle cells to macrophage-like cells in atheromatous lesions. Furthermore, recent studies have demonstrated that some cytokines and growth factors, including the PDGF-BB homodimer and EGF, induce scavenger receptor expression in normal vascular smooth muscle cells through protein kinase C activation (13, 20, 21). Likewise, the present study demonstrated that HB-EGF induced the scavenger receptor expression through protein kinase C activation. Most of the scavenger receptor expression in terms of both mRNA expression and acetylated LDL uptake was suppressed by protein kinase C inhibitors. This result confirms previous reports that the scavenger receptor expression in vascular smooth muscle cells is regulated by protein kinase C.
muscle cells is induced by protein kinase C activation.

It has been speculated that the heparin binding property of HB-EGF may play an important role in the marked enhancement of cell migration and proliferation compared with EGF, which shares the EGF receptor with HB-EGF (1, 7). In fact, we demonstrated that HB-EGF is the most potent growth factor for c-fms expression. A recent report has suggested the possible involvement of HB-EGF in the atherosclerotic process from the evidence of its high expression in atherosclerotic lesions and demonstrated the strong expression of the EGF receptor in intimal smooth muscle cells compared with the extremely low level of EGF receptor expression in medial smooth muscle cells (6). These results suggest that HB-EGF mainly influences functions of smooth muscle cells through the interaction with the EGF receptor in the intima, changing the phenotype to macrophage-like cells by expressing both c-fms and the scavenger receptor. The strong potency of HB-EGF in the regulation of cellular functions, including induction of c-fms and the scavenger receptor, cell migration, and proliferation, suggests its significant involvement in the atherosclerotic process. Although further studies are required to clarify the precise mechanisms of regulation, modifying the expression of HB-EGF as well as PDGF will be a major target for the prevention of the progression of atherosclerosis.

REFERENCES
Induction of Macrophage Colony-stimulating Factor Receptor (c-fms) Expression in Vascular Medial Smooth Muscle Cells Treated with Heparin Binding Epidermal Growth Factor-like Growth Factor

Toshimori Inaba, Shun Ishibashi, Kenji Harada, Jun-ichi Ohsuga, Ken Ohashi, Hiroaki Yagyu, Yoshio Yazaki, Shigeaki Higashiyama, Sumio Kawata, Yuji Matsuzawa and Nobuhiro Yamada

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