Epiregulin as a Major Autocrine/Paracrine Factor Released From ERK- and p38MAPK-Activated Vascular Smooth Muscle Cells

Masanori Takahashi, MD*; Ken’ichiro Hayashi, PhD*; Kenji Yoshida, MD*; Yasuyuki Ohkawa, PhD; Toshi Komurasaki, PhD; Akira Kitabatake, MD, PhD; Akira Ogawa, MD, PhD; Wataru Nishida, MD, PhD; Masahiko Yano, MD, PhD; Morito Monden, MD, PhD; Kenji Sobue, MD, PhD

Background—The coordinated activation of extracellular signal–regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38MAPK) is critical for the induction of vascular and visceral smooth muscle cell (SMC) dedifferentiation. We previously reported that on the forced activation of both MAPKs, visceral SMCs secrete a non–heparin-binding protein factor(s) that is involved in the dedifferentiation of neighboring SMCs. In this study, we sought to identify the dedifferentiation factor(s) derived from vascular SMCs (VSMCs).

Methods and Results—We fractionated the VSMC dedifferentiation factor(s) in the conditioned medium obtained from differentiated VSMCs in which both ERK and p38MAPK were forcibly activated and identified epiregulin as a major autocrine/paracrine factor for VSMC dedifferentiation. The epiregulin-induced VSMC dedifferentiation was mediated through the coordinated activation of ERK and p38MAPK. Unsaturated lysophosphatidic acid and platelet-derived growth factor-BB, which are potent VSMC dedifferentiation factors, rapidly upregulated epiregulin mRNA expression in an ERK- and p38MAPK-dependent manner. Reverse transcriptase–polymerase chain reaction and/or immunohistochemical analyses revealed the restricted expression of epiregulin in human atherosclerotic and balloon-injured rat arteries, in which the phenotypic modulation of medial VSMCs occurred in vivo.

Conclusions—Epiregulin is released from VSMCs primed by atherogenic factors and acts as a major autocrine/paracrine factor for VSMC dedifferentiation. It may be involved in the progression of vascular remodeling such as atherosclerosis. 

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Key Words: vasculature ▪ remodeling ▪ muscle, smooth ▪ atherosclerosis

The phenotypic modulation of vascular smooth muscle cells (VSMCs) from a differentiated state to a dedifferentiated one is critical for the development and progression of vascular remodeling such as atherosclerosis.1 The involvement has been demonstrated of epidermal growth factor (EGF) family members, basic fibroblast growth factor (bFGF), platelet-derived growth factors (PDGFs), insulin-like growth factor (IGF-I), and other growth factors and chemokines in VSMC proliferation and migration.1,2 However, it remains unknown whether any of them are critical for the development and progression of vascular remodeling because the above studies were performed with the use of passaged VSMCs, which show a dedifferentiated phenotype even under quiescent culture conditions.

We established primary culture systems of vascular and visceral SMCs, maintaining a differentiated phenotype.3–5 We used these systems to demonstrate that the IGF-I-stimulated phosphoinositide 3-kinase (PI3-K)/protein kinase B [PKB(Akt)] pathway plays a critical role in maintaining a differentiated SMC phenotype, whereas the coordinated activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (p38MAPK) triggered by PDGF-BB, EGF, bFGF, or unsaturated lysophosphatidic acids (LPAs) induces SMC dedifferentiation.3–5 These findings suggest that the balance of strength between the PI3-K/ PKB(Akt) pathway and the ERK and p38MAPK pathways could determine the SMC phenotype.4,5 We also demonstrated that the ERK- and p38MAPK-activated visceral SMCs secrete a non–heparin-binding protein factor(s) that potently dedifferentiate the neighboring cells.4 In this study, we identified epiregulin as a major factor for VSMC dedifferentiation in the conditioned medium obtained from the ERK- and p38MAPK-activated VSMCs. We also demonstrated the epiregulin-triggered VSMC dedifferentiation and

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From the Department of Neuroscience (K.H., Y.O., W.N., K.S.) and the Department of Surgery and Clinical Oncology (M.Y., M.M.), Osaka University Graduate School of Medicine, Osaka, Japan; the Department of Cardiovascular Medicine, Hokkaido University, Graduate School of Medicine (M.T., A.K.), Sapporo, Japan; the Department of Neurosurgery, Iwate Medical University School of Medicine (K.Y., A.O.), Morioka, Japan; and the Molecular Biology Laboratory, Medical Research Laboratories, Taisho Pharmaceutical Co, Ltd (T.K.), Saitama, Japan.

*These authors contributed equally to this report.

Correspondence to Dr Kenji Sobue, Department of Neuroscience, Osaka University Graduate School of Medicine (D13), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail sobue@nbiochem.med.osaka-u.ac.jp

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the epiregulin expression in unsaturated LPA-primed or PDGF-BB–primed VSMCs in culture and in human atherosclerotic and balloon-injured rat arteries.

**Methods**

**Materials**

Signaling inhibitors were purchased from CalBiochem. Recombinant epiregulin and polyclonal antibodies against human epiregulin and caldesmon (CaD) were used in this study. This anti-epiregulin antibody cross-reacted with rodent epiregulin and also neutralized the epiregulin activity. Polyclonal anti-ERK, anti-p38MAPK, and anti-PKB(Akt) antibodies were purchased from Santa Cruz Biotechnology. Neutralizing anti-EGF antibody was purchased from Upstate. Monoclonal anti-calponin antibody was purchased from Sigma. Monoclonal anti-myosin heavy chain (MHC) SM1 and SM2 antibodies and anti-α-smooth muscle actin (α-SMA) antibody were purchased from Yamasa and DAKO, respectively.

**Primary Culture of Differentiated VSMCs**

Differentiated VSMCs prepared from the rat aortas were cultured on laminin-coated plates in basal medium containing IGF-I (2 ng/mL) for 1 day. The medium was changed to basal medium containing fractions from conditioned medium, growth factors, or 18:1 LPA. Ligand-induced contractility was monitored as described previously. Dedifferentiated VSMCs were prepared by our previous method. 3

**Fractionation of Dedifferentiation Factor(s) in the Conditioned Medium**

According to our previous procedures, expression plasmids carrying constitutively active MEK1 and MKK6 were cotransfected into differentiated VSMCs, and the conditioned medium (CM1) was obtained after 2 days of culture. The expression of active MAPK kinases was confirmed by ERK and p38MAPK assays. A second conditioned medium (CM2) was obtained from the culture supernatant of differentiated VSMCs transfected with control plasmid. CM1 was separated on a heparin-Sepharose column into flow-through and eluted (1.5 mol/L NaCl) fractions. The flow-through fraction was dialyzed against 20 mmol/L Tris-HCl, pH 7.5, and applied to a 1-mL heparin-Sepharose column (HS-FT) but not its eluted fraction. The flow-through and eluted fractions were separated on a heparin-Sepharose column into flow-through and eluted (1.5 mol/L NaCl) fractions. The flow-through fraction was dialyzed against 20 mmol/L Tris-HCl, pH 7.5, and eluted stepwise with 0.1, 0.2, 0.3, 0.4, or 0.5 mol/L NaCl. Next, the flow-through and eluted fractions were dialyzed against the basal medium and then used for VSMC dedifferentiation assays. Cultured VSMCs were treated with inhibitors as described elsewhere. The detection of epiregulin in CM1 and the fractions eluted from the High Q column was performed by immunoprecipitation followed by immunoblotting with an anti-epiregulin antibody.

**Expression of SMC Molecular Markers and Epiregulin**

The total RNAs were extracted from cultured VSMCs, and the expression levels of CaD, calponin, MHC SM1 and SM2, epiregulin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were quantified by reverse transcriptase–polymerase chain reaction (RT-PCR). We first determined the GAPDH mRNA levels in each sample by RT-PCR by using differentially diluted heat-treated, single-stranded cDNA mixtures under different PCR cycles and then amplified the target cDNAs by using defined amounts of heat-denatured, single-stranded cDNA mixtures containing an equal amount of GAPDH cDNA. The PCR products were sampled at indicated cycles, separated on 1.2% agarose gels, and stained with SYBR Green (Molecular Probes). The relative intensities of CaD, calponin, epiregulin, and GAPDH cDNAs were quantified at the number of PCR cycles where each cDNA was linearly amplified (Figure 1A).

**Immunohistochemistry**

Animal experiments were conducted according to the ethical guidelines of Osaka University Medical School. Male SD rats (10 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg). Balloon injury of the left carotid artery was performed as described elsewhere. Segments of human and rat arteries were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut at 6 μm, dewaxed in xylene, rehydrated, permeabilized with proteinase K (DAKO), blocked by 3% hydrogen peroxide, and further blocked with 1% BSA (Sigma) in PBS. The sections were incubated with the indicated antibodies in blocking buffer, and then with anti-mouse Alexa 546 (Molecular Probes) or anti-rabbit EnVision (DAKO) and TSA Fluorescein System (Perkin-Elmer). Serial sections were stained with hematoxylin and eosin.

**Results**

We compared the expression of SMC molecular marker mRNAs in different phenotypes of cultured VSMCs. Figure 1A shows the expression of CaD, calponin, and GAPDH in differentiated and dedifferentiated VSMCs. The intensities of GAPDH cDNA increased linearly in response to increased PCR cycles in both phenotypes of VSMCs, whereas those of cDNAs for h-CaD, h-1-CaD, and calponin increased in differentiated VSMCs under indicated PCR cycles but were less significant in dedifferentiated VSMCs. These results indicated the downregulation of SMC molecular marker expression in dedifferentiated VSMCs. We used this analysis to determine the expression levels of the target molecules.

CM1, obtained from differentiated VSMCs cotransfected with active MEK1 and MKK6, potently induced VSMC dedifferentiation as monitored by the downregulation of SMC molecular marker expression, fibroblast-like shape change, and loss of ligand-induced contractility (Figure 1, B and C), but CM2 did not (data not shown). In the following experiments, we monitored the VSMC phenotype by using these three criteria. Consistent with our previous report on visceral SMCs, treatment of CM1 with heat or trypsin abolished its dedifferentiation activity (data not shown), indicating that cotransfected VSMCs secrete a VSMC dedifferentiation protein factor(s). CM1 markedly activated ERK, p38MAPK, and PKB(Akt), followed by VSMC dedifferentiation (Figure 1D).

We then fractionated and characterized the factor(s) by column chromatographies. The flow-through fraction of a heparin-Sepharose column (HS-FT) but not its eluted fraction...
(HS-EF) showed the dedifferentiation activity (Figure 1, B and C). The activity eluted with 0.3 mol/L NaCl (E3) from a High Q column (Figure 1, B and C). The E3 fraction also activated ERK, p38MAPK, and PKB(Akt) (Figure 1D). A specific inhibitor of the EGF receptor (EGFR) kinase, AG1478 (0.5 μmol/L), blocked these activations (Figure 1D), VSMC dedifferentiation (Figure 1, B and C), and CM1 and E3 induced the tyrosine phosphorylation of EGFR (data not shown). Simultaneous treatment with PD98059 (30 μmol/L) and SB203580 (20 μmol/L) specifically blocked the E3-triggered ERK and p38MAPK activation, resulting in inhibition of the E3-induced VSMC dedifferentiation (Figure 1, B, C, and D). These results suggest that the E3-triggered activation of both MAPKs through the EGFR is critical for VSMC dedifferentiation.

We partially analyzed the effects of EGF family members on visceral SMCs, in which heregulin never activated ERK and p38MAPK; EGF and amphiregulin potently activated ERK and p38MAPK but not PKB(Akt), and epiregulin activated these three protein kinases. In differentiated VSMCs, EGF (Figure 1D) and amphiregulin (data not shown) also activated both MAPKs but not PKB(Akt). The VSMC dedifferentiation factor(s) was not retained on a heparin-Sepharose column (Figure 1, B and C). Thus, the factor(s) might be the EGF family other than EGF, heparin-binding (HB)-EGF, heregulin, and amphiregulin. Among the EGF family members, the elution profile of the factor(s) by High Q column chromatography (Figure 1B) was identical to that of recombinant epiregulin (data not shown). Furthermore, epiregulin was immunoprecipitated from both CM1 and E3 through the use of anti-epiregulin antibody (Figure 2A). By comparing these results with immunoprecipitates obtained with the use of known amounts of recombinant epiregulin, we estimated the concentration of epiregulin in CM1 to be 1.80±0.16 ng/mL. The anti-epiregulin antibody (10 μg/mL) but not nonimmune IgG or anti-EGF antibody (10 μg/mL) completely neutralized the dedifferentiation activity in E3 (Figure 2, B and C), indicating that epiregulin in E3 induces
VSMC dedifferentiation. In accordance with the signaling pathways triggered by CM1 and E3, epiregulin potently activated ERK, p38MAPK, and PKB(Akt) in differentiated VSMCs (Figure 1D). All of the protein kinase activities triggered by epiregulin were blocked by AG1478, and the ERK and p38MAPK activations were sensitive to PD98059 and SB203580, respectively (data not shown). Epiregulin dose- and/or time-dependently downregulated the expression of SMC molecular markers, such as CaD, calponin, and MHC SM1 and SM2 in VSMCs at mRNA and protein levels (Figure 2, D and E). Consistent with the effects of CM1 and E3 on differentiated VSMCs, the epiregulin-triggered downregulation of the SMC molecular marker expression was completely suppressed by AG1478 or both PD98059 and SB203580 (Figure 2D). These results indicate that epiregulin is the major VSMC dedifferentiation factor in the conditioned medium obtained from differentiated VSMCs by the forced activation of ERK and p38MAPK.

We previously reported that unsaturated LPAs and PDGF-BB potently induce VSMC dedifferentiation. Figure 3 shows the rapid expression of epiregulin in VSMCs stimulated by 18:1 LPA; epiregulin mRNA was expressed within 30 minutes after stimulation, and its expression was maintained for more than 24 hours. In contrast, the expression of SMC molecular marker mRNAs except for MHC SM1 mRNA gradually downregulated within 24 hours, indicating that the epiregulin expression is early and sustained events of the phenotypic modulation of VSMCs. The 18:1 LPA-induced expression of epiregulin was partially suppressed by either PD98059 or SB203580 and was completely blocked by both inhibitors (Figure 3). Similar results were observed in VSMCs stimulated with PDGF-BB. Furthermore, AG1478 had no effect on the 18:1 LPA-triggered or PDGF-BB–triggered ERK and p38MAPK activation (data not shown). These results suggest that epiregulin does not act as an inducer for VSMC dedifferentiation but as an autocrine/paracrine factor for the progression of VSMC dedifferentiation.

Figure 2. Identification of epiregulin (ER) as a major factor for VSMC dedifferentiation in CM1 and E3 (A). Basal medium containing epiregulin (ER, 25 ng), CM1, and E3 was immunoprecipitated with an anti-epiregulin antibody (ER Ab) as described elsewhere. Immuno- precipitates (IP) obtained with anti-epiregulin antibody or nonimmune IgG were analyzed by immunoblotting (IB) with anti-epiregulin antibody. Neutralizing effects of anti-epiregulin antibody on E3-triggered VSMC dedifferentiation were monitored by cell morphology; CCH-induced contractility (B) and SMC molecular marker expression were analyzed by RT-PCR (C). Differentiated VSMCs were cultured for 3 days in basal medium with CM1, E3, or IGF-I (I) in the presence of anti-epiregulin and anti-EGF antibodies, nonimmune IgG, vehicle (DMSO, --), AG1478 (AG), or PD98059 and SB203580 (P/S). In ligand-induced contractile assays, arrows indicate contracted cells. Effect of epiregulin on SMC molecular marker expression in differentiated VSMCs (D and E). Differentiated VSMCs were cultured for indicated days in basal medium with IGF-I (I), CM1, or indicated amounts of epiregulin (ER) in the presence of vehicle (DMSO, --), AG1478 (AG), or both PD98059 and SB203580 (P/S). Expression of MHC SM1 and SM2 mRNAs was determined by RT-PCR analyses: MHC SM1(498 bp), 32 cycles; MHC SM2 (396 bp), 36 cycles. Expression of SMC molecular marker proteins was analyzed by immunoblotting (IB). Protein contents in each cell lysate were normalized by ERK protein.

Figure 3. Induction of epiregulin mRNA expression by 18:1 LPA. Differentiated VSMCs were cultured for indicated hours in basal medium containing 18:1 LPA (1 μmol/mL) in the presence of vehicle (DMSO, --), PD98059 (P) or SB203580 (S), or PD98059 and SB203580 (P/S). Size of epiregulin (ER) cDNA and numbers of PCR cycles were 610 bp and 30 cycles, respectively. Progressive changes in SMC molecular marker mRNA expression were monitored by RT-PCR. PCR products were quantified by NIH Image. Epiregulin mRNA levels (lower graphs) were normalized to GAPDH mRNA, and peak value was defined as 1.0. Each value represents mean ± SD of 3 independent experiments.
To address the involvement of epiregulin in vivo, we performed RT-PCR and immunohistochemical analyses of human atherosclerotic arteries (Figures 4 and 5). RT-PCR analyses revealed the expression of epiregulin mRNA in atherosclerotic but not in normal arteries (Figure 4). We determined the expression of α-SMA, calponin, and epiregulin in normal and atherosclerotic arteries with their respective antibodies (Figure 5). In accordance with RT-PCR analyses, epiregulin was only detected in atherosclerotic (n=12) but not in normal (n=4) arteries. Interestingly, most of epiregulin labelings localized to the medial VSMC layer, where the expression of α-SMA and calponin was downregulated. Some of them overlapped with α-SMA labelings. The epiregulin expression was not detected in the neointima. In balloon-injured arteries (Figure 6), epiregulin was expressed within the luminal side of arteries at day 1 and in the inner medial VSMC layer at day 2. Its expression was expanded from the inner medial VSMC layer to the neointima at day 7 but faded away at day 21 (each time point, n=6). Most of epiregulin labelings in balloon-injured arteries localized to the regions where the α-SMA expression was downregulated. In some regions, both of them overlapped. Taken together, these results indicate that epiregulin was initially expressed in the regions where the phenotypic modulation of VSMCs occurred in vivo.

Discussion

In this study, we identified epiregulin as a downstream product of the coordinated activation of ERK and p38MAPK in VSMCs and as an autocrine/paracrine factor for the progression of VSMC dedifferentiation (Figures 1 through 3). We estimated the epiregulin concentration to be 1.80±0.16 ng/mL in CM1 obtained from the supernatant of cultured VSMCs in...
which both ERK and p38MAPK were activated (Figure 2A). This value is consistent with the dose-dependency of recombinant epiregulin required for VSMC dedifferentiation (Figure 2D). Our present study demonstrated that the EGFR serves as the principal receptor for epiregulin-induced VSMC dedifferentiation, because the epiregulin-triggered signaling pathways and VSMC dedifferentiation were completely blocked by AG1478 (Figure 2D) and epiregulin also enhanced the tyrosine phosphorylation of EGFR. EGF potently activated ERK and p38MAPK but not PKB(Akt), mediated through the EGFR, whereas epiregulin activated all of three protein kinases in differentiated VSMCs (Figure 1D). Since epiregulin has broader selectivity for ErbB receptors than EGF, this difference may be due to characteristic features of epiregulin. The epiregulin expression in VSMCs was not an artifact by the forced coexpression of active MEK1 and MKK6 but a naturally occurring event, because unsaturated LPA (Figure 3) or PDGF-BB (data not shown), which induce VSMC dedifferentiation through the ERK and p38MAPK activation, rapidly stimulated the epiregulin expression in VSMCs. Thus, our present study suggests that epiregulin is an autocrine/paracrine factor secreted from atherogenic factor–primed VSMCs and that the released epiregulin further induces the dedifferentiation of neighboring VSMCs.

The expression level of epiregulin mRNA in normal tissues is undetectable, but it is marked in epithelial tumor cell lines, suggesting the involvement of epiregulin in cancer cell proliferation. Taylor et al identified epiregulin as a potent mitogen for passaged (dedifferentiated) VSMCs. We demonstrated the expression of epiregulin in atherosclerotic arteries, where the phenotypic modulation of VSMCs occurred (Figures 4 and 5). Epiregulin was also transiently expressed at early stages of balloon-injured arteries but vanished at a late stage (Figure 6). This is the first finding regarding the epiregulin expression in the vasculature. Thus, the expression of epiregulin in VSMCs was an early event in response to atherogenic stimuli to progress VSMC dedifferentiation. The expression profile of epiregulin in atherosclerotic arteries contrasted with that of HB-EGF, which is mainly detected in the neointima of atherosclerotic arteries and is considered to play a role for proliferation and migration of dedifferentiated VSMCs. Taken together, our results suggest that epiregulin is involved in early stages of VSMC dedifferentiation rather than proliferation and migration of dedifferentiated VSMCs in the vasculature under pathological conditions. Our present study also provides a clue for the molecular mechanism underlying the development of vascular remodeling such as atherosclerosis.

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