Changes in the Balance of Phosphoinositide 3-Kinase/Protein Kinase B (Akt) and the Mitogen-activated Protein Kinases (ERK/p38MAPK) Determine a Phenotype of Visceral and Vascular Smooth Muscle Cells

Ken'ichiro Hayashi, Masanori Takahashi, Kazuhiro Kimura, Wataru Nishida, Hiroshi Saga, and Kenji Sobue

Department of Neurochemistry and Neuropharmacology, Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Abstract. The molecular mechanisms behind phenotypic modulation of smooth muscle cells (SMCs) remain unclear. In our recent paper, we reported the establishment of novel culture system of gizzard SMCs (Hayashi, K., H. Saga, Y. Chimori, K. Kimura, Y. Yamanaka, and K. Sobue. 1998. *J. Biol. Chem.* 273: 28860–28867), in which insulin-like growth factor-I (IGF-I) was the most potent for maintaining the differentiated SMC phenotype, and IGF-I triggered the phosphoinositide 3-kinase (PI3-K) and protein kinase B (PKB(Akt)) pathway. Here, we investigated the signaling pathways involved in de-differentiation of gizzard SMCs induced by PDGF-BB, bFGF, and EGF. In contrast to the IGF-I–triggered pathway, PDGF-BB, bFGF, and EGF coordinately activated ERK and p38MAPK pathways. Further, the forced expression of active forms of MEK1 and MKK6, which are the upstream kinases of ERK and p38MAPK, respectively, induced de-differentiation even when SMCs were stimulated with IGF-I. Among three growth factors, PDGF-BB only triggered the PI3-K/PKB(Akt) pathway in addition to the ERK and p38MAPK pathways. When the ERK and p38MAPK pathways were simultaneously blocked by their specific inhibitors or an active form of either PI3-K or PKB(Akt) was transfected, PDGF-BB in turn initiated to maintain the differentiated SMC phenotype. We applied these findings to vascular SMCs, and demonstrated the possibility that the same signaling pathways might be involved in regulating the vascular SMC phenotype. These results suggest that changes in the balance between the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways would determine phenotypes of visceral and vascular SMCs. We further reported that SMCs cotransfected with active forms of MEK1 and MKK6 secreted a nondialyzable, heat-labile protein factor(s) which induced de-differentiation of surrounding normal SMCs.

Key words: smooth muscle cells • phosphoinositide 3-kinase • mitogen-activated protein kinases • ERK • p38MAPK

Phenotypic modulation of smooth muscle cells (SMCs)[1] is critical in the onset of serious diseases such as atherosclerosis, hypertension, and leiomyogenic tumorigenicity. In the progression of these diseases, SMCs change from a differentiated state to a de-differentiated one (reviewed by Ross, 1993). Differentiated SMCs show a spindle-like shape and organize their unique intracellular structures including well-developed dense membranes, dense bodies, and myofibrils. They also display ligand-induced contraction. In contrast, de-differentiated SMCs lose these characteristic properties. In addition to these morphological and functional alterations, the expression levels and/or the isoforms of several proteins change in the two phenotypes. Therefore, these proteins

Address correspondence to Kenji Sobue, Department of Neurochemistry and Neuropharmacology, Biomedical Research Center, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: 81-6-6879-3680. Fax: 81-6-6879-3689. E-mail: sobue@nbiochem.med.osaka-u.ac.jp

1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CM, conditioned medium; CAT, chloramphenicol acetyltransferase; ERK, extracellular signal-regulated kinase; IGF-I, insulin-like growth factor-I; JNK, c-Jun NH_2-terminal protein kinase; MAPKs, mitogen-activated protein kinases; MBP, myelin basic protein; MT, c-Myc-tag; $p70^{S6K}$, p70 ribosomal S6 kinase; PI, phosphatidylinositol; PI3-K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PS, phosphatidylserine; RSV, Rous sarcoma virus; SMC, smooth muscle cell; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.

© The Rockefeller University Press, 0021-9525/99/05/727/14 $2.00
The Journal of Cell Biology, Volume 145, Number 4, May 17, 1999 727–740
http://www.jcb.org

are used as SMC-specific molecular markers (Owens, 1995; Sobue et al., 1998). For example, the expressions of caldesmon (Yano et al., 1995; Kashiwada et al., 1997), calponin (Gimona et al., 1992; Shanahan et al., 1993), SM22α (Gimona et al., 1992; Shanahan et al., 1993), β-tropomyosin (Kashiwada et al., 1997), and α1 integrin (Glukhova et al., 1993; Obata et al., 1997) at the mRNA and protein levels are upregulated in differentiated SMCs, but downregulated in de-differentiated SMCs. Isoform changes of caldesmon (Ueki et al., 1987), α-tropomyosin (Kashiwada et al., 1997), vinculin/*meta*vinculin (Gimona et al., 1987), and smooth muscle myosin heavy chain (Nagai et al., 1989; Kuro-o et al., 1989) are also controlled by SMC phenotype-dependent alternative splicings. Recently, the transcription machineries of caldesmon (Yano et al., 1995), smooth muscle myosin heavy chain (Katoh et al., 1994; Madsen et al., 1997), SM22α (Solway et al., 1995; Kim et al., 1997) and α1 integrin (Obata et al., 1997) have been partially characterized. However, the molecular mechanisms behind phenotypic modulation of SMCs remain unclear. The slow progress in this area may be due to the plasticity of SMCs. Under conventional culture conditions, SMCs in primary culture display a rapid change in their phenotype (reviewed by Chamley-Campbell et al., 1979). And, SMC-derived clonal cell lines that maintain a fully differentiated phenotype have not yet been established. We have recently established a novel culture system for gizzard SMCs in which they maintain a differentiated phenotype for a long time. Extracellular matrices partially affect the SMC phenotype. Of these, laminin is the most potent for delaying the progression of SMC de-differentiation, but can not maintain a differentiated phenotype for a long culture period, suggesting a requirement for additional factor(s). Among several growth factors and cytokines examined, insulin-like growth factor-I (IGF-I) is the most potent for maintaining the differentiated SMC. In the IGF-I–stimulated culture system, phosphoinositide 3-kinase (PI3-K) and its downstream target, protein kinase B (PKB(Akt)), but not mitogen-activated protein kinases (MAPKs), mediate the critical signaling pathways (Hayashi et al., 1998).

MAPKs have been implicated in the signaling cascades involved in the proliferation and hypertrophy of SMCs (reviewed by Force and Bonventre, 1998). These include extracellular signal-regulated kinase (ERK) and the stress-activated MAPKs, c-Jun NH_2-terminal protein kinase (JNK) and p38MAPK. ERK is activated in response to growth factors, cytokines, and cellular stresses (Ray and Rturgill, 1988; Denharrdt, 1996), and is involved in a variety of biological processes (Force and Bonventre, 1998). Treatment with growth factors such as PDGF, EGF, and basic fibroblast growth factor (bFGF), which induce the proliferation or migration of cultured SMCs, activates ERK. JNK and p38MAPK are also activated by cellular stresses including inflammatory cytokines, heat shock, osmolar stress, ultraviolet irradiation, and inhibition of protein synthesis (Derijard et al., 1994; Kyrian and Avruch, 1996). The ERK and JNK activities are increased in aortic, carotid, and femoral arteries by hypertensive agents, angiotensin II and phenylephrine (Xu et al., 1996). In cultured airway SMCs, endothelin activates both the ERK and JNK signaling pathways, resulting in cell proliferation (Shapiro et al., 1996). ERK (Pyles et al., 1997), ERK/JNK (Hu et al., 1997), or p38MAPK (Larrivee et al., 1998) are enhanced in rat carotid arteries after balloon injury. p38MAPK is also elicited in airway SMCs by PDGF stimulation (Pyne and Pyne, 1997). These findings suggest that some smooth muscle disorders are closely associated with the activation of MAPKs. However, the direct involvement of MAPK pathways in regulating the SMC phenotype has not yet been demonstrated.

We investigated the signaling pathways involved in SMC de-differentiation induced by PDGF-BB, bFGF and EGF, and compared them with the IGF-I–triggered signaling pathway in maintaining a differentiated phenotype of gizzard SMC in culture. Here, we demonstrated the first direct evidence for a mechanism by which the distinctly different signaling pathways regulate the SMC phenotype. Both the ERK and p38MAPK pathways triggered by PDGF-BB, bFGF, and EGF were found to play an essential role in inducing SMC de-differentiation, whereas the PI3-K/PKB(Akt) pathway was critical in maintaining a differentiated state. Interestingly, PDGF-BB only triggered both types of signaling pathways. When the ERK and p38MAPK pathways were blocked by their specific inhibitors, PDGF-BB in turn initiated to maintain a differentiated phenotype of gizzard SMCs. The same signaling pathways involving in the phenotypic determination were observed in vascular SMCs. Thus, changes in the balance between the strengths of the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways would determine phenotypes of visceral and vascular SMCs. We further demonstrated a de-differentiation–inducing factor(s) secreted from SMCs in which both the MAPK pathways were activated by cotransfection with active forms of MEK1 and MKK6.

Materials and Methods

Antibodies

Anti–PI3-K p85 subunit antiserum was purchased from Upstate Biotechnology. Polyclonal antibodies against PKB(Akt), ERK, JNK, p38MAPK, MEK1, and MKK6 were obtained from Santa Cruz Biotechnology. Monoclonal antibodies against c-Myc and Flag were purchased from Santa Cruz Biotechnology and Sigma Chemical Co., respectively.

Plasmids

Construction of the caldesmon promoter plasmid, GP3CAT, was described previously (Yano et al., 1995). The expression vector containing the constitutively active form of the c-Myc–tagged PI3-K p110α subunit (pCMV5p110αact) was kindly provided by Drs. H. Kurosu and T. Katada (Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo). This cDNA was constructed by Hu et al. (1995) and was inserted downstream of the cytomegalovirus promoter, pCMV5. Expression vectors of constitutively active and dominant-negative forms of MEK1 and MKK6, and Flag-tagged ERK2 and Flag-tagged p38MAPK were kindly provided by Dr. K. Sugiyama (Boehringer Ingelheim), Drs. M. Hibi and T. Hirano (Osaka University, Medical School), and Dr. E. Nishida (Graduate School of Science, Kyoto University). Mutant cDNAs of MEK1 and MKK6 were constructed as described elsewhere (Mansour et al., 1994; Raingeaud et al., 1996), and were inserted downstream of the cytomegalovirus promoter of pCS2+ or the SRα promoter of pcDLSRα296. In this study, we used expression vectors constructed in pCS2+ for active and dominant-negative forms of MEK1 (pCS2+MEK1act and pCS2+MEK1DN, respectively) and MKK6 (pCS2+MKK6act and pCS2+MKK6DN, respectively). A PKB(Akt) cDNA was amplified by reverse transcriptase PCR using human placental

mRNA as a template, and the accuracy of its sequence was checked. A PKB(Akt) cDNA thus obtained was inserted downstream of the cytomegalovirus promoter of pCS2+c-Myc–tagged (MT) (pCS2+MT-PKB(Akt)wt for expression of c-Myc–tagged wild-type PKB(Akt). The expression plasmid of c-Myc–tagged constitutively active form of PKB(Akt), pCS2+MT-PKB(Akt)act, was constructed as described previously (Dario et al. 1996).

Cell Culture

Isolated gizzard SMCs were prepared from 15-d-old chick embryo gizzards as described elsewhere (Hayashi et al., 1998), and cultured on laminin-coated six-well plates with the indicated growth factors under kinase inhibited or stimulated conditions. Vascular SMCs were isolated from 5-wk-old rat aortae by enzyme-disperse methods as follows. Aortae were dissected under sterile conditions, minced well with scissors, and incubated at 37°C in 0.1% collagenase for 30 min, followed by incubation in the mixtures of 0.07% collagenase and 0.03% elastase for 90 min. Dispersed single cells were separated from undigested tissues by filtration, and were collected by centrifugation. The cells thus obtained were washed twice with growth factor–free basal medium (DME supplemented with 0.2% BSA), and were cultured in the medium containing IGF-I or PDGF-BB on laminin-coated culture plates. Treatment with specific inhibitors for ERK kinase (MEK1), PD98059 and/or for p38MAPK, SB203580, was performed as follows: gizzard or vascular SMCs were preincubated for 1 h in growth factor–free basal medium (DME supplemented with 0.2% BSA) containing the indicated amounts of inhibitors, and then stimulated with medium containing the indicated growth factors with or without inhibitors.

Ligand-induced contractility of cultured SMCs was monitored as follows. The SMCs were cultured under indicated conditions for 3 d, and then washed with PBS, followed by stimulation with basal culture medium containing 1 mM carbachol for 1 min. Contractility of cultured SMCs was observed with an Olympus microscope, and the same fields before and after carbachol treatment were photographed.

Northern Blotting

2 μg of total RNA from precultured or cultured SMCs under the indicated conditions were separated on 1.0% agarose-formaldehyde denaturing gels, and then transferred to nylon membranes. A caldesmon cDNA (GenBank M28417) fragment (nucleotides 286 to 810) and a calponin cDNA (GenBank M63559) fragment (nucleotides 1 to 867) were used as probes to monitor the expression of respective mRNAs. This caldesmon cDNA fragment, which contains parts of exons 2 and 3a is a common probe for the *h*- and *l*-caldesmons (Hayashi et al., 1991; Hayashi et al., 1992). In our previous studies using specific probes for *h*- or *l*-caldesmon, we demonstrated that the full lengths of *h*- and *l*-caldesmon mRNAs are 4.8 and 4.1 kb, respectively (Kashiwada et al., 1997; Obata et al., 1997). Probes were labeled with ^{32}P on the antisense strands and used for hybridization under the following conditions: 42°C for 16 h in 50% formamide, 6× SSC, 10× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone and 0.02% BSA), 0.5% SDS, and 0.5 mg/ml denatured herring sperm DNA. The blots were washed in 0.1× SSC containing 0.1% SDS at 52°C, and visualized by autoradiography. To quantify the amount of RNA loaded, ribosomal RNAs were stained with 0.02% methylene blue.

Immunoblotting

Total protein of the cell lysates from SMC cultures was separated by SDS-PAGE and transferred to nitrocellulose membranes. Detection of target proteins on the membranes was performed using an ECL Western blotting detection kit (Amersham Pharmacia Biotech) with the indicated polyclonal antibodies.

PI3-K Assay

Phospholipid mixtures (2 mg/ml) containing phosphatidylinositol (PI) and phosphatidylserine (PS) were dried under a stream of nitrogen, and sonicated in 10 mM Hepes (pH 7.4) in a bath sonicator at 0°C for 15 min. 10 μl of the resulting vesicles (PI/PS) were used as a substrate for PI3-K. The preparation of cell extracts and immunoprecipitation for PI3-K were performed at 4°C. The cultured cells were washed three times with ice-cold PBS, and then lysed in 550 μl of lysis buffer (20 mM Tris-HCl [pH 7.5], 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 50 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). After gentle shaking for 30 min, the cell extracts were obtained by centrifugation in a microfuge at 13,000 rpm for 5 min. The amount of PI3-K p85 subunit in the cell extracts was determined by Western blotting using antiserum against the PI3-K p85 subunit. The extracts containing equal amounts of PI3-K p85 subunit were precleaned with control rabbit IgG coupled protein A–Sepharose for 30 min. The PI3-K was immunoprecipitated with antiserum against the PI3-K p85 subunit followed by protein A–Sepharose. The immunoprecipitates were washed twice with lysis buffer, twice with 100 mM Tris-HCl (pH 7.5), 0.5 M LiCl, 1 mM DTT, and 0.2 mM Na_3VO_4, and three times with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, and 0.2 mM Na_3VO_4. All washes were performed at 4°C. The reaction mixtures (50 μl), containing the immunoprecipitates in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 10 μM ATP, 5 μCi γ-[^{32}P]ATP, and 20 μg of PI/PS were incubated at 30°C for 10 min. The reactions were terminated and the lipids were extracted by the addition of $CHCl_3$/MeOH (1:2). The mixture was then vortexed and cleared by centrifugation. The extracted products were separated by thin-layer chromatography in a developing solution composed of $CHCl_3$/MeOH/4 M NH_4OH (9:7:2). The production of phosphatidylinositol-3-phosphate was detected by autoradiography.

Other Protein Kinase Assays

Cell lysis buffers were as follows: 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 50 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin for the ERK and PKB(Akt) assays; 20 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM DTT, 120 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 50 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin for the JNK assays; and 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 2 mM Na_3VO_4, 10 mM β-glycerophosphate, 50 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin for the p38MAPK assays. The cell extracts were immunoprecipitated with specific antibodies against individual protein kinases, and the immunoprecipitates were washed thoroughly with their lysis buffers and then kinase assay buffers, and incubated with their respective substrates and 5 μCi γ-[^{32}P]ATP for 30 min at 30°C. The reaction products were analyzed by 15% SDS-PAGE. Reaction mixtures for the kinase assays were as follows: 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 μM protein kinase A inhibitor, 1 mM DTT, and 25 μg histone H2B for the PKB(Akt) assay; 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 μM protein kinase A inhibitor, 1 mM DTT, and 25 μg myelin basic protein (MBP) for the ERK assay; 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 μM protein kinase A inhibitor, 1 mM DTT, 1 mM Na_3VO_4, and 1 μg GST-Jun (1-79) for the JNK assay; and 20 mM Hepes (pH 7.4), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 μM protein kinase A inhibitor, 2 mM DTT, and 1 μg GST-ATF2 (1-96) for the p38MAPK assay.

Promoter Analysis and Transfection

The caldesmon promoter activity was analyzed using the chloramphenicol acetyltransferase (CAT) construct, GP3CAT, according to the method described previously (Yano et al., 1995; Obata et al., 1997). The SMCs prepared as described above were seeded onto laminin-coated six-well plates, and cultured in the indicated medium for 1 or 3 d. Transfection was carried out using Trans IT™-LT1, polyamine transfection reagents (Pan Vera Corporation). Complex mixtures composed of 10 μg of trans IT™-LT1 reagent and 2 μg of GP3CAT, 1 μg of control plasmid carrying the luciferase gene under the Rous sarcoma virus (RSV) promoter (RSV-luciferase), and 1 μg of either control expression plasmid (pCMV5, pCS2+, or pCS2+MT), expression plasmid carrying a c-Myc–tagged constitutively active form of PI3-K p110α subunit, a c-Myc–tagged wild-type or a constitutively active form of PKB(Akt) (pCMV5p110αact, pCS2+MT-PKB(Akt)wt or pCS2+MT-PKB(Akt)act), or either or both of expression vectors carrying constitutively active and dominant-negative forms of MEK1 (pCS2+MEK1act and pCS2+MEK1DN) and MKK6 (pCS2+MKK6act and pCS2+MKK6DN), were added to the cells in Opti minimum Eagle's medium (GIBCO BRL). After a further 4-h incubation, the medium was replaced with DME supplemented with 0.2% BSA plus 2 ng/ml IGF-I or 20 ng/ml PDGF-BB, and the transfected cells were harvested 48 h later. Standardization of transfection efficiency was performed by measuring luciferase activity as described previously (Yano et al., 1995; Obata et al., 1997). The cell extracts containing equal amounts of luciferase activity were used for the CAT assay. The transfection experiments were repeated at least three times on duplicate cultures with two or

three different plasmid preparations. The CAT activities were quantified by Scanning Imager (Molecular Dynamics).

The effects of forced expression of MEK1 and MKK6 in cultured SMCs were analyzed as follows. The indicated amounts of control expression plasmid and either or both of expression plasmids carrying active or dominant-negative MEK1 and MKK6 were transfected into cultured SMCs together with 1 μg of a reporter plasmid carrying the β-galactosidase gene downstream from the SV-40 early promoter. Total RNA was isolated from the transfected cells and the expression levels of caldesmon and calponin mRNAs were analyzed by Northern blotting as described above. Transfection efficiencies were determined by staining for β-galactosidase activity from the reporter plasmid using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) as a substrate.

Expression of Epitope-tagged Kinases and Kinase Assays

Transfection was carried out as described above in Promoter Analysis and Transfection. In the cases of PI3-K and PKB(Akt) assays, SMCs were transfected with 3 μg of respective expression plasmids of constitutively active form of c-Myc–tagged PI3-K p110α subunit, pCMV5p110αact, or wild-type or constitutive active form of c-Myc–tagged PKB(Akt), pCS2+MT-PKB(Akt)wt or pCS2+MT-PKB(Akt)act. Two micrograms of expression plasmid of each Flag-tagged ERK2 or Flag-tagged p38MAPK was cotransfected with 2 μg of either expression plasmid of active or dominant-negative MEK1 or MKK6, or control plasmid. In both cases, SMCs were cultured under nonstimulated conditions after transfection. 2 d later, SMCs were stimulated under indicated conditions. The cell extracts containing the equal amounts of proteins were precleaned with control mouse IgG coupled protein G–Sepharose for 30 min and immunoprecipitated with monoclonal antibody against c-Myc or Flag followed by protein G–Sepharose. The kinase activities were determined as described above in PI3-K assay and other protein kinase assays.

Characterization of Conditioned Medium

Conditioned medium obtained from SMCs transfected with both expression plasmids carrying active MEK1 and MKK6 was filtered through a 0.22-μm membrane. The conditioned medium was heated to 100°C for 15 min or treated with trypsin (30 μg/ml) for 3 h at 30°C, followed by the addition of trypsin inhibitor at a 10-fold excess. The heat- or trypsin-treated conditioned medium was dialyzed against DME supplemented with 0.2% BSA at 4°C for 16 h, and adjusted to the concentration of IGF-I to 2 ng/ml. The SMCs were cultured in these medium for 3 d. Heparin–Sepharose affinity chromatography was carried out as follows. One ml of 50% slurry of heparin–Sepharose (Amersham Pharmacia Biotech) equilibrated with PBS was added to 20 ml of the conditioned medium and gently agitated for 5 h at 4°C. The mixture was poured over a 1-ml Prep Column (Bio-Rad Labs.), and the follow through fraction (non–heparin-binding) was collected. The column was rinsed with 10 vol of PBS and eluted stepwise with 1 ml of PBC containing NaCl (0.5, 1.0, and 1.5 M). Each fraction was collected and desalted by dialysis against DME supplemented with 0.2% BSA through a membrane of 3-kD cutoff (Spectrum). Aliquots of each fraction were diluted (1:4) with DME supplemented with 0.2% BSA and then added to SMC cultures. Treatment with specific inhibitor of EGF receptor kinase, AG1478, was performed as follows: SMCs cultured for 2 d under IGF-I–stimulated conditions were preincubated for 1 h in DME supplemented with 0.2% BSA containing 1 μM AG1478, and then stimulated with the conditioned medium containing the same concentration of this drug for 3 d. Total RNAs from cultured SMCs were extracted and the expression patterns of caldesmon and calponin mRNAs were analyzed by Northern blotting. Control medium was obtained from culture supernatant of SMCs transfected with expression plasmid alone.

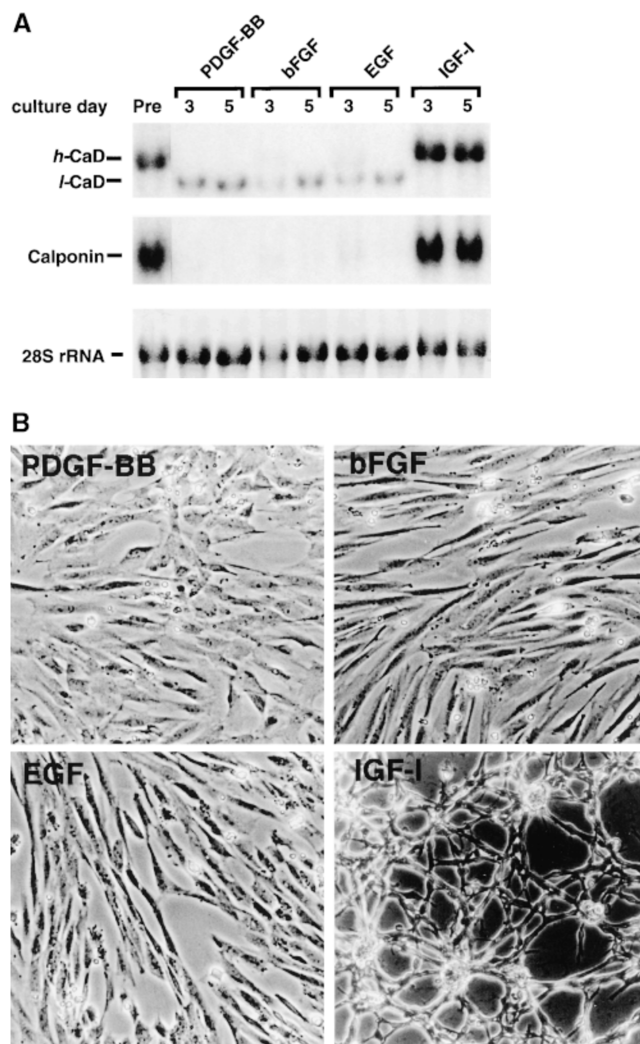


Figure 1. Effects of PDGF-BB, bFGF, EGF, and IGF-I on a phenotype of cultured gizzard SMCs. (A) Expression of caldesmon mRNAs (top panel; *h*-caldesmon mRNA [*h*-CaD, 4.8 kb] and *l*-caldesmon mRNA [*l*-CaD, 4.1 kb]) and calponin mRNA (middle panel; 1.8 kb) in SMCs stimulated with indicated growth factors. The bottom panel shows the 28S rRNA stained by methylene blue. Gizzard SMCs were cultured on laminin-coated plates in DME supplemented with 0.2% BSA for 24 h, and then stimulated with the same medium containing PDGF-BB (20 ng/ml), bFGF (10 ng/ml), EGF (10 ng/ml), or IGF-I (2 ng/ml) for 3 or 5 d. Caldesmon and calponin mRNAs in freshly isolated SMCs (Pre) and cultured SMCs under various conditions were analyzed by Northern blotting. (B) Comparison of cell morphology of SMCs under PDGF-BB, bFGF, EGF, or IGF-I–stimulated conditions for 5 d. The results are shown from four independent experiments.

Results

Different Downstream Signaling Pathways Triggered by PDGF-BB, bFGF, EGF, and IGF-I

We have recently established a novel culture system of gizzard SMCs in which they maintain a differentiated phenotype for a long culture period. Of growth factors and cytokines examined, IGF-I is the most potent for maintaining the differentiated SMC phenotype as defined by the expression of SMC-specific molecular markers, cell morphology, and function (Hayashi et al., 1998). On the other hand, PDGF-BB, bFGF, and EGF potently induce SMC de-differentiation (Fig. 1). In 3- and 5-d–cultured SMCs stimulated with PDGF-BB, bFGF, or EGF, *h*-caldesmon mRNA converts to *l*-caldesmon mRNA, and total *h*- and *l*-caldesmon mRNAs decrease to 20% of the levels

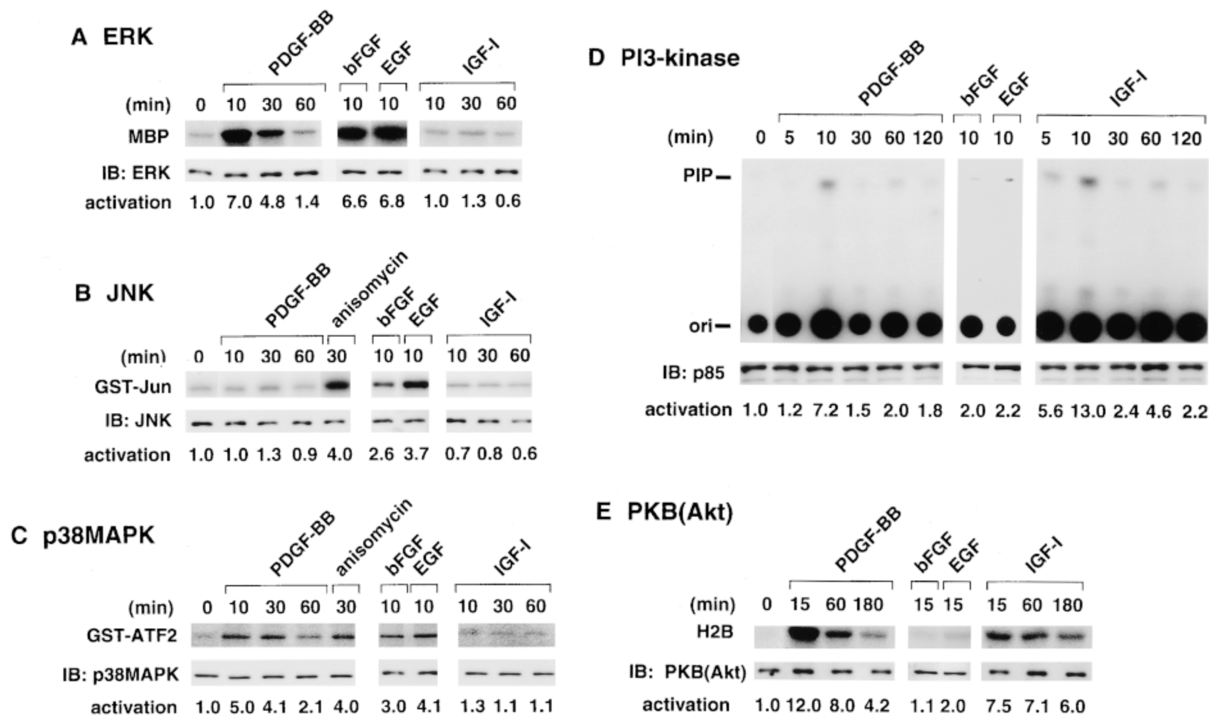


Figure 2. Characterization of the downstream signaling pathways triggered by PDGF-BB, bFGF, EGF, and IGF-I in SMCs. Gizzard SMCs were plated on laminin and cultured in DME supplemented with 0.2% BSA for 24 h, and then stimulated with the following growth factors for the indicated times: 20 ng/ml PDGF-BB, 10 ng/ml bFGF, 10 ng/ml EGF, 2 ng/ml IGF-I, or 10 μg/ml anisomycin. The cells were lysed and the extracts were assayed for the kinase activities of ERK (A), JNK (B), p38MAPK (C), PI3-K (D), and PKB(Akt) (E) as described in Materials and Methods. The left-hand lanes (0 min) in A–E were the controls without stimulation. Immunoblottings were performed to determine the amounts of kinase proteins in the cell lysates. The top and middle panels show the results of kinase assays and immunoblottings (IB), respectively. The relative activation rates normalized to respective control are shown in the bottoms. The representative results are shown from five independent experiments.

seen in precultured SMCs. Calponin mRNA is also downregulated to a negligible level (Fig. 1 A). However, the levels of *h*-caldesmon and calponin mRNAs in cultured SMCs under IGF-I–stimulated conditions are identical with those seen in precultured cells (Fig. 1 A). Similar results are obtained using α- and β-tropomyosins (Kashiwada et al., 1997) and α1 integrin (Obata et al., 1997), which are also SMC-specific molecular markers (data not shown). With regard to cell morphology and function, cultured SMCs under IGF-I–stimulated conditions showed a spindle-like shape, formed a meshwork structure, and displayed carbachol-induced contraction. In contrast, SMCs stimulated with PDGF-BB, bFGF, or EGF showed a fibroblast-like shape and lost the contractility (Figs. 1 B and 4), indicating that PDGF-BB, bFGF, and EGF are potent factors for inducing SMC de-differentiation.

Our previous studies revealed that the PI3-K/PKB(Akt) pathway triggered by IGF-I plays a critical role in maintaining the differentiated SMC phenotype (Hayashi et al., 1998). To investigate the downstream signaling pathways involving in SMC de-differentiation triggered by PDGF-BB, bFGF, or EGF, we analyzed several kinases including ERK, JNK, p38MAPK, PI3-K, and PKB(Akt). PDGF-BB, bFGF, and EGF all activated ERK (Fig. 2 A) and p38MAPK (Fig. 2 C). Their maximum activations were found at 10 min after stimulation. bFGF and EGF also activated JNK, whereas PDGF-BB did not (Fig. 2 B). IGF-I had no effect on ERK (Fig. 2 A), JNK (Fig. 2 B), and p38MAPK (Fig. 2 C). These data suggest that growth factors inducing SMC de-differentiation coordinately activate the ERK and p38MAPK pathways.

As demonstrated previously (Hayashi et al., 1998), IGF-I potently activated PI3-K (Fig. 2 D) and PKB(Akt) (Fig. 2 E); the maximum activation of PI3-K was achieved at 10 min after IGF-I stimulation and this activation reduced thereafter, while the activation of PKB(Akt) by IGF-I (2 ng/ml) lasted for more than 180 min. The activation of PI3-K and PKB(Akt) by IGF-I was suppressed by specific PI3-K inhibitors, wortmannin or LY249002 (data not shown), indicating that the PKB(Akt) activation exclusively depends on the PI3-K activity. No significant activation of PI3-K and PKB(Akt) was observed in SMCs stimulated by either bFGF or EGF (Fig. 2, D and E). Among the three growth factors inducing SMC de-differentiation, PDGF-BB was the only one that could activate PI3-K and PKB(Akt) (Fig. 2, D and E). The PKB(Akt) activation by PDGF-BB was more potent than that by IGF-I at 15 min after growth factor stimulation, whereas this activation was transient, but retained at a substantial level for 180 min. By contrast, the PKB(Akt) activation by IGF-I was sustained at a high level for more than 180 min. These results suggest the possibility that in addition to PI3-K,

PDGF-BB activates PKB(Akt) mediated through another unknown cascade.

Dual Function of PDGF-BB on Gizzard SMC Phenotype

It is curious that PDGF-BB, which is a potent factor inducing SMC de-differentiation (Fig. 1), triggered the dual signaling pathways mediated through both PI3-K/PKB(Akt) and two MAPKs, ERK and p38MAPK (Fig. 2). To simplify the PDGF-BB–triggered signaling pathways, we examined the effects of specific MAPK inhibitors, PD98059 for ERK kinase (MEK1) and SB203580 for p38MAPK, on the PDGF-BB–stimulated SMC phenotype. Either PD98059 or SB203580 specifically inhibited the PDGF-BB–induced activation of ERK or p38MAPK, respectively, to near basal levels (Fig. 3 A), but had no effect on PI3-K and PKB(Akt) (data not shown). Treatment with either PD98059 or SB203580 only slightly suppressed the PDGF-BB–induced isoform conversion of caldesmon mRNA and downregulation of caldesmon and calponin mRNAs (Fig. 3 B). However, simultaneous treatment with both drugs strongly suppressed the PDGF-BB–induced SMC de-differentiation as monitored by the expression of caldesmon and calponin mRNAs (Fig. 3 B). In addition to these molecular events, both drugs could also rescue the morphological alteration from a spindle-like shape to a fibroblast-like shape change and a loss of contractility induced by PDGF-BB (Fig. 4). As a control, treatment with individual drug resulted in less significant effect on cell morphology and function. Table I shows the effects of PD98059 and/or SB203580 on carbachol-stimulated contractility of SMCs under various culture conditions. Further, both drugs only slightly delayed the induction of SMC de-differentiation by bFGF or EGF, but did not prevent SMC de-differentiation (data not shown).

Table I. Effects of MAPK Inhibitors on the Contractile Response of Cultured SMCs

Culture conditions	Contracted cells
	%
IGFI	92.9 ± 2.0
PDGF-BB	0
PDGF-BB + PD	1.8 ± 0.7
PDGF-BB + SB	9.0 ± 1.0
PDGF-BB + PD/SB	92.4 ± 3.1

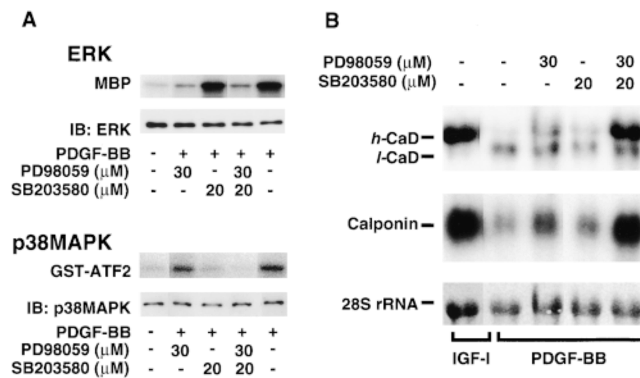


Figure 3. The effect of blocking the ERK and p38MAPK signalings on the expression of caldesmon and calponin mRNAs in gizzard SMCs under PDGF-BB–stimulated conditions. (A) Inhibition of PDGF-BB–induced activation of ERK and p38MAPK by PD98059 and/or SB203589. The SMCs plated on laminin were cultured in DME supplemented with 0.2% BSA for 24 h, and then pretreated with either PD98059 (30 μM) or SB203580 (20 μM), or both drugs for 1 h, followed by PDGF-BB (20 ng/ml) stimulation in the presence of vehicle alone or indicated drugs for 10 min. The representative data are shown from three independent experiments. (B) The expression of caldesmon and calponin mRNAs was analyzed by Northern blotting as shown in Fig. 1 A. Total RNAs were isolated from 3-d–cultured SMCs plated on laminin under indicated conditions, and then analyzed by Northern blotting using caldesmon and calponin cDNA fragments as probes. Culture conditions were as follows: IGF-I (2 ng/ml), PDGF-BB (20 ng/ml), PDGF-BB (20 ng/ml) in the presence of either PD98059 (30 μM) or SB203580 (20 μM) or both drugs. The results are shown from four independent experiments.

Promoter analyses of the caldesmon gene further support these findings (Fig. 5). We used the caldesmon promoter/CAT construct, GP3CAT, which produces the high promoter activity in differentiated SMCs (Yano et al., 1995). The promoter activity in SMCs stimulated by PDGF-BB reduced to 30% of that by IGF-I (Fig. 5 C). Even under PDGF-BB–stimulated conditions, inhibition of both the ERK and p38MAPK pathways by their specific inhibitors or the forced expression of active PI3-K (Fig. 5 A) or active PKB(Akt) (Fig. 5 B) could protect such reduction (Fig. 5 C). These results suggest that PDGF-BB displays the dual function in maintaining the differentiated SMC phenotype mediated through the PI3-K/PKB(Akt) pathway and inducing SMC de-differentiation by the ERK and p38MAK pathways. Thus, changes in the balance between the strengths of the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways would determine the SMC phenotype.

Direct Involvement of ERK and p38MAPK Inducing Gizzard SMC De-differentiation

The MAPK signaling cascades are involved in a variety of cell functions (Force and Bonventre, 1998). Dual phosphorylation on Thr and Tyr within the Thr-Xaa-Tyr motif catalyzed by MAPK kinases is essential for MAPK activation (Davis, 1994). MEK1 and MKK6 are specific upstream kinases for ERK and p38MAPK, respectively (Cohen, 1997). To investigate the direct involvement of ERK and p38MAPK in SMC de-differentiation, we examined the effects of active or dominant-negative MEK1 and/or MKK6 on the caldesmon promoter activity in SMCs under IGF-I–stimulated conditions. We determined the respective MAPK kinase activity by in vitro kinase assay (Fig. 6 A). In this experiment, expression plasmids carrying active or dominant-negative MAPK kinases were cotransfected with expression plasmid carrying Flag-tagged ERK or Flag-tagged p38MAPK into cultured SMCs, and Flag-tagged proteins were immunoprecipitated from the cell lysates with anti-Flag monoclonal antibody. The ERK or p38MAPK activities were potently enhanced in SMCs cotransfected with active MEK1 or MKK6 under nonstimulated or PDGF-BB–stimulated conditions (Fig. 6 A, a

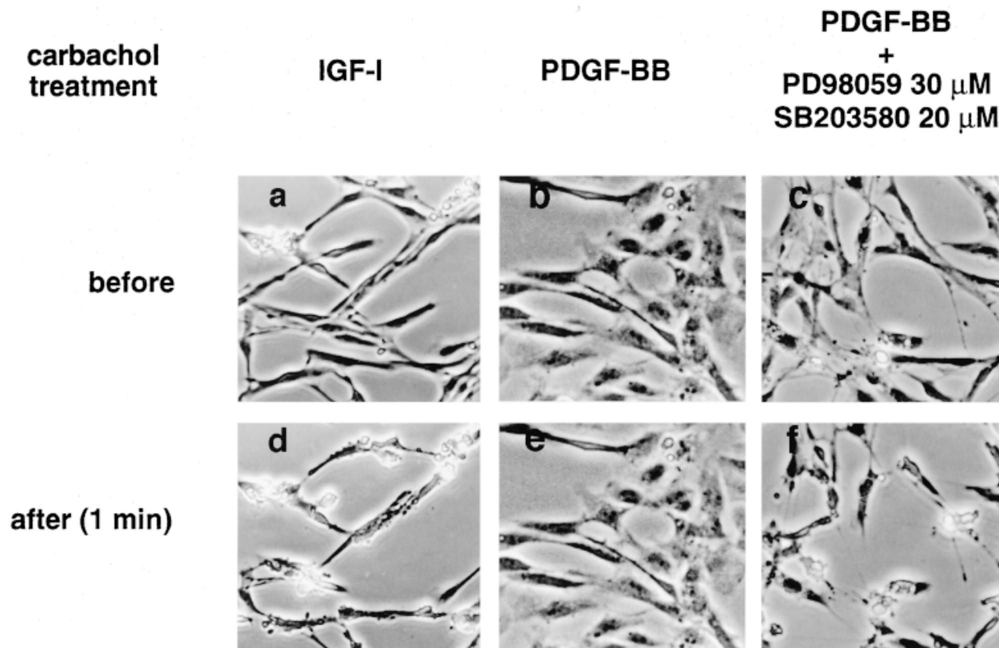


Figure 4. Rescue of PDGF-BB–induced SMC de-differentiation by blocking the ERK and p38MAPK pathways. Gizzard SMCs plated on laminin were stimulated with 2 ng/ml IGF-I (a and d), 20 ng/ml PDGF-BB (b and e), or 20 ng/ml PDGF-BB in the presence of both PD98059 (30 μM) and SB203580 (20 μM) (c and f) for 3 d. Ligand-induced contractility was monitored by the addition of carbachol (1 mM) for 1 min. Photographs show cultured SMCs before (a, b, and c) and after (d, e, and f) carbachol treatment. The data are presented from five independent experiments.

and b). Even when SMCs were stimulated by PDGF-BB, their enhancements were strongly abolished by the forced expression of dominant-negative MEK1 or MKK6 (Fig. 6 A, a and b). The expressions of active and dominant-negative MEK1 or MKK6 proteins were confirmed by immunoblotting (data not shown). The caldesmon promoter activity in differentiated SMCs under IGF-I–stimulated conditions was analyzed by the forced expression of active

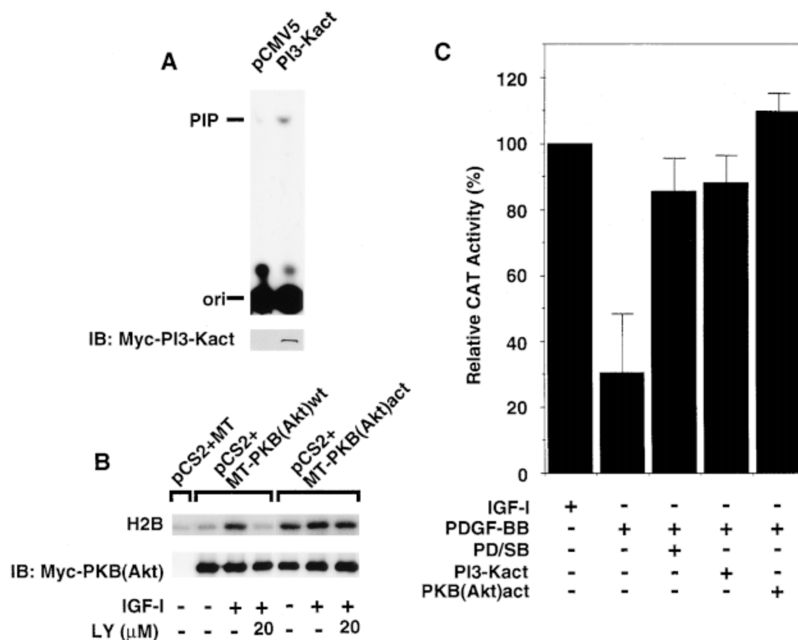


Figure 5. Regulation of the caldesmon promoter activity mediated through the PI3-K/PKB(Akt) pathway and the ERK and p38MPAK pathways. (A) The PI3-K activity in nonstimulated SMCs transfected with control plasmid (pCMV5) and expression plasmid of c-Myc–tagged active PI3-K p110α subunit, pCMV5p110αact (PI3-Kact). Gizzard SMCs transfected with indicated plasmids were cultured under nonstimulated conditions, and the PI3-K activity was determined by immunoprecipitation with anti–c-Myc monoclonal antibody, followed by in vitro kinase assays as described in Materials and Methods. (B) The PKB(Akt) activity in SMCs transfected with control plasmid, pCS2+MT, expression plasmid of c-Myc–tagged wild-type PKB(Akt), pCS2+MT-PKB(Akt)wt, or expression plasmid of c-Myc–tagged active PKB(Akt), pCS2+MT-PKB(Akt)act. The SMCs were cultured under nonstimulated conditions for 2 d after transfection, and then half of the cultures were stimulated by 2 ng/ml IGF-I with or without treatment of 20 μM LY294002. PKB(Akt) assays were carried out as described above. In A and B, the top and bottom panels are the results of kinase assay and immunoblotting (IB) to determine the amounts of kinase proteins in the cell lysates, respectively. The kinase activities are shown from three independent experiments. (C) Effects of PDGF-BB– or IGF-I–triggered signalings on the caldesmon promoter activity. The promoter construct of caldesmon, GP3CAT, was transfected into 3-d–cultured SMCs under the following conditions: DME supplemented with 0.2% BSA alone or BSA plus 20 ng/ml PDGF-BB. The GP3CAT (2 μg) was cotransfected with RSV-luciferase (1 μg) and control plasmid (1 μg), expression plasmid of c-Myc–tagged active PI3-K p110α (PI3-Kact, 1 μg), or expression plasmid of c-Myc–tagged active PKB(Akt) (PKB(Akt)act, 1 μg), respectively. After transfection, SMCs were stimulated with 2 ng/ml IGF-I, 2 ng/ml IGF-I plus 20 μM LY294002, 20 ng/ml PDGF-BB, or 20 ng/ml PDGF-BB plus PD98059 (30 μM) and SB203580 (20 μM). The promoter activity was assayed at 48 h after transfection as described in Materials and Methods. The relative promoter (CAT) activities were normalized to the activity in culture SMCs under IGF-I–stimulated conditions, which was defined as 100%. Each value represents the average ± SD of three independent experiments. A promoterless control CAT plasmid (pUC0CAT) did not show detectable CAT activity under the same conditions (data not shown).

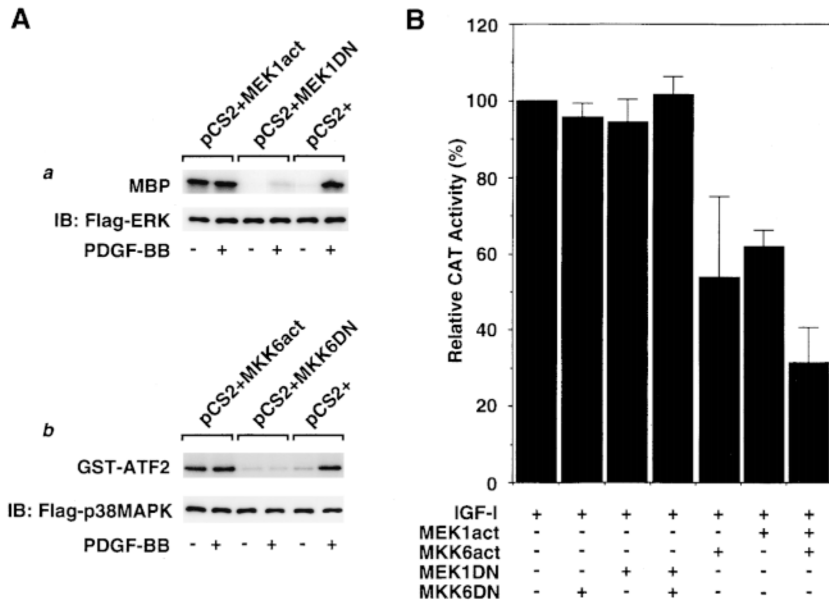


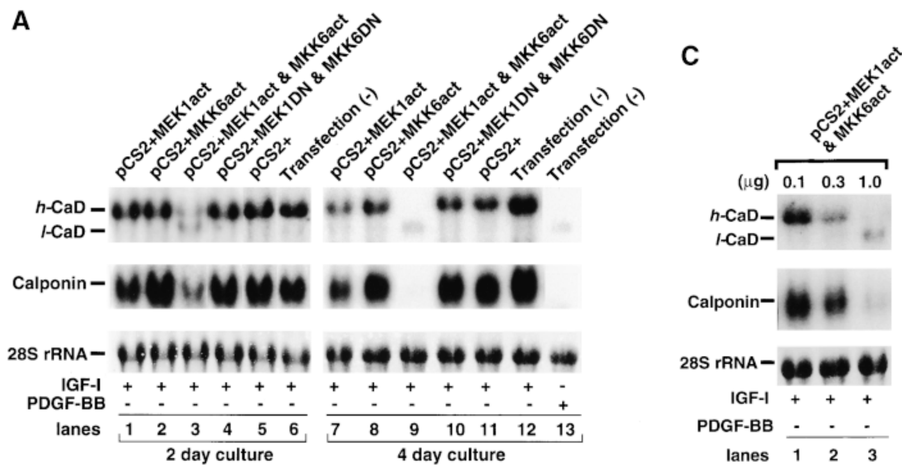
Figure 6. Inhibition of the caldesmon promoter activity in gizzard SMCs by the forced expression of active MEK1 and MKK6. (A) Effects of active and dominant-negative MEK1 or MKK6 on the kinase activities of ERK (a) and p38MAPK (b) in cultured SMCs under non-stimulated and PDGF-BB–stimulated conditions. The SMCs were cotransfected with 2 μg each of expression plasmid of active or dominant-negative MEK1 and/or MKK6 or control plasmid together with 2 μg Flag-tagged ERK2 or Flag-tagged p38MAPK. The SMCs were cultured under nonstimulated conditions for 2 d, and half of the cultures were stimulated with 20 ng/ml PDGF-BB for 10 min. The cells were lysed and subjected to kinase assays after immunoprecipitation with anti-Flag monoclonal antibody. The top and bottom panels are the results of kinase assay and immunoblotting (IB) to determine the amounts of Flag-tagged kinase proteins in the cell lysates. The representative data are from two independent experiments. (B) Effects of the forced expression of active or dominant-negative MEK1 and/or MKK6 on the caldesmon promoter activity. The SMCs were cotransfected with GP3CAT (2 μg), RSV-luciferase (1 μg), and either or both expression vectors carrying active or dominant-negative MEK1 and/or MKK6 (1 μg). The total amounts of transfected plasmids were adjusted to 5 μg by the addition of control vector, pCS2+. The promoter activities were determined as described in the legend of Fig. 5. The relative promoter activities were normalized to the activity in cultured SMCs under IGF-I–stimulated conditions without expression vectors carrying MEK1 or MKK6, which was defined as 100%. Each value represents the average ± SD of three independent experiments.

or dominant-negative MAPK kinases (Fig. 6 B). The promoter activity was not affected by either or both dominant-negative MEK1 and/or MKK6. Transfection with either active MEK1 or MKK6 significantly reduced the caldesmon promoter activity, while cotransfection with both active kinases further suppressed the promoter activity. Since the SV-40 promoter was not affected by the forced expression of MEK1 and/or MKK6 (data not shown), suppression of the caldesmon promoter activity by active MEK1 and MKK6 was specific. These results indicate that the ERK and p38MAPK pathways are directly involved in the induction of SMC de-differentiation.

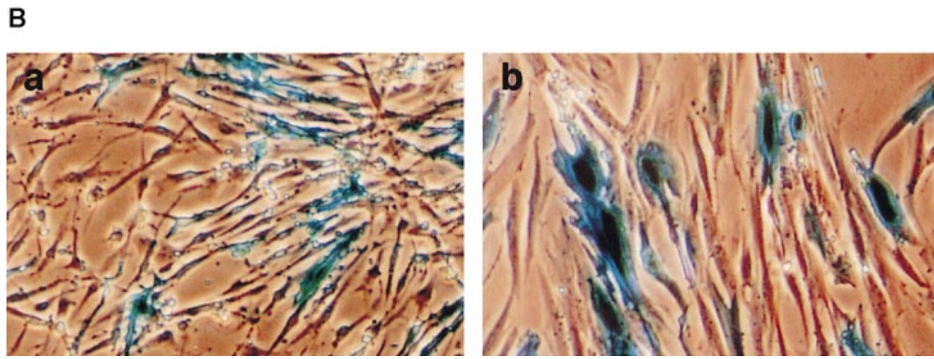
Detection of De-differentiation-inducing Factor(s) from Gizzard SMCs Cotransfected with Active MEK1 and MKK6

We further examined the effects of active and dominant-negative MEK1 or MKK6 on the endogenous expression of caldesmon and calponin mRNAs and on cell morphology. Transfection with either active kinase alone or cotransfection with dominant-negative kinases had less significant effects on caldesmon and calponin mRNAs in 2- or 4-d–cultured SMCs (Fig. 7 A, lanes 1, 2, 4–8, and 10–12). Even under IGF-I–stimulated conditions, cotransfection of active MEK1 and MKK6 potently induced isoform conversion of caldesmon mRNA and downregulation of caldesmon and calponin mRNAs. These changes progressed during culture; the endogenous expressions of caldesmon and calponin mRNAs in 4-d–cultured SMCs after cotransfection were comparable to those seen in 4-d–cultured SMCs stimulated by PDGF-BB (Fig. 7 A, lanes 3, 9, and 13). These results indicate that after cotransfection with active MEK1 and MKK6, almost all of cultured SMCs alter their phenotype to de-differentiation. To compare transfection efficiency and cell morphology, cultured SMCs stimulated by IGF-I were transfected with control plasmid (Fig. 7 B, a) or both expression plasmids carrying active MEK1 and MKK6 (Fig. 7 B, b) together with β-galactosidase expression plasmid. As revealed by β-galactosidase staining, transfection efficiencies were ~25%. The SMCs transfected with control vector remained to show a spindle-like shape (Fig. 7 B, a). In the case of SMCs cotransfected with active MEK1 and MKK6, all of the β-galactosidase-stained and -unstained cells converted from a spindle-like shape to a fibroblast-like shape (Fig. 7 B, b). The transfection efficiency of both active MEK1 and MKK6 was correlated with SMC-specific marker gene expression and cell morphology (Fig. 7, C and D). These data suggest that SMCs cotransfected with active MEK1 and MKK6 secrete some factor(s) which induces de-differentiation of surrounding normal SMCs.

To further characterize such a factor(s), conditioned medium (CM1) obtained from SMCs cotransfected with expression plasmids carrying active MEK1 and MKK6 was prepared. Fig. 8 A shows the expression of caldesmon and calponin mRNAs in 3-d–cultured SMCs stimulated with the conditioned medium. Even when SMCs were stimulated by IGF-I, the conditioned medium potently induced isoform conversion of caldesmon mRNA and downregulation of caldesmon and calponin mRNAs (Fig. 8 A, lane 1), whereas control medium (CM2) obtained from SMCs



B



D

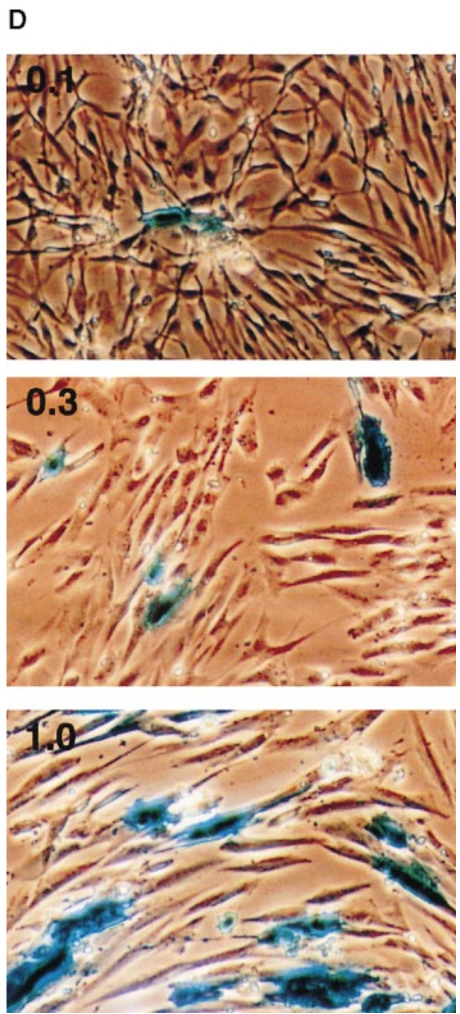


Figure 7. Detection of de-differentiation–inducing factor(s) from SMCs cotransfected with active MEK1 and MKK6. (A) Effects of the forced expression of active or dominant-negative MEK1 and/or MKK6 on the endogenous expression of caldesmon and calponin mRNAs. Gizzard SMCs were transfected with 1 μg of indicated expression plasmids, and then cultured under IGF-I–stimulated conditions for 2 d (lanes 1–5) and for 4 d (lanes 7–11). The total amounts of transfected plasmids were adjusted to 2 μg by the addition of pCS2+. The SMCs were also cultured under IGF-I–stimulated conditions (2 ng/ml) without transfection for 2 d (lane 6) and 4 d (lane 12), or under PDGF-BB–stimulated conditions (20 ng/ml) without transfection for 4 d (lane 13). Caldesmon and calponin mRNAs in cultured SMCs were analyzed by Northern blotting as shown in Fig. 1 A. (B) Transfection efficiency and comparison of cell morphology between SMCs transfected with control plasmid (a) and expression plasmids carrying active MEK1 and MKK6 (b). The SMCs were transfected with pCS2+ (2 μg) and pSVβ-galactosidase (1 μg) (a) or with pCS2+MEK1act (1 μg), pCS2+MKK6act (1 μg), and pSVβ-galactosidase (1 μg) (b), and then cultured under IGF-I–stimulated conditions. At 4 d after transfection, β-galactosidase activity was visualized using X-gal as a substrate. (C and D) Dose-dependent effect of transfection with active MEK1 and MKK6 on the SMC phenotype. The SMCs were transfected with the indicated amounts of pCS2+MEK1act and pCS2+MKK6act together with pSVβ-galactosidase (1 μg), and then cultured for 4 d under IGF-I–stimulated conditions. Transfection efficiency was increased in increasing amounts of transfected plasmids as follows: lane 1 in C and top panel in D, <1%; lane 2 in C and middle panel in D, 5%; and lane 3 in C and bottom panel in D, 25%. The expression of caldesmon and calponin mRNAs (C) and cell morphology (D) of transfected SMCs are shown. The representative data are shown from four (A and B) or three (C and D) independent experiments.

transfected with control plasmid alone did not (Fig. 8 A, lane 2). Further, the conditioned medium enhanced the ERK, JNK, and p38MAPK activities (Fig. 8 B). These results exclude the possibility of retransfection with residual expression vectors, because MEK1 and MKK6 proteins derived from expression plasmids were not detected in cultured SMCs by immunoblotting (data not shown). Thus, this study revealed that the forced activation of both ERK and p38MAPK in SMCs induces the secretion of some factor(s) which initiates de-differentiation of surrounding normal SMCs in a paracrine manner. Heat or trypsin treatment of the conditioned medium completely abolished the activity inducing SMC de-differentiation (Fig. 8, lanes 3 and 4), suggesting that the factor(s) is a protein in nature. The conditioned medium was further fractionated using a heparin–Sepharose column. The flow through (non–heparin-binding) fraction retained the potent de-differentiation activity, while the eluted fraction by 0.5, 1.0, and 1.5 M NaCl did not (Fig. 8 A, lanes 5–8). PDGFs and bFGF show heparin-binding abilities; the former was eluted with 0.5 M NaCl and the latter with 1.5 M NaCl (data not shown). By contrast, EGF is known as a non–heparin-binding growth factor, suggesting a candidate for the active protein factor. A specific inhibitor for EGF receptor kinase, AG1478, only slightly inhibited the conditioned medium-induced de-differentiation, but this effect was less significant (Fig. 8 A, lane 9). Therefore, the active protein factor(s), which induces SMC de-differentiation, in the conditioned medium is considered to be different from PDGFs, bFGF, and EGF.

Signaling Pathways in Regulating the Vascular SMC Phenotype

To examine whether vascular SMCs could be regulated by the same signaling pathways as revealed in gizzard SMCs, we first applied our culture system of gizzard SMCs to vascular SMCs. We isolated rat vascular SMCs by the enzyme-disperse method, and cultured them on laminin-coated plates under IGF-I–stimulated conditions. Because of difficulty to obtain a lot of cell numbers from rat aortae, we observed cell morphology and ligand-induced contractility to determine the vascular SMC phenotype (Fig. 9). Vascular SMCs could also maintain a spindle shape for more than 2 weeks under IGF-I–stimulated conditions, and showed ligand-induced contractility (Fig. 9, a and f). A blockade of the PI3-K pathway by LY294002 resulted in a morphological change from a spindle shape to a fibroblast-like shape and a loss of contractility (Fig. 9, b and g). Inconsistent with the case of gizzard SMCs, PDGF-BB rapidly induced de-differentiation of vascular SMCs as monitored by cell morphology and ligand-induced contractility (Fig. 9, c and h). Even under PDGF-BB–stimulated conditions, simultaneous treatment with PD98059 and SB203580 could retain a spindle shape and contractility (Fig. 9, d and i). As a control, treatment with each drug individually was less significant effect on the PDGF-BB–induced de-differentiation (data not shown). Furthermore, the conditioned medium obtained from gizzard SMCs transfected with both active MEK1 and MKK6 remarkably induced de-differentiation of vascular SMCs (Fig. 9, e and j). Therefore, these data also suggest that the PI3-K–mediated signaling pathway plays a vital role in maintaining a differentiated phenotype of vascular SMCs and the ERK and p38 MAPK pathways are coordinately involved in de-differentiation of vascular SMCs.

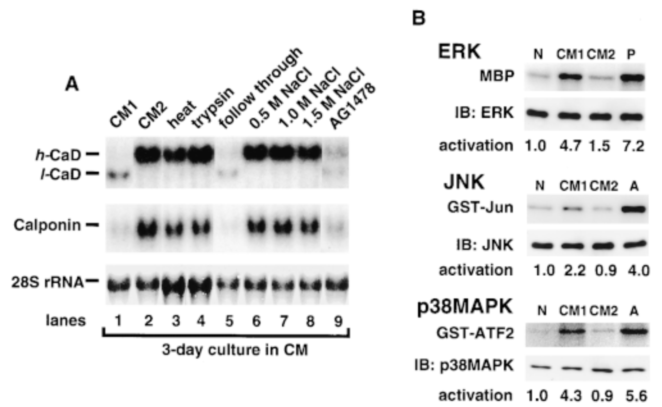


Figure 8. Characterization of SMC de-differentiation–inducing factor(s). (A) Gizzard, SMCs were transfected with pCS2+MEK1act (1 μg) and pCS2+MKK6act (1 μg) or with pCS2+ (2 μg), and were cultured for 3 d. Each culture medium was collected; CM1 from SMCs transfected with pCS2+MEK1act and pCS2+MKK6act and CM2 from SMCs transfected with pCS2+. Then, SMCs were cultured under following conditions for 3 d: CM1 (lane 1), CM2 (lane 2), heat-treated CM1 (lane 3), trypsin-treated CM1 (lane 4), follow through (lane 5), and eluted (lanes 6–8) fractions of heparin-affinity column chromatography of CM1, or CM1 plus 1 μM AG1478 (lane 9). Caldesmon and calponin mRNAs in cultured SMCs were analyzed by Northern blotting as shown in Fig. 1 A. (B) Activation of ERK, JNK, and p38MAPK by the conditioned medium. Gizzard SMCs were stimulated under the following conditions: nonstimulation (N), CM1 for 10 min, CM2 for 10 min, PDGF-BB (20 ng /ml) for 10 min (P), or anisomycin (10 μg/ml) for 30 min (A). Then, the cells were lysed and subjected for kinase assays, ERK (top panel), JNK (middle panel), and p38MAPK (bottom panel) as described in Materials and Methods. (A). The data are representative of three (A) or two (B) independent experiments.

Discussion

Under pathological conditions, phenotype of SMCs can change from a differentiated state to a de-differentiated state in vivo and in vitro. During de-differentiation, SMCs show dramatic and irreversible alterations in their cell shape, function, and expression of SMC-specific molecular markers. Long spindle-shaped cells change to fibroblast-like cells, accompanied by losses in a ligand-induced contractility and SMC-specific molecular marker expression. Since there has not been a primary culture system available for SMCs or SMC-derived cell lines in which they can maintain a fully differentiated phenotype, the intracellular signaling pathways regulating the SMC phenotype have not been well characterized. Recently, we established a novel culture system in which gizzard SMCs can maintain a differentiated phenotype for a long culture time (Hayashi et al., 1998). In this culture system, IGF-I is the most potent for maintaining the differentiated SMC phenotype,

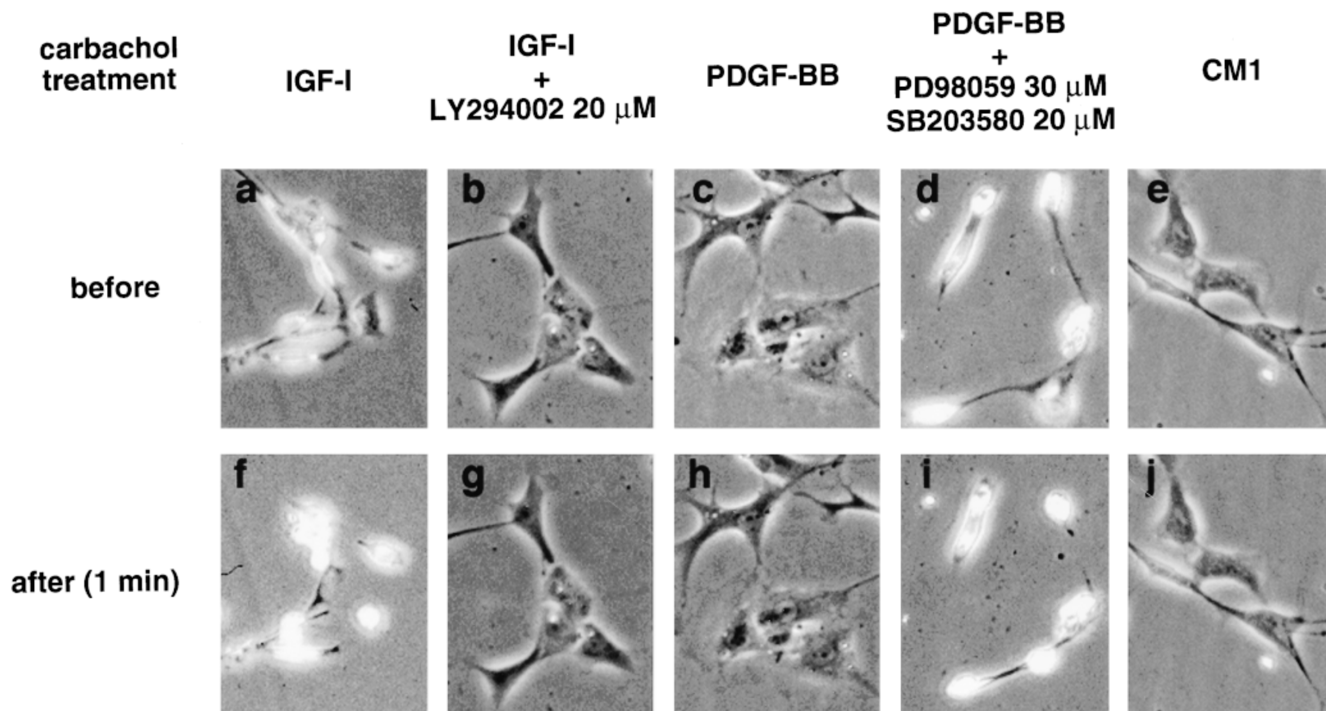


Figure 9. Analyses of signaling pathways in regulating the vascular SMC phenotype. Rat vascular SMCs were cultured on laminin in the presence of IGF-I (20 ng/ml) for 2 d, and then stimulated with following conditions for 3 d: 20 ng/ml IGF-I (a and f), 20 ng/ml IGF-I plus 20 μM LY294002 (b and g), 20 ng/ml PDGF-BB (c and h), 20 ng/ml PDGF-BB plus PD98059 (30 μM) and SB203580 (20 μM) (d and i), and the conditioned medium (CM1) from cultured gizzard SMCs transfected with pCS2+MEK1act and pCS2+MKK6act (e and j). Ligand-induced contractility was monitored as described in the legend of Fig. 4. Photographs show SMCs before (a–e) and after (f–j) carbachol treatment. The representative data are shown from five independent experiments.

and the IGF-I–triggered signaling pathway, PI3-K/PKB-(Akt), plays a critical role in this maintenance. Here, we investigated the signaling pathways inducing SMC de-differentiation and compared them with the PI3-K/PKB-(Akt) pathway.

It has been reported that PDGF, EGF, bFGF, or angiotensin II enhance cell proliferation or hypertrophy through the activation of the ERK signaling cascade in passaged vascular SMCs (Force and Bonventre, 1998). MAPKs such as JNK and p38MAPK are also activated in response to various cellular stresses (Derijard et al., 1994; Kyrian and Avruch, 1996). Angiotensin II and phenylephrine, which induce acute hypertension, enhance the ERK and JNK activities in aortic, carotid and femoral arteries (Xu et al., 1996), and endothelin activates both of these kinases in proliferative airway SMCs (Shapiro et al., 1996). p38MAPK, ERK, and/or JNK are also activated by balloon injury of carotid arteries (Hu et al., 1997; Pyles et al., 1997; Larrivee et al., 1998). These findings suggest a close association of MAPK cascades with smooth muscle disorders. However, the direct involvement of these signaling cascades in regulating the SMC phenotype has been unknown. As a first step, we used a novel culture system of gizzard SMCs and demonstrated that activations of the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways are directly involved in maintaining SMC differentiation and inducing SMC de-differentiation, respectively. This conclusion is based on the following findings. First, the signaling pathways in regulating the phenotypic determination of gizzard SMCs were distinctly different; the PI3-K/PKB(Akt) pathway played a critical role in maintaining the differentiated SMC phenotype (Figs. 2 and 5) and the ERK and p38MAPK pathways triggered by PDGF-BB, bFGF, and EGF were closely associated with SMC de-differentiation (Figs. 1 and 2). Second, among the three growth factors inducing SMC de-differentiation, PDGF-BB only triggered both the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways. When both the MAPK pathways were blocked by their specific inhibitors, PD98059 and SB203580, or when SMCs were transfected with active PI3-K or PKB(Akt), PDGF-BB in turn initiated to maintain the differentiated SMC phenotype (Figs. 3–5 and Table I). Third, even when SMCs were cultured under IGF-I–stimulated conditions, the forced activation of both the MAPK pathways by the coexpression of active MEK1 and MKK6 potently induced SMC de-differentiation (Fig. 6). Fourth, SMCs cotransfected with active MEK1 and MKK6 secreted a nondialyzable and heat-labile protein factor(s), which induced de-differentiation of surrounding normal SMCs (Figs. 7 and 8). Finally, the same signaling pathways as described above were observed to be involved in regulating the vascular SMC phenotype (Fig. 9).

Since IGF-I enhances the proliferation and migration of cultured vascular SMCs (Clemmons 1985; Bornfeldt et al., 1990; Cercek et al., 1990; Delafontaine et al., 1991; Born-

feldt et al., 1994), it has been considered to be an important growth factor in the progression of atherosclerosis. These findings are, however, obtained using passaged SMCs. In this paper, we demonstrated using a novel SMC culture system that IGF-I solely triggers the PI3-K/PKB(Akt) pathway, but not the MAPK pathways (Fig. 2). Further, IGF-I did not affect the proliferation of SMCs (data not shown). These properties of IGF-I signaling made it possible to maintain the differentiated SMC phenotype for more than 2 wk in primary culture. Since rapamycin had no effect on the differentiated SMC phenotype under IGF-I–stimulated conditions (Hayashi et al., 1998), p70 ribosomal S6 kinase ($p70^{S6K}$) is unlikely to be a downstream target of PI3-K/PKB(Akt). Further study is required to identify the downstream targets of PI3-K/PKB(Akt) in SMCs. It has been reported that IGF-I and its downstream signaling, PI3-K/PKB(Akt), play a role in protection against programmed cell death (Alessi and Cohen 1998). We observed neither a significant decrease in cell numbers nor DNA fragmentation in our SMC cultures even under nonstimulated conditions or in the presence of IGF-I neutralizing antibodies (data not shown). Therefore, cultured SMCs might secrete anti-apoptotic factor(s) in the absence of IGF-I, and the maintenance of the differentiated SMC phenotype by IGF-I would be distinct from an anti-apoptotic action. In passaged SMCs, IGF-I enhanced the JNK and p38MAPK activities (data not shown). These results suggest that the downstream signalings of IGF-I might be modulated during cell passage. Actually, differentiated SMCs rapidly change their phenotype under serum-stimulated conditions (Kashiwada et al., 1997; Hayashi et al., 1998). Since passaged SMCs do not represent a stable differentiated state, studies reported previously might not be able to reveal the IGF-I's function and signaling in differentiated SMCs. In this study, we used SMCs that showed well-characterized and stable phenotypes. We analyzed the relationship between the modulation of SMC phenotype and cell proliferation. Although serum-induced SMC de-differentiation was concomitant with cell proliferation, other growth factors that trigger SMC de-differentiation, such as PDGF-BB, bFGF, and EGF, did not significantly induce SMC proliferation (data not shown). This result also suggests that SMC de-differentiation is not essentially associated with cell proliferation.

It has been reported that de-differentiated SMCs produce and secrete PDGF which further promotes cell proliferation and migration in an autocrine/paracrine manner (Sjolund et al., 1988). PDGF is also known to promote the activation of ERK and p38MAPK in passaged SMCs (Bornfeldt et al., 1994; Pyne and Pyne, 1997). In our culture system, PDGF-BB triggered the dual signaling pathways mediated by PI3-K/PKB(Akt) and two MAPKs, ERK and p38MAPK. Under these culture conditions, PDGF-BB stimulation resulted in SMC de-differentiation. When the two MAPK pathways were blocked by their specific inhibitors, PD98059 and SB203580, PDGF-BB stimulation, in turn, initiated to maintain SMC differentiation (Figs. 3 and 4). bFGF and EGF are known to activate ERK and also to induce proliferation of SMCs (Berrou et al., 1996; Jones et al., 1997; Yu et al., 1997; Miyamoto et al., 1998). In our culture system, both growth factors activated ERK and p38MAPK and potently induced SMC de-differentiation (Figs. 1 and 2). However, PD98059 and SB203580 could not prevent such de-differentiation (data not shown). This is because the signaling pathway mediated by PI3-K/PKB(Akt) was not activated by bFGF or EGF (Fig. 2). In the present culture system, bFGF and EGF also activated JNK, but IGF-I and PDGF-BB did not (Fig. 2). It is, therefore, unlikely that JNK is involved in regulating the SMC phenotype. PDGF-BB reduced the caldesmon promoter activity and this reduction could be overcome by the forced expression of active PI3-K or PKB(Akt), or by treatment with both PD98059 and SB203580 (Figs. 3–5 and Table I). Further, the activation of ERK and p38MAPK by the forced expression of both active MEK1 and MKK6 could overcome the PI3-K/PKB(Akt) pathway triggered by IGF-I, resulting in the induction of SMC de-differentiation (Fig. 6). These data support our hypothesis that the SMC phenotype would be determined by the balance between the strengths of the signaling pathways mediated by PI3-K/PKB(Akt) and by ERK and p38MAPK.

Curiously, even though the transfection efficiencies of both expression vectors carrying active MEK1 and MKK6 were only 25%, almost all of SMCs came to de-differentiate as monitored by cell morphology and endogenous expression of SMC-specific molecular markers (Fig. 7). These findings indicate that the production and secretion of some de-differentiation-inducing factor(s) occur in SMCs in which both the ERK and p38MAPK pathways are constitutively activated. We have not yet identified such a factor(s), but the conditioned medium from these cells activated three MAPK pathways (ERK, p38MAPK, and JNK). The activating factor(s) was a heat-labile, non–heparin-binding protein factor (Fig. 8). From these biochemical properties (Fig. 8), we excluded the possibility that PDGF-BB, bFGF, and EGF would be the main factor inducing SMC de-differentiation in the conditioned medium. Further study is required to identify such a factor(s). Anyway, this study provides a evidence that only a small portion of de-differentiated SMCs secretes a protein factor(s) for the surrounding normal SMCs to be de-differentiated. These findings could be helpful to understand the progression of smooth muscle disorders such as atherosclerosis.

We then applied a culture system of gizzard SMCs to that of vascular SMCs and investigated the signaling pathways in regulating the vascular SMC phenotype (Fig. 9). Like gizzard SMCs, IGF-I potently maintained a differentiated phenotype of vascular SMCs and a specific PI3-K inhibitors, LY24002, prevented this IGF-I's action. Treatment with two MAPK pathway inhibitors, PD98059 and SB203580, could rescue the PDGF-BB–induced de-differentiation of vascular SMCs. Further, the conditioned medium obtained from gizzard SMCs also induce de-differentiation of vascular SMCs. These results strongly suggest that a culture system of gizzard SMCs is applicable for that of vascular SMCs and that the distinct signaling pathways mediated by PI3-K/PKB(Akt) and two MAPKs are also involved in the phenotypic determination of vascular SMCs.

Our results presented here are summarized in Fig. 10. The signaling pathway mediated by PI3-K/PKB(Akt) is

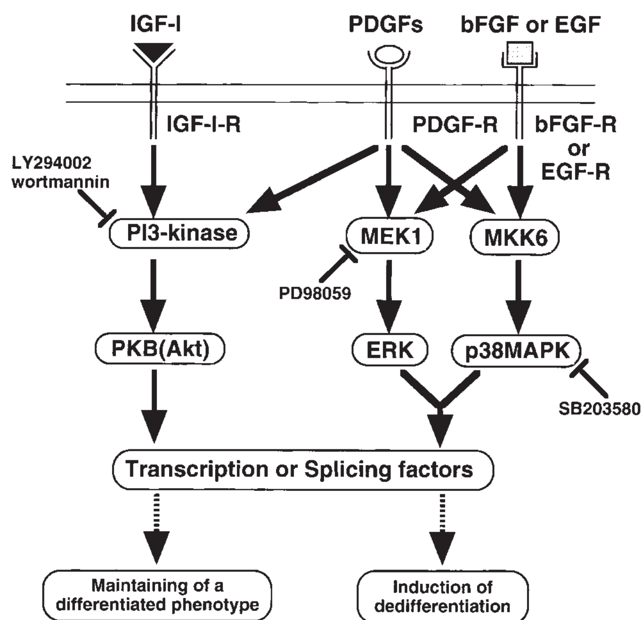


Figure 10. Distinct signaling pathways are directly involved in the phenotypic determination of visceral and vascular SMCs. Maintenance of a differentiated phenotype of SMCs depends on the PI3-K/PKB(Akt) pathway. In contrast, the coordinate activation of the ERK and p38MAPK pathways induces SMC de-differentiation. IGF-I, which is a potent factor for maintaining the differentiated SMC phenotype, activates the signaling pathway mediated through PI3-K/PKB(Akt), but not MAPKs. Blocking the PI3-K/PKB(Akt) pathway with specific inhibitors of PI3-K, LY249002, or wortmannin, induces SMC de-differentiation. Potent SMC de-differentiation-inducing factors, PDGF-BB, bFGF, and EGF, all activate the ERK and p38MAPK pathways. bFGF and EGF do not enhance the PI3-K/PKB(Akt) pathway, whereas, PDGF-BB does activate it. Thus, PDGF-BB triggers the dual signaling pathways, PI3-K/PKB(Akt) and two MAPKs. When the ERK and p38MAPK pathways were simultaneously blocked by their specific inhibitors, PD98059 and SB203580, PDGF-BB in turn initiates to induce maintaining SMC differentiation. Therefore, the SMC phenotype would be determined by the balance between the strengths of the PI3-K/PKB(Akt) pathway and the ERK and p38MAPK pathways.

required to maintain a differentiated phenotype of visceral and vascular SMCs, while the activation of ERK and p38MAPK leads to SMC de-differentiation of both types of SMCs. Thus, the signaling pathways in regulating the phenotypic determination are considered to be essentially the same in visceral and vascular SMCs, and the SMC phenotype would be regulated by the balance between the strengths of these signaling pathways. Although visceral and vascular SMCs originate from different precursors, our present findings provide a further insight in the common molecular mechanism of phenotypic determination of two types of SMCs. This is because visceral and vascular SMCs have common characteristics with respect to cell structure, function, and expression of molecular markers as follows. The main function of both types of SMCs is contraction, which is Ca^{2+}-dependent and controlled by a myosin-linked, actin-linked dual regulation (Sobue et al., 1988, 1991). Both types of SMCs are rich in myofibrils and are organized in a three-dimensional direction with two prominent electron dense structures such as the dense body in the cytoplasm and the dense membrane (dense plaque) in cell–cell contact. Further, contractile and cytoskeletal proteins are also specifically expressed in and serve as specific molecular markers for differentiated SMCs. The expression patterns of these molecular markers are identical in both visceral and vascular SMCs and their expression mechanisms, including transcription and splicing, are also regulated in common ways (Owens 1995; Sobue et al., 1998). Further studies are required to understand the detailed signaling pathways in regulating the vascular SMC phenotype and the functional linkage between such signaling pathways and SMC-specific gene regulation machineries, and to apply these findings to SMC disorders.

We thank Drs. H. Kurosu and T. Katada (Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo) for providing a constitutive active form of PI3-kinase p110α subunit cDNA, and also thank Dr. K. Sugiyama (Boehringer Ingelheim), Drs. M. Hibi and T. Hirano (Osaka University, Medical School), and Dr. E. Nishida (Graduate School of Science, Kyoto University) for kindly providing expression vectors carrying active and negative forms of MEK1 and MKK6, or Flag-tagged ERK2 and p38MAPK.

This work was supported by Grants-in-aid for COE Research (to K.S.) and in part by Grants-in-aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

Received for publication 20 November 1998 and in revised form 30 March 1999.

References

Alessi, D.R., and P. Cohen. 1998. Mechanism of activation and function of protein kinase B. *Curr. Opin. Genet. Dev.* 8:55–62.

Berrou, E., M. Fontenay-Roupie, R. Quarck, F.R. McKenzie, S. Levy-Toledano, G. Tobelem, and M. Bryckaert. 1996. Transforming growth factor β1 inhibits mitogen-activated protein kinase induced by basic fibroblast growth factor in smooth muscle cells. *Biochem. J.* 316:167–173.

Bornfeldt, K.E., H.L. Arnqvist, and G. Norstedt. 1990. Regulation of insulin-like growth factor-I gene expression by growth factors in cultured vascular smooth muscle cells. *J. Endocrinol.* 125:381–386.

Bornfeldt, K.E., E.W. Rainco, T. Nateano, L.M. Groves, E. Krebs, and R. Ross. 1994. Insulin-like growth factor-I and platelet-derived growth factor-BB induce directed migration of human arterial smooth muscle cells via signaling pathways that are distinct from those of proliferation. *J. Clin. Invest.* 93: 1266–1274.

Cercek, B., M.C. Fishbein, J.S. Forrester, R.H. Helfant, and J.A. Fagin. 1990. Induction of insulin-like growth factor I messenger RNA in rat aorta after balloon denudation. *Circ. Res.* 66:1755–1760.

Chamley-Campbell, J., G.R. Campbell, and R. Ross. 1979. The smooth muscle cell in culture. *Physiol. Rev.* 59:1–61.

Clemmons, D.R. 1985. Variables controlling the secretion of a somatomedin-like peptide by cultured porcine smooth muscle cells. *Circ. Res.* 56:418–426.

Cohen, P. 1997. The search for physiological substrates of MAP and SAP kinases in mammalian cells. *Trends Cell Biol.* 7:353–361.

Dario, R.A., A. Mirjana, C. Barry, P. Cron, M. Nick, P. Cohen, and B.A. Hemmings. 1996. Mechanism of activation of protein kinase B by insulin and IGF-I. *EMBO (Eur. Mol. Biol. Organ.) J.* 23:6541–6551.

Davis, R.J. 1994. MAPKs: new JNK expands the group. *Trends Biochem. Sci.* 19:470–473.

Delafontaine, P., H. Lou, and R.W. Alexander. 1991. Regulation of insulin-like growth factor I messenger RNA levels in vascular smooth muscle cells. *Hypertension.* 18:724–747.

Denharrdt, D.T. 1996. Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell. *Biochem. J.* 318:729–747.

Derijard, B., M. Hibi, I.H. Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R.J. Davis. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell.* 76:1025–1037.

Force, T., and J.V. Bonventre. 1998. Growth factors and mitogen-activated protein kinases. *Hypertension.* 31:152–161.

Gimona, M., Furst, D.O., and J.V. Small. 1987. Metavinculin and vinculin from mammalian smooth muscle: bulk isolation and characterization. *J. Muscle Res. Cell Motil.* 8:329–341.

Gimona, M., M.P. Sparrow, P. Strasser, M. Herzog, and J.V. Small. 1992. Calponin and SM 22 isoforms in avian and mammalian smooth muscle. Ab-

sence of phosphorylation *in vivo. Eur J Biochem.* 205:1067–1075.

Glukhova, M., V. Koteliansky, C. Fondacci, F. Marotte, and L. Rappaport. 1993. Laminin variants and integrin laminin receptors in developing and adult human smooth muscle. *Dev. Biol.* 157:437–447.

Hayashi, K., Y. Fujio, I. Kato, and K. Sobue. 1991. Structural and functional relationships between *h*- and *l*-caldesmons. *J. Biol. Chem.* 266:355–361.

Hayashi, K., H. Yano, T. Hashida, R. Takeuchi, O. Takeda, K. Asada, E. Takahashi, I. Kato, and K. Sobue. 1992. Genomic structure of the human caldesmon gene. *Proc. Natl. Acad. Sci. USA.* 89:12122–12126.

Hayashi, K., H. Saga, Y. Chimori, K. Kimura, Y. Yamanaka, and K. Sobue. 1998. Differentiated phenotype of smooth muscle cells depends on signaling pathways through insulin-like growth factors and phosphatidylinositol 3-kinase. *J. Biol. Chem.* 273:28860–28867.

Hu, Q., A. Klippel, A.J. Muslin, W.J. Fantl, and L.T. Williams. 1995. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science.* 268:100–102.

Hu, Y., L. Cheng, B.W. Hochleitner, and Q. Xu. 1997. Activation of mitogen-activated protein kinases (ERK/JNK) and AP-1 transcription factor in rat carotid arteries after balloon injury. *Arterioscler. Thromb. Vasc. Biol.* 17: 2808–2816.

Jones, P.L., K.N. Cowan, and M. Rabinnovitch. 1997. Tenascin-C, proliferation and subendothelial fibronectin in progressive pulmonary vascular disease. *Am. J. Pathol.* 150:1349–1360.

Kashiwada, K., W. Nishida, K. Hayashi, K. Ozawa, Y. Yamanaka, H. Saga, T. Yamashita, M. Tohyama, S. Shimada, K. Sato, and K. Sobue. 1997. Coordinate expression of α-tropomyosin and caldesmon isoforms in association with phenotypic modulation of smooth muscle cells. *J. Biol. Chem.* 272: 15396–15404.

Katoh, Y., E. Loukianov, E. Kopras, A. Zilberman, and M. Periasamy. 1994. Identification of functional promoter elements in the rabbit smooth muscle myosin heavy chain gene. *J. Biol. Chem.* 269:30538–30545.

Kim, S., H.S. Ip, M.M. Lu, C. Clendenin, and M.S. Parmacek. 1997. A serum response factor dependent transcriptional regulatory program identifies distinct smooth muscle cell sublineages. *Mol. Cell. Biol.* 17:2266–2278.

Kuro-o, M., R. Nagai, H. Tsuchimochi, H. Katoh, Y. Yazaki, A. Ohkubo, and F. Takaku. 1989. Developmentally regulated expression of vascular smooth muscle myosin heavy chain isoforms. *J. Biol. Chem.* 264:18272–18275.

Kyrian, J.M., and J. Avruch. 1996. Sounding the alarm: protein kinase cascades activated by stress inflammation. *J. Biol. Chem.* 271:24313–24316.

Larrivee, J.F., D.R. Bachvarov, F. Houle, J. Landry, J. Huot, and F. Marceau. 1998. Role of the mitogen-activated protein kinases in the expression of the kinin B1 receptors induced by tissue injury. *J. Immunol.* 160:1419–1426.

Madsen, C.S., J.C. Hershey, M.B. Hautmann, S.L. White, and G.K. Owens. 1997. Expression of the smooth muscle myosin heavy chain gene is regulated by a negative-acting GC-rich element located between two positive-acting serum response factor-binding elements. *J. Biol. Chem.* 272:6332–6340.

Mansour, S.J., W.T. Matten, A.S. Hermann, J.M. Candia, S. Rong, K. Fukasawa, G.F. Vande Woude, and N.G. Ahn. 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science.* 265: 966–970.

Miyamoto, T., I. Leconte, J.L. Swain, and J.C. Fox. 1998. Autocrine FGF signaling is required for vascular smooth muscle cell survival in vitro. *J. Cell Phyiol.* 117:58–67.

Nagai, R., M. Kuro-o, P. Babij, and M. Periasamy. 1989. Identification of two types of smooth muscle myosin heavy chain isoforms by cDNA cloning and immunoblot analysis. *J. Biol. Chem.* 264:9734–9737.

Obata, H., K. Hayashi, W. Nishida, T. Momiyama, and K. Sobue. 1997. Smooth muscle cell phenotype-dependent transcriptional regulation of the α1 integrin gene. *J. Biol.Chem.* 272:26648–26651.

Owens, G.K. 1995. Regulation of differentiation of vascular smooth muscle cells. *Physiol. Rev.* 75:487–517.

Pyles, J.M., K.L. March, M. Franklin, K. Mehdi, R.K. Wilensky, and L.P. Adam. 1997. Activation of MAP kinase in vivo follows balloon overstretch injury of porcine coronary and carotid arteries. *Circ. Res.* 81:904–910.

Pyne, N.J., and S. Pyne. 1997. Platelet-derived growth factor activates a mammalian Ste20 coupled mitogen-activated protein kinase in airway smooth muscle. *Cell. Signal.* 9:311–317.

Raingeaud, J., A.J. Whitmarsh, T. Barrett, B. Derijard, and R.J. Davis. 1996. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.* 16: 1247–1255.

Ray, L.B., and T.W. Rturgill. 1988. Insulin-stimulated microtuble-associated protein kinase is phosphorylated on tyrosine and threonin *in vivo. Proc. Natl. Acad. Sci. USA.* 85:3753–3757.

Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature.* 362:801–809.

Shanahan, C.M., P.L. Weissberg, and J.C. Necalfe. 1993. Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. *Cir. Res.* 73:193–204.

Shapiro, P.S., J.N. Evans, R.J. Davis, and J.A. Posada. 1996. The seven-transmembrane-spanning receptors for endothelin and thrombin cause proliferation of airway smooth muscle cells and activation of the extracellular regulated kinase and c-Jun NH_2-terminal kinase groups of mitogen activated protein kinases. *J. Biol. Chem.* 271:5750–5754.

Sjolund, M., U. Hedin, T. Sejersen, C.H. Heldin, and J. Tyberg. 1988. Arterial smooth muscle cells express platelet-derived growth factor (PDGF) A chain mRNA, secrete a PDGF-like mitogen, and bind exogenous PDGF in a phenotype- and growth state-dependent manner. *J. Cell. Biol.* 106:403–413.

Sobue, K., and J.R. Sellers. 1991. Caldesmon a novel regulatory protein in smooth muscle and nonmuscle actomyosin system. *J. Biol. Chem.* 266:12115–12118.

Sobue, K., K. Kanda, T. Tanaka, and N. Ueki. 1988. Caldesmon: a common actin-ligand regulatory protein in the smooth muscle and non-muscle contractile system. *J. Cell. Biochem.* 37:317–325.

Sobue, K., K. Hayashi, and W. Nishida. 1998. Molecular mechanism of phenotypic modulation of smooth muscle cells. *Horm. Res.* 50:15–24.

Solway, J., J. Seltzer, F.F. Samaha, S. Kim, L.E. Alger, Q. Niu, E.E. Morrisey, and M.S. Parmacek. 1995. Structure and expression of a smooth muscle cell-specific gene, SM22α. *J. Biol. Chem.* 270:13460–13469.

Ueki, N., K. Sobue, K. Kanda, T. Hada, and K. Higashino. 1987. Expression of high and low molecular weight caldesmons during phenotypic modulation of smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* 84:9049–9053.

Yano, H., K. Hayashi, T. Momiyama, H. Saga, M. Haruna, and K. Sobue. 1995. Transcriptional regulation of the chicken caldesmon gene. Activation of gizzard-type caldesmon promoter requires a CArG box-like motif. *J. Biol. Chem.* 270:23661–23666.

Yu, S.M., L.M. Hung, and C.C. Lin. 1997. CGMP-elevating agents suppress proliferation of vascular smooth muscle cells by inhibiting the activation of epidermal growth factor signaling pathway. *Circ.* 95:1269–1277.

Xu, Q., Y. Liu, M. Gorospe, R. Udelsman, and N.J. Holbrook. 1996. Acute hypertension activates mitogen-activated protein kinases in arterial wall. *J. Clin. Invest.* 97:508–514.