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) 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

Abbreviations used in this paper: bFGF, basic fibroblast growth factor; CM, conditioned medium; CAT, chloramphenicol acetyltransferase; E.R.K., 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.
are used as SM C-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), SM 22α (Gimona et al., 1992; Shanahan et al., 1993), β-tropomyosin (Kashiwada et al., 1997), and α1 integrin (Gluhova et al., 1993; O’bata 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/metavinculin (Gimona et al., 1987), and smooth muscle myosin heavy chain (Nagai et al., 1989; Kuro-o et al., 1989) are also controlled by SM C phenotype-dependent alternative splicings. Recently, the transcription machineries of caldesmon (Yano et al., 1995), smooth muscle myosin heavy chain (Kato et al., 1994; Madsen et al., 1997), SM 22α (Solway et al., 1995; Kim et al., 1997) and α1 integrin (O’bata et al., 1997) have been partially characterized. However, the molecular mechanisms behind the phenotypic modulation of SM C remain unclear. The slow progress in this area may be due to the plasticity of SM C. Under conventional culture conditions, SM C in primary culture display a rapid change in their phenotype (reviewed by Chamley-Campbell et al., 1979). And, SM C-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 SM C in which they maintain a differentiated phenotype for a long time. Extracellular matrices partially affect the SM C phenotype. Of these, laminin is the most potent for delaying the progression of SM C 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-1 (IGF-1) is the most potent for maintaining the differentiated SM C. In the IGF-1-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 (H yashi et al., 1998).

MAPKs have been implicated in the signaling cascades involved in the proliferation and hypertrophy of SM C (reviewed by Force and Bonventre, 1998). These include extracellular signal-regulated kinase (ERK) and the stress-activated MAPKs, c-jun NH2-terminal protein kinase (JNK) and p38 MAPK. ERK is activated in response to growth factors, cytokines, and cellular stresses (Ray and R turgill, 1988; D enhar dt, 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 SM C, activates ERK, JNK and p38 MAPK. ERK are also activated by cellular stresses including inflammatory cytokines, heat shock, osmolar stress, ultraviolet irradiation, and inhibition of protein synthesis (Derijard et al., 1994; K yrian and A vruch, 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 SM C, 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 p38 MAPK (Larrivee et al., 1998) are enhanced in rat carotid arteries after balloon injury. p38 MAPK is also elicited in airway SM C 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 SM C phenotype has not yet been demonstrated.

We investigated the signaling pathways involved in SM C 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 SM C in culture. Here, we demonstrated the first direct evidence for a mechanism by which the distinctly different signaling pathways regulate the SM C phenotype. Both the ERK and p38 MAPK pathways triggered by PDGF-BB, bFGF, and EGF were found to play an essential role in inducing SM C 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 p38 MAPK pathways were blocked by their specific inhibitors, PDGF-BB in turn initiated to maintain a differentiated phenotype of gizzard SM C. The same signaling pathways involving in the phenotypic determination were observed in vascular SM C. Thus, changes in the balance between the strengths of the PI3-K/PKB(Akt) pathway and the ERK and p38 MAPK pathways would determine phenotypes of visceral and vascular SM C. We further demonstrated a de-differentiation-inducing factor(s) secreted from SM C in which both the MAPK pathways were activated by cotransfection with active forms of MEK1 and MKK6.

Materials and Methods

Antibodies

A nti-P13-K p85 subunit antiserum was purchased from Upstate Biotechnology. Polyclonal antibodies against PKB(Akt), ERK, JNK, p38 MAPK, 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 P13-K p110 (pCMV5p110act) 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 p38 MAPK were kindly provided by Dr. K. Sugiyama (Boehringer Ingehelm), Drs. M. Hibi and T. Hirano (Osaka University, Medical School), and Dr. E. Nishida (Graduate School of Pharmaceutical Sciences, University of Tokyo). Mu tant cDNAs of MEK1 and MKK6 were constructed as described elsewhere (Manour et al., 1994; Rainea ud et al., 1996), and were inserted downstream of the cytomegalovirus promoter of pCS2+ or the SR alpha promoter of pCDLRα296. 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 cytomegalo-
virus promoter of pcS2+ (pcS2+ c-myc-tagged (MT)) (pcS2+ M-TPS B/A-1 RNA for expression of c-Myc-tagged wild-type PKB (A). The expression plasmid of c-Myc-tagged constitutively active form of PKB (A) (pcS2+ M-TP B/A) 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 lamin-
in-coated six-well plates with the indicated growth factors in kinase

inhibited or stimulated conditions. Vascular SMCs were isolated from 5-wk-old rat aorta by enzyme-dispersion methods as follows. Aorta 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. Disspersed 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 (GF-1 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%

a new antibody for p38MAPK. The reaction mixtures (50 μl), containing the immunoprecipitates in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl2, 0.5 mM EGTA, 10 μM ATP, 50 μg/l [32P]ATP, and 20 μg of PI3-K were incubated at 30°C for 10 min. The reactions were terminated and the lipids were extracted by the addition of CHCl3/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 CHCl3/MeOH/H4M NH4OH (9:7:2). The production of phosphotyrosinol-3-phosphate was detected by autoradiography.

Other Protein Kinase Assays
Cell lysates were prepared 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 the 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 fragment, which contains parts of exons 2 and 3a is a common probe for c-Myc–tagged (MT) (pCS2+ M-TP B/A-1) assay; 20 mM Tris-

HCl (pH 7.5), 10% glycerol, 1 mM DTT, 120 mM NaCl, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 1 mM β-glycerophosphate, 50 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin for the p38MAPK assay. The reaction mixtures (50 μl), containing the PKB (Akt) was immunoprecipitated with a c-Myc–tagged wild-type PI3-K p85 subunit were precleaned with control rabbit IgG coupled 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 EDTA, 1 mM EGTA, and three times with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, and 0.2 mM Na3VO4. Aliquots of the washed immunoprecipitates were loaded onto 10% SDS-PAGE, and the protein bands were detected using an ECL Western blotting detection kit (Amerosh Pharmacia Biotech) with the indicated polyclonal antibodies.

Immunoblotting
Total protein of the cell lysates from SMC cultures was separated by SDS-

PAGE and transferred to nitrocellulose membranes. Detection of target proteins was performed using an ECL Western blotting detection kit (Amerosh 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 soni-
dared 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 MgCl2, 1 mM CaCl2, 1% NP-40, 50 mM NaF, 1 mM Na3VO4, 50 μg/ml PM SF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). A 1 μl aliquot of the clarified material was then added to 20 μl of 100 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM β-glycerophosphate, 50 μg/ml PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin for the PI3-K assay. The cell extracts were immunoprecipitated with specific antibodies against individual protein kinases, and the immunoprecipitates were washed thoroughly with their lysis buffers and kinase assay buffers, and incubated with their respective substrates and 5 μCi γ-[32P]ATP for 30 min at 30°C. The reaction products were analyzed by 15% SDS-PAGE. The reaction mixtures for the kinase assays were as fol-

ows: 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 μg/ml 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 MgCl2, 1 μg/ml protein kinase A inhibitor, 1 mM DTT, 25 μg myelin basic protein (MBP) for the ERK assay; 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 μg/ml protein kinase A inhibitor, 1 mM DTT, 1 mM Na3VO4, and 1 μg GST-Jun (1-79) for the JNK ass-

ay; and 20 mM Hepes (pH 7.4), 20 mM MgCl2, 20 mM β-glycerophos-
phate, 1 μg/ml protein kinase A inhibitor, 1 mM DTT, and 1 μg GST-ATF2 (1-96) for the p38 MAPK assay.

Promoter Analysis and Transfection
The caldesmon promoter reporter 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, pcSC2+ 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 (A) (pCMV5-PKB) assay, pcS2+ M-TPS B/A-1 assay, or an empty vector (pCMV5-MPK), expression vector carrying constitutively active and dominant-negative forms of MEK1 (pCS2+M-EK1act and pCS2+M-EK1DN) and MKK6 (pCS2+ M-K6act and pCS2+ M-K6DN), were added to the cells in Opti

minimum Eagle’s medium (GIBCO BRL). A cell line was transfected for 6 h incubation, then harvested and lysed for Western blotting detection of MEK supplemented with 0.2% BSA plus 2 ng/ml IGF-1 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; O bata 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 or p110α subunit, pcDMV Sp110αact, 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 precleared 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. 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. 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.

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 SM C phenotype as defined by the expression of SM C-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 SM Cs stimulated with PDGF-BB, bFGF, or EGF, h-caldesmon mRNA converts to l-caldesmon mRNA and total h- and l-caldesmon mRNA decreases to 20% of the levels...
seen in precultured SMCs. Calponin mRNA is also down-regulated to a negligible level (Fig. 1A). 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. 1A). 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. 1B 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 SM C 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. 2A) and p38MAPK (Fig. 2C). Their maximum activations were found at 10 min after stimulation. bFGF and EGF also activated JNK, whereas PDGF-BB did not (Fig. 2B). IGF-I had no effect on ERK (Fig. 2A), JNK (Fig. 2B), and p38MAPK (Fig. 2C). 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. 2D) and PKB(Akt) (Fig. 2E); 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 LY294002 (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, PD 98059 for ERK kinase (MEK1) and SB 203580 for p38MAPK, on the PDGF-BB–stimulated SMC phenotype. Either PD 98059 or SB 203580 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 PD 98059 or SB 203580 only slightly suppressed the PDGF-BB–induced isoform conversion of caldesmon mRNA and downregulation of caldesmon and calponin mRNA (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 mRNA (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 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 PD 98059 and/or SB 203580 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).

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 p38MAPK 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

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Contracted cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>92.9 ± 2.0</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>0</td>
</tr>
<tr>
<td>PDGF-BB + PD</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>PDGF-BB + SB</td>
<td>9.0 ± 1.0</td>
</tr>
<tr>
<td>PDGF-BB + PD/SB</td>
<td>92.4 ± 3.1</td>
</tr>
</tbody>
</table>

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 PD 98059 and/or SB 203580. The SMCs plated on laminin were cultured in DME supplemented with 0.2% BSA for 24 h, and then pretreated with either PD 98059 (30 µM) or SB 203580 (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 mRNA was analyzed by Northern blotting as shown in Fig. 1 A. Total RNA was 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 PD 98059 (30 µM) or SB 203580 (20 µM) or both drugs. The results are shown from four 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 form of PI3-K (PI3-Kact) or PKB(Akt) (PKB(Akt)act) (Fig. 5 A and B). Gizzard SMCs transfected with control plasmid (pCS2+MT) or expression plasmids of c-Myc–tagged active PI3-K p110α subunit, pCMV5p110αact (PI3-Kact). 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 assays 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), expression plasmid of c-Myc-tagged wild-type PKB(Akt) (PKB(Akt)wt), expression plasmid of c-Myc-tagged active PKB(Akt) (PKB(Akt)act), or expression plasmid of c-Myc–tagged active PKB(Akt) in the presence of both 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).
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 isof orm 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 isof orm conversion of caldesmon mRNA and downregulation of caldesmon and calponin mRNAs (Fig. 8 A, lane 1), whereas control medium (CM2) obtained from SMCs

The Journal of Cell Biology, Volume 145, 1999

734
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 p38 MAPK 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 p38 MAPK 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 cells from rat aorta, 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 the 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). 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-

**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. 1A. (B) Activation of ERK, JNK, and p38 MAPK 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.

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.

**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 SM C-specific molecular markers. Long spindle-shaped cells change to fibroblast-like cells, accompanied by losses in a ligand-induced contractility and SM C-specific molecular marker expression. Since there has not been a primary culture system available for SMCs or SM C-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 (Hayaishi et al., 1998). In this culture system, IGF-I is the most potent for maintaining the differentiated SM C phenotype,
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 dedifferentiation 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). A 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).

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; Börnfeldt et al., 1990; Cercek et al., 1990; Delafontaine et al., 1991; Börn-
p70 ribosomal S6 kinase (p70S6K) is unlikely to be a down-
mycin had no effect on the differentiated SMC phenotype
made it possible to maintain the differentiated SMC phe-
(data not shown). These properties of IGF-I signaling
2). Further, IGF-I did not affect the proliferation of SMCs
SMC culture system that IGF-I solely triggers the PI3-
feldt et al., 1994), it has been considered to be an impor-
tion (Figs. 3 and 4). bFGF and EGF are known to activate
sclerosis. It has been reported that de-differentiated SMCs pro-
duced and secrete PDGF which further promotes cell pro-
from an anti-apoptotic action. In passaged SMGs, IGF-I
enhanced the JNK and p38 MAPK activities (data not shown).
These results suggest that the downstream signal-
ings of IGF-I might be modulated during cell passage. A
tually, differentiated SMGs rapidly change their pheno-
type under serum-stimulated conditions (Kashiwada et al.,
1997; Hayashi et al., 1998). Since passaged SMGs 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 SMGs. In this study, we
used SMGs that showed well-characterized and stable phe-
notypes. We analyzed the relationship between the modu-
lation of SMG phenotype and cell proliferation. A thought
serum-induced SMG de-differentiation was concomitant
with cell proliferation, other growth factors that trigger
SMG de-differentiation, such as PDGF-BB, bFGF, and
EGF, did not significantly induce SMG proliferation (data
not shown). This result also suggests that SMG de-dif-
terntion is not essentially associated with cell prolifera-
It has been reported that de-differentiated SMGs pro-
duce and secrete PDGF which further promotes cell prolif-
eration and migration in an autocrine/paracrine manner
(Sjolund et al., 1988). PDGF is also known to promote the
activation of ERK and p38 MAPK in passaged SMGs
(Bornfeldt et al., 1994; Pyne and Pyne, 1997). In our cul-
ture system, PDGF-BB triggered the dual signaling path-
ways mediated by PI3-K/PkB (Akt) and two MAPKs,
ERK and p38 MAPK. Under these culture conditions,
PDGF-BB stimulation resulted in SMG de-differentiation.
When the two MAPK pathways were blocked by their
specific inhibitors, PD98059 and SB 203580, PDGF-BB
stimulation, in turn, initiated to maintain SMG differen-
tiation (Figs. 3 and 4). bFGF and EGF are known to activate
ERK and also to induce proliferation of SMGs (Berrou et al.,
1996; Jones et al., 1997; Y u et al., 1997; Miyamoto et al.,
1998). In our culture system, both growth factors activated
ERK and p38 MAPK and potently induced SMG de-dif-
terntiation (Figs. 1 and 2). However, PD98059 and
SB 203580 could not prevent such de-differentiation (data
not shown). This is because the signaling pathway medi-
ated 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 SMG 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
SB 203580 (Figs. 3–5 and Table I). Further, the activation of
ERK and p38 MAPK 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 SMG de-differentiation (Fig. 6). These data
support our hypothesis that the SMG phenotype would be
determined by the balance between the strengths of the
signaling pathways mediated by PI3-K/PkB (Akt) and by
ERK and p38 MAPK.
Curiously, even though the transfection efficiencies of
both expression vectors carrying active MEK1 and MKK6
were only 25%, almost all of SMGs came to de-differen-
tiate as monitored by cell morphology and endogenous
expression of SMG-specific molecular markers (Fig. 7).
These findings indicate that the production and secre-
tion of some de-differentiation-inducing factor(s) occur in
SMGs in which both the ERK and p38 MAPK 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, p38 MAPK,
and JNK). The activating factor(s) was a heat-labile, non–
heparin-binding protein factor (Fig. 8). From these bio-
chemical properties (Fig. 8), we excluded the possibility
that PDGF-BB, bFGF, and EGF would be the main factor
inducing SMG de-differentiation in the conditioned me-
dium. Further study is required to identify such a factor(s).
Anyway, this study provides a evidence that only a small
portion of de-differentiated SMGs secretes a protein fac-
tor(s) for the surrounding normal SMGs to be de-differen-
tiated. These findings could be helpful to understand the
progression of smooth muscle disorders such as athero-
sclerosis.
We then applied a culture system of gizzard SMGs to
that of vascular SMGs and investigated the signaling path-
ways in regulating the vascular SMG phenotype (Fig. 9).
Like gizzard SMGs, IGF-I potently maintained a differen-
tiated phenotype of vascular SMGs and a specific PI3-K
inhibitors, LY 24002, prevented this IGF-I’s action. Treat-
ment with two MAPK pathway inhibitors, PD98059 and
SB 203580, could rescue the PDGF-BB–induced de-differen-
tiation of vascular SMGs. Further, the conditioned me-
dium obtained from gizzard SMGs also induce de-differen-
tiation of some de-differentiation-inducing factor(s) occur in
SMGs in which both the ERK and p38 MAPK 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, p38 MAPK,
and JNK). The activating factor(s) was a heat-labile, non–
heparin-binding protein factor (Fig. 8). From these bio-
chemical properties (Fig. 8), we excluded the possibility
that PDGF-BB, bFGF, and EGF would be the main factor
inducing SMG de-differentiation in the conditioned me-
dium. Further study is required to identify such a factor(s).
Anyway, this study provides a evidence that only a small
portion of de-differentiated SMGs secretes a protein fac-
tor(s) for the surrounding normal SMGs to be de-differen-
tiated. These findings could be helpful to understand the
progression of smooth muscle disorders such as athero-
sclerosis.
Our results presented here are summarized in Fig. 10.
The signaling pathway mediated by PI3-K/PKB (Akt) is
Distinct signaling pathways are directly involved in the phenotypic determination of visceral and vascular SMCs. Maintenance of a differentiated phenotype of SM Cs 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, LY 249002, or wortmannin, induces SM de-differentiation. Potent SM de-differentiation-inducing factors, PDGF-B-B, bFGF, and EGF, all activate the ERK and p38MAPK pathways. bFGF and EGF do not enhance the PI3-K/PKB (Akt) pathway, whereas, PDGF-B-B does activate it. Thus, PDGF-B-B 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, PD 98059 and SB 203580, PDGF-B-B in turn initiates to induce maintaining SMC differentiation. Therefore, the SMC phenotype would be determined by the balance between the strengths of these signaling pathways. PI3-K/PKB (Akt) pathway and the ERK and p38MAPK pathways.

References


Sobue, K., H. Hayashi, and W. Nishida. 1998. Molecular mechanism of pheno-


Xu, Q., Y. Liu, M. Gorospe, R. Udelsman, and N.J. Holbrook. 1996. Acute hy-