APEG-1, a Novel Gene Preferentially Expressed in Aortic Smooth Muscle Cells, Is Down-regulated by Vascular Injury*

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Despite the importance of phenotypic alterations in arterial smooth muscle cells (ASMC) during the pathogenesis of arteriosclerosis, little is known about genes that define differentiated ASMC. Using differential mRNA display, we isolated a novel gene preferentially expressed in the rat aorta and termed this gene APEG-1. The cDNA of rat APEG-1 contained an open reading frame encoding 113 amino acids, which would predict a basic protein of 12.7 kDa. The amino acid sequence of rat APEG-1 was highly conserved among human and mouse homologues (97 and 98%, respectively). Using an APEG-1 fusion protein containing an N-terminal c-Myc tag, we identified APEG-1 as a nuclear protein. By in situ hybridization, APEG-1 mRNA was expressed in rat ASMC. Although APEG-1 was expressed highly in differentiated ASMC in vivo, its expression was quickly down-regulated in dedifferentiated ASMC in culture. In vivo, APEG-1 mRNA levels decreased by more than 80% in response to vascular injury as ASMC changed from a quiescent to a proliferative phenotype. Taken together, these data indicate that APEG-1 is a novel marker for differentiated ASMC and may have a role in regulating growth and differentiation of this cell type.

Arterial smooth muscle cells (ASMC) constitute the major portion of the blood vessel wall, and their main function is to regulate vascular tone. By controlling vascular tone, ASMC have an important role in regulating blood pressure. Fully differentiated ASMC in normal vessel walls proliferate at an extremely low rate and express unique contractile proteins, ion channels, and signaling molecules required for their contractile function (1). In contrast to skeletal and cardiac muscle cells (2, 3), ASMC are not terminally differentiated in adult animals. In response to vascular injuries caused by smoking, hypercholesterolemia, hyperhomocysteinemia, hypertension, or trauma (such as balloon angioplasty), ASMC de-differentiate and change from a quiescent and contractile phenotype to a proliferative and synthetic phenotype (1, 4–9). This proliferation of vascular smooth muscle cells is one of the most prominent features of arteriosclerosis, the leading cause of death in developed countries (4). Similar alterations in ASMC phenotypes occur during angiogenesis in solid tumors and may have an important role in determining tumor growth rate of these tumors (10–12). Despite the importance of alteration of ASMC phenotypes in vascular diseases, little is known about molecular mechanisms regulating differentiation of this cell type (1). This is due, at least in part, to a lack of differentiation markers of ASMC.

Several genes encoding proteins specific to smooth muscle cells, including smooth muscle a-actin, calponin, SM-22, and caldesmon, have been used as markers for differentiated smooth muscle cells (13–18). However, their expression is not limited to vascular smooth muscle cells. Recently, a homeobox gene gax has been shown to be highly expressed in vascular smooth muscle cells and may regulate their proliferation (19, 20). Yet, high level expression of gax has also been detected in the heart, indicating that gax is expressed both in smooth as well as striated muscles. To date, no marker specific to differentiated ASMC has been identified.

To identify genes preferentially expressed in differentiated ASMC, we performed differential mRNA display using RNA samples extracted from different rat organs. Here we report the isolation of a novel gene, APEG-1, that is preferentially expressed in the rat ASMC in vivo. The amino acid sequence of APEG-1 is highly conserved among human, rat, and mouse homologues. Despite the absence of a classical nuclear localization signal, APEG-1 is a nuclear protein. APEG-1 is highly expressed in differentiated arterial smooth muscle cells in vivo. However, its expression is quickly down-regulated in cultured arterial smooth muscle cells during the first three passages. In vivo, APEG-1 mRNA levels decreased as ASMC changed from a quiescent to a proliferative phenotype in response to vascular injury, suggesting that APEG-1 may have a role in regulating the growth and differentiation of this cell type.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Rat aortic smooth muscle cells were harvested from the thoracic aorta of adult male Sprague-Dawley rats (200–250 g) by enzymatic digestion, according to the method of Gunther et al. (21). COS-7 cells were obtained from the American Type Cell Culture Collection. U2OS cells were kindly provided by Dr. T.-P. Yao (Dana-Farber Cancer Institute). All cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Life Sciences, Lenexa, KS) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 4 mM l-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified incubator (37 °C, 5% CO2), with the exceptions that rat aortic smooth muscle cells were further supplemented with 10 μM HEPES (pH 7.4) in the medium, and COS-7 were supplemented with 10% Serum Plus (HyClone, Logan, UT) instead of fetal calf serum.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U57097, U57098, and U57099 for rat, mouse, and human APEG-1, respectively.

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† The abbreviations used are: ASMC, arterial smooth muscle cells; APEG-1, aortic preferentially expressed gene-1; RT-PCR, reverse transcription-polymerase chain reaction; Tricine, N-[2-hydroxy-1,1-bis (hydroxymethyl)ethyl]glycine.
RNA Extraction and Northern Blot Analysis—Total RNA from cultured cells was purified by guanidinium/CsCl ultracentrifugation (22). Rat tissues were obtained from adult male Sprague-Dawley rats (Charles River, Wilmington, MA), and the carotid arteries were obtained from rats that had been subjected to balloon angioplasty (Zivic-Miller, Xenionople, PA). RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose filters. The filters were then hybridized with a [α-32P]dCTP-labeled, random primed APEG-1 cDNA probe as described (22, 23). The hybridized filters were washed in 30 mM sodium chloride, 3 mM sodium citrate, and 0.1% SDS at 55°C and exposed to Kodak XAR film at ~80°C. To correct for differences in RNA loading, the blots were hybridized with an 18S RNA oligonucleotide (24). The filters were also exposed to a phosphorscreen, and radioactivity was measured on a PhosphorImager by using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Cloning and Sequencing of Rat (r)APEG-1, Mouse (m)APEG-1, and Human (h)APEG-1—To identify genes preferentially expressed in the aorta, we performed differential mRNA display on RNA samples prepared from rat aorta (after removal of adventitia), brain, skeletal muscle, esophagus, heart, and intestine, essentially as described by Liang et al. (24, 25). One of the cloned cDNA fragments, termed APEG-1, encoded a novel sequence, and this cDNA fragment was used to isolate the full-length clone from a rat aortic cDNA library. The rAPEG-1 was used to screen a human aortic cDNA library to obtain the human sequence. The mouse cDNA encoding the open reading frame was first amplified from mouse aortic RNA by reverse transcription polymerase chain reaction (RT-PCR) with primers conserved between the rat and human sequences. Using nested primers designed according to the open reading frame of rAPEG-1, 1332 bp were obtained by 3' rapid amplification of cDNA ends. Both strands of the entire rAPEG-1, mAPEG-1, and hAPEG-1 DNAs were sequenced at least once by the dideoxy chain termination sequencing method.

In Situ Hybridization—rAPEG-1 mRNA was detected in situ as described (26) with minor modifications. We perfused adult male Sprague-Dawley rats with 4% paraformaldehyde in phosphate-buffered saline. Organs were then postfixed with 4% paraformaldehyde, soaked in 30% sucrose, embedded in optimum cutting temperature compound, and frozen in isopentane at −80°C. Tissue sections were cut at a thickness of 5 microns. A 382-bp DNA fragment from the rAPEG-1 cDNA 3'-untranslated region (927–1308 bp) was subcloned into pCDNA3 vector in both orientations and linearized by XhoI digestion to generate cRNA probes by T7 RNA polymerase. In situ hybridization was carried out with [α-32P]UTP-labeled sense or antisense cRNA probes on tissue sections at 50°C. After hybridization the tissue sections were washed under moderately stringent conditions as described (26). Dried tissue sections were subjected to autoradiography with Kodak NTB2 emulsion (Eastman Kodak Co.) for 5 days at 4°C. Specific APEG-1 signal was observed in the slides hybridized with antisense riboprobe. Hybridization with APEG-1 sense riboprobe was performed to control for nonspecific hybridization background. Counterstaining was performed with hematoxylin and eosin.

Cellular Localization of APEG-1—To construct the expression plasmid c-myc-rAPEG-1/pCR3, we added in frame a c-Myc peptide tag (EQKLISEED) to the rAPEG-1 sequence. This hybrid DNA fragment was then cloned into the eukaryotic expression vector pCR3 (Invitrogen, San Diego, CA). COS-7 cells were transiently transfected with the c-myc-rAPEG-1 expressing plasmid by the DEAE-dextran method with minor modifications (27). The U2OS cells were transiently transfected by the calcium phosphate method (22). Twenty-four hours after transfection, cells were transfected to two-well chamber slides. The cells were fixed with 4% paraformaldehyde in phosphate-buffered saline after growing for an additional 24 h. Immunostaining was performed with an anti-c-Myc monoclonal antibody (9E10, Oncogene, Cambridge, MA) followed by a rhodamine-conjugated goat anti-mouse IgG secondary antibody (Sigma). Nuclear DNA counterstaining was performed with Hoechst 33258 at a concentration of 1 μg/ml.

In Vitro Transcription and Translation—The entire rAPEG-1 cDNA was subcloned into the pSP72 vector. In vitro transcription was performed using a large-scale T7 transcription system (Novagen, Madison, WI) in the presence of [α-32P]GTP. The in vitro transcribed mRNA was translated in an in vitro translation system of wheat germ extract (Promega, Madison, WI) in the presence of [α-32P]methionine. The translated proteins were resolved on a 10% Tris-SDS-polyacrylamide gel (28), and the dried gel was subjected to autoradiography.

Reverse Transcription-Polymerase Chain Reaction and Southern Analysis—RNA from rat ASM at passages 1, 2, and 3 as well as from rat aorta were used for reverse transcription and PCR with the following rAPEG-1-specific primers: 108F, AGTACCTGACGGCGCCGCAG-GAGTTC and 1144R: TGTGAAAGAGGCGCACCCTGTGTC. The underlined nucleotide sequences were modified to create a NotI restriction enzyme site for cloning. Reverse transcription reactions were carried out with the Moloney murine leukemia virus enzyme as recommended by Life Technologies, Inc. One-fourth of the reaction was then used for PCR with 5 units of Taq DNA polymerase (Boehringer Mannheim) in a 100-μl reaction. After 30 cycles of reaction, 10 μl of the product was separated on a 1% agarose gel and visualized with ethidium bromide. DNA was then transferred to nitrocellulose membrane, hybridized with a [α-32P]dCTP-labeled rAPEG-1 probe, and subjected it to autoradiography on Kodak XAR film.

RESULTS

Cloning and Tissue Expression Pattern of APEG-1—Using RNA samples obtained from rat brain, aorta, skeletal muscle, esophagus, heart, and intestine, we performed differential mRNA display to isolate genes preferentially expressed in the...
of an arteriole located in the adventitial cell layer. Smooth muscle cells, but not in the surrounding adventitial layer of aorta hybridized with antisense (C) and sense (D) riboprobes. Specific hybridization is seen in the arterial smooth muscle cells, but not in the surrounding adventitial tissues. Magnification: × 600.

**FIG. 2.** Analysis of rAPEG-1 expression in rat aorta by in situ hybridization. In situ hybridization experiments were performed using (35S)UTP-labeled APEG-1 antisense (A and C) and sense (B and D) riboprobes on adjacent sections of rat aorta as described under "Experimental Procedures." A and B, cross-sections of a rat aorta hybridized with antisense (A) and sense (B) riboprobes. Specific hybridization (black dots) is seen in the arterial smooth muscle cells, but not in the endothelial cell layer. C and D, cross-sections of an arteriole located in the adventitial layer of aorta hybridized with antisense (C) and sense (D) riboprobes. Specific hybridization is seen in the arterial smooth muscle cells, but not in the surrounding adventitial tissues. Magnification: × 600.

A unique band (flanked by dots, Fig. 1A) was detected only in the aortic sample by a 5′ arbitrary primer (5′ TGC-CCAGCTG 3′) and the anchoring primer T12VG. Using the cDNA amplified from the DNA eluted from this band as a probe and RNA samples prepared from rat organs in Northern analysis, an intense message of 1.3 kilobases was detected in the aorta (Fig. 1B). A much weaker signal was detected in the brain and testis. This message was barely detectable in organs rich in striated muscle (skeletal muscle, heart, and esophagus) or visceral smooth muscle (esophagus and intestine). Even among vascular tissue, APEG-1 was expressed at much higher levels in aorta than in the vena cava. Because of its preferential expression in the aorta, we termed this gene APEG-1. Since the aorta is composed of three layers, we prepared RNA from adventitia, media, and media plus intima. No APEG-1 expression was detected in adventitia, but it was expressed at similar levels in media alone after removal of endothelial cells, and media plus intima rich in endothelial cells (Fig. 1C), indicating that this gene was expressed mainly in ASMC of the media. To confirm the expression of APEG-1 in ASMC, we performed in situ hybridization. Compared with the background signal of the sense probe (Fig. 2, B and D), rat aorta hybridized with the APEG-1 antisense probe showed intense signal limited to ASMC in the medial layer of aorta (Fig. 2A) and ASMC in an arteriole (Fig. 2C).

Sequence Analysis of Rat, Human, and Mouse APEG-1 cDNA—Using the amplified APEG-1 cDNA fragment as a probe to screen a rat aortic cDNA library, we isolated a 1308-bp, full-length APEG-1 cDNA clone (Fig. 3A). The longest open reading frame of APEG-1 cDNA encoded 113 amino acids that would predict a protein with a molecular mass of 12.7 kDa and an estimated pI of 9.1. The nucleotide sequence flanking the putative initiation codon complied with the Kozak consensus sequence for initiation of translation (29). A GenBank search failed to identify published sequences homologous to APEG-1, indicating that APEG-1 is a novel gene. Motif search of the APEG-1 peptide sequence revealed an RGD domain at positions 85 to 87 (in bold, Fig. 3A). Although it is not clear whether the APEG-1 RGD domain is functional, the RGD domain has been implicated as the binding site for integrins (30, 31). There are a few potential serine or threonine phosphorylation sites.

To confirm the putative open reading frame, we performed in vitro transcription and translation by use of a prokaryotic expression plasmid pSP72 containing the full-length APEG-1 cDNA. The 1.3-kilobase mRNA (Fig. 3B) generated from in vitro transcription was used as the template for in vitro translation with wheat germ extract. The major translated product has a molecular mass of 12.7 kDa, consistent with the predicted size of APEG-1 protein (Fig. 3C).

To determine whether APEG-1 was conserved across species, we isolated the human and mouse APEG-1 homologues. A comparison of the human and rat open reading frames revealed 90% identity at the cDNA level and 97% identity at the amino acid level (Fig. 4, A and B). Comparison of the open reading frames of mouse and rat APEG-1 revealed 95% identity at the cDNA level and 98% identity at the amino acid level (Fig. 4, A and B). Thus, APEG-1 is highly conserved across species.

Cellular Localization of APEG-1—To determine the cellular localization of APEG-1 protein, we first generated a plasmid, c-myc-rAPEG-1/pCR3, that would express a fusion protein of APEG-1 with an N-terminal c-Myc tag. COS-7 cells were then transiently transfected with the c-myc-rAPEG-1/pCR3 plasmid and immunostained with a monoclonal anti-c-Myc antibody, 9E10. c-Myc-tagged APEG-1 protein was expressed predominantly in the nuclei of transfected COS-7 cells (Fig. 5, A and B). We have also performed the same experiment in U2OS cells and found that APEG-1 localized to the nuclei in these cells as well (Fig. 5, C and D).

Down-regulation of APEG-1 mRNA Levels in ASMC in Culture—Although a high level of APEG-1 mRNA was detected in ASMC in vivo (Figs. 1, B and C, and 2A), this mRNA was undetectable by Northern analysis in primary rat ASMC in culture (data not shown). To determine the time course of down-regulation of APEG-1 mRNA in ASMC in culture, we performed reverse transcription PCR with APEG-1-specific primers and RNA prepared from rat ASMC at passages 1 to 3. The intensity of the band amplified from passage 1 rat ASMC was much weaker than that in the aortic tissue after staining with ethidium bromide (Fig. 6). The bands from passage 2 or 3 ASMC became barely detectable only after hybridization to an
APEG-1 probe in Southern analysis (Fig. 6). Thus, APEG-1 mRNA was quickly down-regulated and disappeared in dedifferentiated rat ASMC in culture.

In response to vascular injury, ASMC dedifferentiate in vivo from a quiescent and contractile to a proliferative and synthetic phenotype. To determine whether the APEG-1 mRNA level would be down-regulated in dedifferentiated ASMC in vivo, we performed Northern analysis on RNA samples obtained from carotid arteries previously injured by balloon angioplasty. It has been shown previously that maximal ASMC proliferation occurs in the media at 48 h and in the neointima at 96 h (32).

APEG-1 mRNA levels were markedly down-regulated to 18 and 20% of control 2 and 5 days after injury, respectively (Fig. 7). This down-regulation was followed by a recovery to 40% of control at day 8. These data suggest that APEG-1 mRNA is down-regulated in dedifferentiated ASMC both in vitro and in vivo.

**DISCUSSION**

Using differential mRNA display, we have isolated a novel gene, APEG-1, that is preferentially expressed in ASMC.

APEG-1 encodes a 12.7-kDa protein (Fig. 3). Its predicted protein sequence reveals an RGD domain and no known nuclear localization signals (33). Since an RGD domain is the potential binding site for integrins, this finding would implicate APEG-1 as a membrane protein. However, c-Myc-tagged APEG-1 is localized to the nucleus (Fig. 5). This nuclear localization is not due to the c-Myc tag, because c-Myc-tagged cyclin B protein is expressed exclusively in the cytoplasm (data not shown), as reported previously (34). An as yet unidentified nuclear localization signal may be responsible for its nuclear localization. Alternatively, APEG-1 may dimerize with another protein with a nuclear localization signal and then be transported to the nucleus.

Trans-Acting factors are nuclear proteins that have important roles in regulating proliferation and differentiation, as well as in determination of cell lineage. For example, members of the MyoD family are expressed only in skeletal muscle cells and define lineage, inhibit proliferation, and promote differentiation of this cell type (35–37). Despite the importance of ASMC in regulating vascular tone and in the pathogenesis of vascular diseases, no ASMC-specific nuclear proteins have been identified (1).

**Fig. 4. Evolutionary conservation of APEG-1.** 
A, the amino acid sequences of human, rat, and mouse APEG-1 are shown. A consensus sequence derived from these three species is shown below the protein sequences. The consensus sequences indicated by uppercase are conserved among all three species, sequences indicated by lowercase are conserved between two species. B, the sequence identities between species are shown in percentages at the nucleotide level, both the open reading frame (ORF) and the full-length cDNA (full length), and at the amino acid (Protein) level.
Despite high level expression of need to be down-regulated to allow this phenotypic change. Genes important for maintaining the differentiated state may pathogenesis of vascular diseases and angiogenesis (1, 4, 9). expressed in ASMC.

Fig. 5. Cellular localization of rAPEG-1 protein. The c-myc-rAPEG-1 expressing plasmid was transiently transfected into the COS-7 cells (A and B) and the U2OS cells (C and D). The expressed c-Myc-rAPEG-1 fusion protein was detected by immunocytochemistry with a monoclonal antibody (9E10) against the c-Myc peptide (A and C), and the nuclear DNA counterstaining was performed with Hoechst 33258 (B and D) to locate the cell nuclei. A and B, transfected COS-7 cells expressed the c-Myc-rAPEG-1 fusion protein predominantly in the nuclei (A), as demonstrated by the colocalization of fusion protein with the nuclear DNA counterstaining (B). C and D, transfected U2OS cells expressed the majority of c-Myc-rAPEG-1 fusion protein in their nuclei (C), as demonstrated by the distribution of the expressed fusion protein and the nuclear DNA counterstaining (D). Magnification: × 600.

Fig. 6. Down-regulation of rAPEG-1 expression in early passages of rat ASMC in culture. RNA samples from cultured rat ASMC at passages 1, 2, and 3 were used in RT-PCR with specific primers as described under “Experimental Procedures.” Rat aortic RNA was included in this experiment to serve as a positive control. After gel electrophoresis, the RT-PCR products were visualized by ethidium bromide staining under UV light, and a picture was taken for a record (RT-PCR). Southern analysis was performed by transferring the RT-PCR products to a nitrocellulose membrane that was then hybridized to a [32P]dCTP-labeled rAPEG-1 cDNA probe (Southern). In contrast to its readily detectable expression in the aorta (Aorta), APEG-1 expression is strongly down-regulated in early passages of rat ASMC in culture (P1, P2, and P3). In addition, there is a further down-regulation of APEG-1 expression from passage 1 (P1) to passage 2 (P2) by Southern analysis.

APEG-1 appears to be expressed only in highly differentiated ASMC in normal vessel walls and its down-regulation serves as a sensitive marker for dedifferentiated ASMC in vivo. This is further supported by our in vivo finding that APEG-1 mRNA levels in the carotid artery were markedly down-regulated to ~18–20% of control after balloon injury when ASMC dedifferentiated (Fig. 7).

Takén together, our data indicate that APEG-1 is a novel marker for differentiated ASMC. The immediate down-regulation of this nuclear protein by phenotypic change suggests that it may have a role in regulating growth and differentiation of this cell type. Further work aimed at cloning the APEG-1 gene and its 5′-flanking sequence will allow us to identify ASMC-specific cis-acting elements and trans-acting factors that may prove useful for expressing foreign genes in the blood vessel wall.

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expressed in ASMC.

Alteration in ASMC phenotypes has an important role in the pathogenesis of vascular diseases and angiogenesis (1, 4, 9). Genes important for maintaining the differentiated state may need to be down-regulated to allow this phenotypic change. Despite high level expression of APEG-1 in differentiated ASMC in normal aorta, APEG-1 mRNA levels decreased rapidly and disappeared in dedifferentiated ASMC in culture even in early passage (Fig. 6). It has been shown that ASMC grown on Matrigel exhibit a more differentiated phenotype with a low proliferative index and enhanced levels of the smooth muscle isoform of α-actin (38, 39). However, APEG-1 mRNA did not reappear in ASMC grown on Matrigel (data not shown). Thus,
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