Inhibition of Angiogenesis and Vascular Leakiness by Angiopoietin-Related Protein 4

Yasuhiro Ito, Yuichi Oike, Kunio Yasunaga, Koichi Hamada, Keishi Miyata, Shun-ichiro Matsumoto, Sumio Sugano, Hidenobu Tanihara, Yasuhiko Masuho, and Toshio Suda

Department of Cell Differentiation, The Sakaguchi Laboratory, School of Medicine, Keio University, Tokyo 160-8582, Japan [Y. I., Y. O., T. S.]; Department of Cell Differentiation, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto 860-0811, Japan [K. H., K. M.]; Molecular Medicine Laboratories, Yamanouchi Pharmaceutical Co., Ltd., Tsukuba 305-8585, Japan [K. Y., S. M.]; Helix Research Institute, Inc., Kisarazu 292-0812, Japan [S. M., Y. M.]; Department of Ophthalmology, Kumamoto University School of Medicine, Kumamoto 860-0811, Japan [Y. I., H. T.]; and Department of Virology, Institute of Medical Science, University of Tokyo, Tokyo 113-8655, Japan [S. S.]

ABSTRACT

Angiopoietins and angiopoietin-related proteins (ARPs) have been shown to regulate angiogenesis, a process essential for various neovascular diseases including tumors. Here, we identify ARP4/fasting-induced adipose factor/PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR gamma angiopoietin-related as a novel antiangiogenic modulatory factor. We hypothesized that ARP4 may regulate angiogenesis. In vitro experiments using purified recombinant ARP4 protein revealed that ARP4 markedly inhibited the proliferation, chemotaxis, and tube formation of endothelial cells. Moreover, using corneal neovascularization and Miles permeability assays, we found that both vascular endothelial growth factor-induced in vivo angiogenesis and vascular leakiness were significantly inhibited by the addition of ARP4. Finally, we found remarkable suppression of tumor growth within the dermal layer associated with decreased numbers of invading blood vessels in transgenic mice that express ARP4 in the skin driven by the keratinocyte promoter. These findings demonstrate that ARP4 functions as a novel antiangiogenic modulatory factor and indicate a potential therapeutic effect of ARP4 in neoplastic diseases.

INTRODUCTION

Blood vessel formation, which is required for embryogenesis, also contributes to several pathophysiological conditions such as neovascularization in tumorigenesis and inflammatory and ischemic disease in adulthood. Recent studies show that many critical growth factors regulate blood vessel formation and that the actions of these factors are carefully orchestrated in terms of time, space, and dose to form a vascular network (1, 2). Among them, endothelial-restricted receptors and their ligands are critical for such events. In particular, receptor tyrosine kinases, such as VEGF receptors and Tie receptors, play pivotal roles in blood vessel formation in both embryogenesis and pathophysiological conditions (3, 4). Tie2 ligands, angiopoietin-1, -2, -3, and -4, regulate both dynamic and quiescent states of endothelial cells through competitive interaction. Among the angiopoietins, angiopoietin-1 is critical for modulation of angiogenesis, playing complementary and coordinated roles with VEGF in vascular development (4).

To understand the roles of angiopoietin family proteins in the vascular system, we cloned four structurally homologous proteins containing characteristic coiled-coil domains within the NH2 terminus and a fibrinogen-like domain in the COOH terminus. Among them, ARP4 (human ARP4, GenBank accession no. AB054540; mouse ARP4, GenBank accession no. AB054540) was identical to FIAF/PGAR, which has recently been found to be a PPAR target gene (5, 6). However, the role of ARP4 in angiogenesis has not been fully elucidated. Several lines of recent evidence indicate that ARPs also play important roles in regulating angiogenesis. For example, ANGPTL3, a member of the ARP family, exhibits biological activity as an angiogenic factor (7, 8). In contrast, ARP1/angioarrestin inhibits proliferation, migration, and tube formation of endothelial cells and significantly reduces HT1080 tumor nodule formation (9). Furthermore, recent reports indicate that ARP4/FIAF/PGAR expression is lost in two gastric cancer cell lines and reduced in primary gastric cancers (10). Therefore, we hypothesized that ARP4 could play important roles in modulating angiogenesis in tumorigogenesis.

In this study, we show that ARP4 inhibits proliferation, migration, and tubule formation of endothelial cells and reduces vascular leak. These conditions are found in multiple abnormalities of tumor vessels, and they influence neovascularization, which is required for both primary tumor growth and metastatic growth. Taken together, our findings indicate that ARP4 is a novel negative angiogenic regulator that may be useful in treating neovascular diseases such as cancer.

MATERIALS AND METHODS

Cells. HUVECs (BioWhittaker, Inc., Walkersville, MD) and HDMVECs (BioWhittaker, Inc.) were cultured in EGM-2 medium (Clonetics, San Diego, CA). bEND3 (11), mouse brain capillary endothelial cells, were cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (JRH Biosciences, Lenexa, KS).

Expression, Purification, and Characterization of Recombinant ARP4 Protein. The coding region of ARP4 fused at the COOH terminus to the FLAG epitope was subcloned into PCPE4 (Invitrogen, Groningen, the Netherlands). HEK293 cells were cultured at 37°C in humidified 5% CO2/air in DMEM supplemented with 10% FBS and transfected with pcPE4-ARP4-FLAG using FuGENE 6 (Roche Diagnostics, Mannheim, Germany). After transfection, HEK293 cells were selected in 300 μg/ml Hygromycin B (Life Technologies, Inc.) for 5 days, and the conditioned medium was collected and filtered with a 0.22-μm pore size filter (Millipore Co., Bedford, MA). To purify ARP4-FLAG fusion protein, it was transferred to an anti-FLAG antibody (M2) affinity gel (Sigma, St. Louis, MO). After washing the gel with PBS, protein was eluted by adding Gly-HCl (pH 3.0) and immediately neutralized with Tris-HCl (pH 8.0). The protein was visualized by SDS-PAGE with Coomassie Brilliant Blue Staining (Wako, Osaka, Japan). Western blot was performed using horseradish peroxidase-conjugated anti-FLAG antibody (M2, 1:500; Sigma). Development of peroxidase activity was detected using 

Received 3/29/03; revised 6/24/03; accepted 7/30/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by Grants-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture of Japan, by the Yamanouchi Foundation for Research on Metabolic Disorders, by a Grant-in-Aid from the Tokyo Biochemical Research Foundation, and by the Nateglilde Memorial Toyoshima Research and Education Fund.

2 These authors contributed equally to this work.

3 To whom requests for reprints should be addressed, at Department of Cell Differentiation, The Sakaguchi Laboratory, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Phone: 81-3-5363-3473; Fax: 81-3-5363-3474; E-mail: sudato@sc.itc.keio.ac.jp.

4 The abbreviations used are: VEGF, vascular endothelial growth factor; ARP, angiopoietin-related protein; FIAF, fasting-induced adipose factor; PPAR, peroxisome proliferator-activated receptor; PGAR, PPARγ angiopoietin-related; Tg, transgenic; HUVEC, human umbilical vein endothelial cell; HDMVEC, human dermal microvascular endothelial cell; FBS, fetal bovine serum; BrDUrd, 5-bromo-2′-deoxyuridine; ANGPTL, angiopoietin-like; K14, keratin 14; PECAM-1, platelet/endothelial cell adhesion molecule 1.
the enhanced chemiluminescence detection system (ECL; Amersham Biosciences Corp., Piscataway, NJ).

Cell Proliferation Assay. HUVECs and HDMVECs were cultured on collagen-coated 96-well plates (Becton Dickinson Labware, Bedford, MA) at a density of $5 \times 10^3$ cells/well in 100 $\mu$l of EBM-2 (Clonetics) containing 0.5% FBS. After 24-h starvation at 37°C, the cells were washed twice with serum-free medium and incubated in fresh medium containing 0.5% FBS and VEGF (10 ng/ml; Pepro Tech EC Inc., London, United Kingdom) with or without various concentrations of ARP4. The BrdUrd incorporation assay was performed according to the manufacturer’s instructions (Roche Diagnostics). Cells were incubated with BrdUrd for 24 h. Each experiment was performed in triplicate and repeated three times.

Cell Migration Assay. A migration assay was performed by using Transwell polycarbonate membrane filters with 8.0-$\mu$m pore size (Costar, Cambridge, MA; Ref. 12). The lower surface of the membranes was precoated with 0.1% gelatin and dried overnight at room temperature. The coated membranes were washed in PBS and dried. EBM-2 containing 0.5% FBS with or without the indicated factors was loaded in the lower wells, and $3 \times 10^5$ HUVECs, which were serum-starved for 24 h and suspended in 100 $\mu$l of EBM-2 medium, were inoculated onto each upper well. DMEM was used instead of EBM-2 medium for bEND3 cells ($3 \times 10^5$). After a 4-h incubation at 37°C, the membranes were fixed with 100% methanol and stained with Giemsa solution. The cells on the upper surface of the membranes were removed with cotton swabs. Cells migrating to the lower surface of the membrane were counted in three independent fields under a microscope. The number of cells counted in untreated samples was represented as 100%. Each experiment was performed in triplicate and repeated three times.

Cell Tubule Formation Assay. Ninety-six-well culture dishes were coated with 80 $\mu$l/well Matrigel (Becton Dickinson) on ice, and Matrigel was allowed to solidify for 30 min at 37°C. HUVECs ($1 \times 10^5$) in 100 $\mu$l of EGM-2 containing 2% FBS and 10 ng/ml VEGF and 10 ng/ml basic fibroblast growth factor (Pepro Tech EC Inc.) with or without various concentrations of ARP4 per well were seeded onto each Matrigel-coated well. After a 24-h incubation at 37°C, endothelial tubule formation was photographed under phase-contrast microscopy. To quantify the length of tubular structures, tubule length was measured with NIH Image software. Tubules shorter than 100 $\mu$m were excluded from the measurement. Each experiment was performed in triplicate and repeated three times.

Mouse Corneal Assay. The corneal assay was performed as described previously (13, 14). In brief, under sterile conditions, slow-release pellets were prepared incorporating VEGF (150 ng) alone, ARP4 (1 $\mu$g) alone, and both VEGF (150 ng) and ARP4 (1 $\mu$g) in a casting solution of an ethynil-vinyl copolymer (Elvac-40; DuPont, Wilmington, DE) in 10% methylene chloride. After anesthesia with sodium pentobarbital (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), the pellet was implanted into the corneal micropocket of male 8-week-old C57BL/6 mice (SLC, Shizuoka, Japan). Erythromycin ophthalmic ointment was applied to each eye after implantation of the pellet. The eyes were examined by a slit-lamp biomicroscope (Kowa Co., Ltd., Nagoya, Japan) each day after pellet implantation. The neovascular response was measured as the maximal vessel length from limbal vasculature toward the pellet ($R_v$; in mm) and the contiguous circumferential zone of clock-hours of neovascularization ($R_z$; in mm). The neovascular area was calculated by the following formula: area (in mm$^2$) = $0.2 \times \pi \times R_v \times R_z$ (15). Each experiment was performed in triplicate and repeated three times.

Vascular Permeability Assay. The vascular permeability assay was performed as described previously (16). Male 8-week-old BALB/c mice (SLC) were anesthetized, and 100 $\mu$l of 1% Evans Blue dye (Wako) were injected into tail vein. Five min after injection, VEGF (50 ng) alone, a combination of ARP4 (1 $\mu$g) and VEGF (50 ng) in 20 $\mu$l of PBS, or PBS alone was injected intradermally at adjacent locations on the back. Extravasation of Evans Blue dye was recorded at 15 and 30 min from the underside of the back skin surrounding the injected site. Thirty min after intradermal injection, the mouse was perfused from the left ventricle with 1% paraformaldehyde in citrate buffer at pH 3.5. Skin surrounding the injected site was removed in the same square, gently blotted, and weighed, and Evans Blue dye was extracted from skin with 1 ml of formamide. The amount of extravasated dye was measured with a spectrophotometer (610 nm) and expressed as the content of dye per 1 mg wet weight of tissue. Each experiment was performed in triplicate and repeated three times.

Fig. 1. Recombinant mouse ARP4-FLAG protein. After transfection of HEK293 cells with mouse ARP4 cDNA with a 3’-terminal extension encoding a FLAG tag, mouse ARP4-FLAG protein was detected in culture supernatants by SDS-PAGE staining with Coomasie Brilliant Blue (two left panels) and Western blot analysis with an anti-FLAG antibody (two right panels) with or without 2-mercaptoethanol (2ME). Each lane contained approximately 1 $\mu$g of purified protein (two left panels). Lanes contain approximately 10, 30, and 100 ng of purified protein (two right panels). Arrows indicate the monomer form of ARP4-FLAG protein. Open arrowheads indicate the multimer form of ARP4-FLAG protein.

Fig. 2. ARP4 inhibits endothelial cell proliferation. After 24 h of starvation, HUVECs and HDMVECs were treated with VEGF (10 ng/ml) alone or in combination with concentrations of ARP4 ranging from 0.1 to 2 $\mu$g/ml and incubated with BrdUrd for 24 h ($n = 3$). BrdUrd incorporation with VEGF alone was considered as maximum incorporation, and the percentage of inhibition was calculated. The error bars represent mean values ± SD.

Fig. 3. ARP4 inhibits endothelial cell migration. A total of $3 \times 10^5$ HUVECs or mouse bEND3 cells/well were inoculated into the upper chamber of a Transwell, and VEGF (10 ng/ml) and ARP4 (0.1, 0.5 and 1 $\mu$g/ml) were added to the lower chamber. Endothelial cells were incubated for 4 h at 37°C ($n = 3$). Endothelial cell migration with VEGF was considered as maximum migration, and the percentage of inhibition was calculated. The error bars represent mean values ± SD.
Generation and Identification of K14-ARP4 Tg Mice. To generate Tg mice that express ARP4 under control of the K14 promoter (K14-ARP4 Tg mice), a transgene was generated by inserting an XbaI fragment containing mouse ARP4 cDNA at the XbaI site of the expression vector under the regulation of the human K14 promoter (17). DNA containing the transgene was prepared for pronuclear injections as described previously (18). A total of 250 pronuclear injections in C57BL/6 mice were performed, resulting in 27 pups. PCR analysis of genomic DNA was performed with forward (5'-H11032-GCTCCTGGGCAACGTGCTGG-3' /H11032) and reverse (5'-H11032-TTGGGAGTCAAGCAAATGAG-3'/H11032) primers in a reaction consisting of 5 min at 95°C, followed by 30 cycles of denaturing at 95°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 60 s. A final 7-min extension was done at 72°C. Northern
blot analysis was performed using [α-32P]dCTP-labeled mouse ARP4 cDNA probes generated by random priming using a BcaBEST Labeling Kit (Takara Shuzo Co., Ltd., Shiga, Japan). Membranes blotted with 20 μg of total RNA extracted from mouse skin using TRIZol (Life Technologies, Inc.) were used. The mice were housed in environmentally controlled rooms of the Laboratory Animal Research Center of Keio University under the guidelines of Keio University for animal and recombinant DNA experiments. To detect ARP4 protein in sections, we prepared antimouse ARP4 polyclonal antibodies that were produced by immunizing rabbits with a synthetic peptide corresponding to amino acids 84–97 of mouse ARP4 (KGKDAPFKDSEDRV). Fixed sections from back skin in K14-ARP4 Tg mice were stained with a 1:200 diluted antimouse ARP4 antibody.

**LECTIN STAINING FOR WHOLE MOUNTS OF EAR SKIN.** Lectin staining of whole mount of ear skin with fluorescein-labeled *Lycopersicon esculentum* lectin (Vector Laboratories, Burlingame, CA) was performed as described elsewhere (16). Stained samples were analyzed using a fluorescence microscope (Olympus, Tokyo, Japan), and vascular density of the samples was assessed by the Chalkley Grid Method (19).

**IN VIVO CRT93 XENOGRAFT TUMOR MODELS.** CRT93 murine colorectal tumor cells were cultured in DMEM supplemented with 10% FBS. Cells were trypsinized, washed with PBS, and resuspended in 300 μl of Matrigel at 1 × 10^6 cells. Mice were inoculated intradermally in the flank. Five weeks after implantation, mice were sacrificed, and tumors were removed. Tumor volumes were estimated by the formula \( V = \pi h \times a \times b \), where the short diameter of tumor is represented as \( a \), and the long diameter of the tumor is represented as \( b \). Tumors were then prepared for immunostaining with a rat antimouse PECAM-1 monoclonal antibody to evaluate capillary formation. The secondary antibody was a horseradish peroxidase-conjugated antirat immunoglobulin antibody (Biosource, Camarillo, CA). Five random photographs per tumor were taken to quantify the tumor capillary vessel areas using NIH Image software.

**STATISTICS.** Statistical evaluation of data was conducted using Student’s t test for per-comparison analysis.

**RESULTS**

**ARP4 Is a Secreted and Oligomerized Protein.** To analyze the biological function of ARP4, we prepared recombinant protein from the conditioned medium of HEK293 cells transfected with ARP4 cDNA. SDS-PAGE and Western blot analysis revealed that a major mouse and human ARP4 product greater than 50 kDa was detected in the culture medium and present at a low levels in the cell lysate. Under nonreducing conditions (without 2-mercaptoethanol), ARP4 oligomerized unexpectedly, as do other proteins containing coiled-coil motifs such as angiopoietins (Fig. 1; data not shown). These results indicate that ARP4 is a secreted and oligomerized protein, as has been reported previously for the angiopoietin family (20–22).

**ARP4 Inhibits Proliferation, Chemotactic Activity, and Tubule Formation of Vascular Endothelial Cells.** VEGF-induced angiogenesis and vascular leakiness occurs in the process of tumorigenesis. Therefore, we analyzed the role of ARP4 signaling in the vasculature by asking whether ARP4 affected VEGF-induced proliferation of HUVECs and HDMVECs. Addition of VEGF increased BrdUrd incorporation in HUVECs and HDMVECs, whereas VEGF-induced BrdUrd incorporation was inhibited in a dose-dependent manner by the addition of ARP4 (Fig. 2).

Next, we investigated whether ARP4 affected VEGF-induced chemotaxis of HUVECs and bEND3 cells. VEGF promoted migration of HUVECs and bEND3 cells into a microchemotaxis membrane. Such VEGF-induced chemotaxis was inhibited by the addition of ARP4 in a dose-dependent manner (Fig. 3). Treatment with ARP4 alone did not affect the basal level of migration of HUVECs and bEND3 cells.

We investigated the effect of ARP4 on tubule formation of HUVECs on Matrigel. HUVECs cultured on Matrigel undergo morphological changes with formation of a tubule, which possesses a lumen surrounded by endothelial cells adhering to another (Fig. 4A). We found that ARP4 inhibited tubule formation of HUVECs in a dose-dependent manner (Fig. 4, B–E). These tubular formations were quantified with NIH Image software by pixels (Fig. 4F). Taken together, the results of the above in vitro analyses indicate that ARP4 directly inhibits the proliferation, chemotaxis, and tubule formation of endothelial cells.

**ARP4 Inhibits in Vivo Neovascularization.** Based on our findings in vitro, we investigated the effect of ARP4 on endothelial cells in in vivo angiogenesis using a corneal neovascularization assay in the mouse. Five days after implantation of a pellet containing VEGF alone, growth of new capillary vessels from the corneal limbus toward the pellet was detected (Fig. 5A), whereas corneal neovascularization toward a pellet containing both VEGF and ARP4 was significantly inhibited (Fig. 5B). The maximal vessel length, clock hours, and vessel area, which are parameters of corneal neovascularization, were markedly inhibited compared with VEGF-induced neovascularization (Fig. 5, D–F). No growth of new capillary vessels was observed in cornea-implanted pellets containing ARP4 alone (Fig. 5C). These results demonstrate that corneas implanted with pellets containing
ARP4 in addition to VEGF exhibit a significant reduction in angiogenesis, relative to those induced by VEGF alone.

**ARP4 Suppresses Vascular Leakage in the Miles Assay.** Vessel leakiness is one of multiple abnormalities seen in tumor vessels, which influences angiogenesis, tumor growth, and metastasis. We therefore estimated the effect of ARP4 on the permeability of blood vessels using the Miles assay, in which extravasation of Evans Blue dye from vessels in the back skin of a mouse can be quantified. We found that ARP4 has a suppressive effect on VEGF-induced microvascular permeability (Fig. 6A). Quantitative analysis revealed that about 4.1-fold less leakage occurred in skin injected with both ARP4 and VEGF compared with skin injected with VEGF only (Fig. 6B). These results show that the presence of ARP4 led to a significant reduction in blood vessel permeability induced by VEGF.

**K14-ARP4 Tg Mice Suppressed Tumor Growth and Neovascularization in an Intradermal Grafted Tumor Model.** Several lines of *in vitro* experiments in this study characterized ARP4 as an antiangiogenic factor. Tg mice expressing a gene targeted to the epidermis under the control of keratinocyte-specific promoter K14 represent a powerful strategy to investigate whether a targeted gene affects angiogenesis (16, 23, 24). To further investigate the roles of ARP4 *in vivo*, we therefore generated K14-ARP4 Tg mice, which express ARP4 in basal epidermis (Fig. 7A). From 27 pups, we identified 3 Tg mice by PCR analysis (data not shown). Subsequently, we selected two independent K14-ARP4 Tg mouse lines expressing ARP4 in the skin as determined by Northern blot analysis (Fig. 7B). ARP4 transgene was expressed in the basal keratinocytes of the epidermis and the hair follicle outer sheath (Fig. 7C). K14-ARP4 Tg mice were grossly normal, and their skin color was also normal (Fig. 7, D and E). Histological analysis of the skin tissues of 3-month-old K14-ARP4 Tg mice showed no alteration in the thickness and morphology of both the epidermis and dermis (Fig. 7F). Fluorescein-labeled *L. esculentum* lectin staining revealed that the vessels in the ear skin of K14-ARP4 Tg mice resembled vessels in the ear skin of control mice in numbers, density, morphology, and size (Fig. 7, G and H). Based on these observations, these two lines were chosen for further study, and both showed essentially equivalent phenotypes.

We analyzed whether ARP4 from keratinocytes would suppress tumor growth using an intradermal CMT93 xenografted tumor model. Five weeks after intradermal CMT93 tumor cell inoculation, the size

---

**Fig. 7.** Generation and characterization of K14-ARP4 Tg mice. A, schematic representation of the transgene used to generate K14-ARP4 Tg mice. *K14* promoter, intron, and *pA* indicate the human K14 promoter, rabbit β-globin intron, and a polyadenylation signal derived from the K14 gene, respectively. *B*, expression of ARP4 mRNA in the skin of K14-ARP4 Tg mice was detected by Northern blot analysis. Arrowhead indicates the ARP4 transcript. Hybridization with a murine glyceraldehyde-3-phosphate dehydrogenase probe was used as a control. C, immunohistochemical analysis of K14-ARP4 Tg mice with anti-ARP4 antibody. Expression of ARP4 mRNA in the skin of K14-ARP4 Tg mice was detected by Northern blot analysis. Arrowhead indicates the ARP4 transcript. Hybridization with a murine glyceraldehyde-3-phosphate dehydrogenase probe was used as a control. D, expression of ARP4 mRNA in the skin of K14-ARP4 Tg mice was detected by Northern blot analysis. Arrowhead indicates the ARP4 transcript. Hybridization with a murine glyceraldehyde-3-phosphate dehydrogenase probe was used as a control.
Fig. 8. ARP4 Tg mice suppressed the growth of grafted CMT93 tumor cells intradermally. A, grafted grown tumor volume 5 weeks after intradermal inoculation of CMT93 tumor cells (1 × 10^7 cells) into K14-ARP4 Tg mice (n = 5). B, PECAM-1 staining of invaded capillary vessels into grafted tumors in K14-ARP4 Tg mice and control littermates. Control litttermates inoculated with CMT93 tumor cells had many blood vessels exhibiting large lumens. In contrast, K14-ARP4 Tg mice inoculated with CMT93 tumor cells had fewer vessels and smaller lumens. Scale bar, 50 μm. C, capillary blood vessel area in grafted tumors of K14-ARP4 Tg and control mice was measured using NIH Image software (n = 5). Bars represent the mean ± SD.

of tumors seen in K14-ARP4 Tg mice was significantly smaller than that seen in control littermates (Fig. 8A). We found that the number of invaded capillary vessels in K14-ARP4 Tg mice was markedly decreased and that capillary lumens were significantly smaller compared with those of control littermates by PECAM-1 immunostaining (Fig. 8, B and C). Taken together, these findings suggest that ARP4 suppresses tumor growth by its antiangiogenic activity.

**DISCUSSION**

ARP4 expression was recently found to be reduced in two gastric cancer cell lines (MKN28 and MKN74) in association with methylation of CpG islands in the 5' region of ARP4 (10). A reduced expression level of ARP4 was also observed in primary gastric cancer, but not in normal tissue (10). We have also detected a marked reduction of ARP4 expression in many tumor cells compared with normal tissues (data not shown). These findings indicate an inverse correlation between ARP4 expression and tumorigenesis. Recent findings also indicate that down-regulation of expression of antiangiogenic factors, such as maspin (25) and thrombospondin-1 (26), correlates with tumor progression and metastasis. Based on these observations, we hypothesized that ARP4 signaling might negatively regulate tumorigenesis. Indeed, we found that ARP4 inhibited the proliferation, chemotaxis, and tubule formation of endothelial cells, supporting such a role.

Furthermore, we found that ARP4 had a potential inhibitory effect on VEGF-induced vascular leakage in vivo. Because many tumor cells secrete VEGF, plasma leakage is a key feature of new vessels in tumorigenesis (27). This finding also indicates that ARP4 could negatively regulate angiogenesis in tumorigenesis. This antiangiogenic effect is similar to that of angiopoietin-1, which potentially reduces both VEGF- and inflammation-induced plasma leakage (16). Angiopoietins and ARPs have similar structures exhibiting coiled-coil domains and fibrinogen-like domains in which an essential binding site for Tie2 receptor exists. ANGPTL3 (7, 8), ARP1/angioarestin (9, 28), and ARP2 (28), which are ARP family members, act on the endothelial cells to regulate angiogenesis, although previous reports revealed that ARP family members in general bound to neither Tie1 nor Tie2 receptor. Therefore, it is necessary to identify the ARP4 receptor and determine not only where it is expressed but how it signals to address the antiangiogenic and antipermeability mechanism of ARP4. Such characterization should clarify how decreases in ARP4 expression could potentially lead to tumor formation and promote vascular leakage.

Our cloned ARP4 is identical to the previously reported FIAF (5) and PGAR (6) proteins. FIAF/PGAR is a PPAR target gene. Investigators of FIAF/PGAR propose that those proteins may play a role in regulating systemic lipid metabolism or glucose homeostasis. PPARγ is expressed in endothelial cells, and PPARγ ligands, inducers of FIAF/PGAR/ARP4 expression, are potent inhibitors of endothelial tube-like structures and proliferation in vitro and also suppress VEGF-induced angiogenesis in vivo (29). Moreover, PPARγ ligands induce endothelial cell apoptosis in vitro (30) and reduce the expression of metalloproteinases, such as matrix metalloproteinase 9 (31), which are implicated in tumor angiogenesis and invasion (32). Furthermore, PPARγ ligands can inhibit primary tumor growth and metastasis (33–35) through inhibition of their proliferation induced by growth factors or induction of apoptosis and fibrosis of injected tumor cells (32). In this study, we found significantly suppressed growth of grafted tumor cells and decreased numbers of invading blood capillary vessels in grafted tumors in the dermal layer of K14-ARP4 Tg mice relative to control littermates. Given that angiogenesis is essential for tumor growth, these findings also suggest that ARP4 may function in tumorigenesis as a negative modulator by suppressing tumor angiogenesis. During preparation of this manuscript, ANGPTL4, which is identical to ARP4, was reported to have a proangiogenic effect and to be induced under hypoxic conditions (36). Although we also confirmed that ARP4 mRNA was induced in endothelial cells under hypoxic conditions (data not shown), we could not find a proangiogenic effect of ARP4 by any in vitro experiments. Moreover, we generated Tg mice expressing ARP4 in the epidermis (K14-ARP4 Tg) to examine whether ARP4 acts in angiogenesis. Because both VEGF and Ang1 are proangiogenic factors, we therefore compared skin color and vasculature in the dermis of K14-ARP4 Tg mice with those of K14-VEGF and K14-Ang1 Tg mice (both gifts from Dr. George D. Yancopoulos; Regeneron Pharmaceuticals, Tarrytown, NY), which had shown angiogenic activity in their dermal layer (16). This investigation revealed no alteration in skin color or in the number and size of microvessels in the dermis of K14-ARP4 Tg mice compared with their controls, whereas both K14-VEGF and K14-Ang1 Tg mice were grossly red, and K14-VEGF Tg mice showed an increased number of microvessels, and K14-Ang1 Tg mice showed an enlarged size of microvessels in their dermis (Ref. 16; data not shown) as reported...
elsewhere. These findings suggest that ARP4 is not a proangiogenic factor, but an antiangiogenic factor. Critical to understanding the function of a novel ligand is identification and characterization of its cognate receptor. The fibroblastic-like domain at the COOH terminus of angiopoietins is a binding site for the Tie2 receptor. ARP4 also contains this domain, suggesting that ARP4 could be a ligand for either Tie1 or Tie2. However, we observed that, like other ARPs, ARP4 did not bind to either immobilized Tie1-Fc or Tie2-Fc protein by a BLAcore binding assay (data not shown). We need further investigation to identify the ARP4 receptor to understand the function of ARP4 in endothelial cells.

In summary, we demonstrate here that ARP4 inhibits proliferation, migration, and tube formation of endothelial cells in vitro and VEGF-induced angiogenesis and vascular leakage in vivo. Because several lines of evidence indicate that both angiogenesis and vascular leakiness play a crucial role in the process of growth of solid tumors and metastasis, we hypothesize that ARP4 might play an important role as a negative regulator in tumor angiogenesis. Neovascularization and plasma leakage are common key features of rheumatoid arthritis, diabetic retinopathy, and cancers. Our in vitro and in vivo functional analyses provide insight into the role of ARP4 in the vascular system as an antiangiogenic regulator. These findings suggest that ARP4 could act as a therapeutic agent not only in tumors but also in various pathologic conditions related to neovascularization.

ACKNOWLEDGMENTS

We thank T. Ota, T. Isogai, T. Keida, Y. Saito, and M. Kitamura for experimental assistance and Dr. Y. Suzuki for providing a full-length cDNA library.

REFERENCES

Inhibition of Angiogenesis and Vascular Leakiness by Angiopoietin-Related Protein 4

Yasuhiro Ito, Yuichi Oike, Kunio Yasunaga, et al.


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/63/20/6651

Cited articles

This article cites 35 articles, 21 of which you can access for free at:
http://cancerres.aacrjournals.org/content/63/20/6651.full.html#ref-list-1

Citing articles

This article has been cited by 41 HighWire-hosted articles. Access the articles at:
/content/63/20/6651.full.html#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.