A number of studies have identified a role for plasminogen activator inhibitor-1 (PAI-1) in regulating angiogenesis, although results from these investigations have been controversial. Among key cellular components of an angiogenic vessel are endothelial cells (ECs), which are known to express several components of the fibrinolytic system, including PAI-1. Thus, alterations in expression of this protein may have direct effects on cell functions involved in vascular development. In this study, ECs were isolated from sections of murine arterial trees from wild-type and PAI-1-deficient mice, and low passage (passes 3–4) homogeneous subpopulations of these cells were obtained by immunomagnetic absorption to antibodies against CD105/CD106. The homogeneity of these cells was further assessed by immunohistochemistry and quantitative real-time reverse transcription-PCR analysis of a number of EC markers. Comparative analyses of EC proliferation (one event associated with angiogenesis) in wild-type and PAI-1-deficient ECs demonstrated enhanced rates of cell growth for PAI-1-deficient cells relative to wild-type cells. Additional studies demonstrated similar levels of both vascular endothelial growth factor (VEGF) mRNA and protein and enhanced levels of VEGF receptor-1 (Flt-1) mRNA in PAI-1-deficient cells relative to wild-type cells. Immunohistochemical analyses indicated that phosphorylation of Akt was also enhanced in PAI-1-deficient cells, implicating VEGF-induced cell signaling alterations in PAI-1-deficient cells, the result of which may contribute to alterations in cell proliferation.

The development of transgenic and targeted gene-altered mice has allowed for the ability to identify and characterize the roles of individual genes in physiological and pathophysiological events. However, although much has been learned utilizing these mice, technological challenges arise due to the small size of the animal and limited tissue availability, which have hampered further cell-based in vitro investigations.

Recent studies have implicated a role for plasminogen activator inhibitor-1 (PAI-1) in angiogenesis. Our laboratory has demonstrated that host PAI-1 deficiency decreases angiogenesis in tumor- and non-tumor-based models (1, 2). Another study has demonstrated that human endothelial cell (EC) migration is stimulated by the urokinase-PAI-1 complex derived from endometrial stromal cells (3). Additionally, a lack of PAI-1 has been shown to correlate with diminished expression of vascular endothelial growth factor (VEGF) in the affected tissue (2, 4, 5). In one of these studies (5), aortic smooth muscle cells transfected with PAI-1 antisense RNA resulted in diminished expression of PAI-1 concomitant with a decrease in VEGF expression. This indicates that PAI-1 could potentially regulate, either directly or indirectly, the expression of the pro-angiogenic growth factor (VEGF). Additionally, increases in PAI-1 mRNA have been shown to be localized to cells at the edge of wounded bovine microvascular and aortic ECs and is inhibited by antibodies to basic fibroblast growth factor (bFGF) (6). In contrast, PAI-1 has been shown to inhibit bFGF-induced angiogenesis in a chicken chorioallantoic membrane assay (7). One study utilizing adenoviral delivery of two mutant forms of PAI-1 to PAI-1-deficient (PAI-1+/−) mice demonstrated that PAI-1 facilitates tumor angiogenesis by inhibiting proteolytic activity rather than by interacting with vitronectin, indicating that excess plasmin proteolysis prevents the stable assembly of tumor vessels (8). However, other investigations utilizing PAI-1 variants ascribed this activity to both antiprotease and vitronectin binding functions (7). Interestingly, in vitro studies utilizing aortic rings from PAI-1+/− mice cultured in collagen or Matrigel demonstrated enhanced capillary sprouting (9). Therefore, the function of PAI-1 in neovessel formation remains controversial.

We believed that in vitro studies of specific properties of isolated ECs related to angiogenesis would be valuable in identifying functions of PAI-1 in this process. This work presents a characterization of such cells isolated from wild-type and PAI-1+/− aortas and compares their growth factor-induced proliferative capacities as one step in the angiogenic process. In this study, low passage ECs were isolated from the thoracic aortas of WT and PAI-1+/− mice, and the comparative proliferative properties of these cells and potential mechanisms are described herein.
FIG. 1. Frequency histograms of WT ECs stained with calcein and labeled with a rat antibody to CD105 (a and b) or CD106 (c and d), followed by Alexa 647-conjugated goat anti-rat antibody. A gate was set on calcein-positive cells (live cells; a and c), and cells positive for CD105 (b) or CD106 (d) within the gated calcein-positive population were calculated utilizing the Agilent Technologies Model 2100 bioanalyzer software program. A negative control (minus primary antibody (green peak)) (e) demonstrated that secondary antibody was not associated with the gated calcein-positive population of cells. Blue peak represents addition of primary antibody.
EXPERIMENTAL PROCEDURES

Mice

PAI-1−/− mice have been described previously (10, 11). These mice have been backcrossed at least seven generations (F7) into the C57BL/6J strain. WT C57BL/6J (>F7) mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were used as controls. All animals were housed in micro-isolation cages on a 12-h light/dark cycle with controlled temperature and humidity and had access to food and water ad libitum. Male mice between 8 and 12 weeks of age were exclusively employed for this work.

Harvesting of Aortic Trees

WT and PAI-1−/− male mice (six mice/genotype/preparation) were anesthetized intraperitoneally with a rodent mixture of 0.015 mg of xylazine, 0.075 mg of ketamine, and 0.0025 mg acepromazine/eq of body weight. An incision was made into the peritoneal cavity and diaphragm. A bilateral thoracotomy was then performed to expose the lungs and heart. An incision was made into the right atrium, and the animal was perfused through the left ventricle with sterile saline. The rib cage was then cut at the base of the throat to expose the thymus, which was removed to visualize the aortic arch. Fat was excised from the aortic tree starting at the arch and then working posteriorly. The aortic tree was freed from attachment to the heart, carotids, and subclavian artery and, in the periphery, the aortic arch, from the kidneys and iliac branches. The aortic tree was then placed in sterile saline to remove debris and blood and transferred to complete medium (RPMI 1640 medium (Sigma), 20% fetal bovine serum (Mediatech, Inc., Herndon, VA), 50 µg/ml endothelial growth factor supplement (BD Biosciences), 2 mM l-glutamine (BioWhittaker, Inc., Walkersville, MD), 1% antibiotic/antimyotic mixture (100 units of penicillin, 0.1 mg of streptomycin, 0.25 µg of amphotericin B; Sigma), 0.1 mM amino acids (Invitrogen), and 1 µl/ml β-mercaptoethanol (Invitrogen) and kept on ice until further processing.

Isolation of ECs from Aortic Tissue

The initial harvesting of ECs was as described previously (12) with the exception that the cells were maintained on a collagen-coated surface at all times due to the potential effects of plastic and other surfaces on the trans-differentiation of these cells (13). For the initial plating, a collagen mixture was prepared by the addition of 9 ml of collagen type I rat tail (1 mg/ml; BD Biosciences) to 1 ml of sterile 0.1× NaOH, 3.5 ml of 0.8× Dulbecco’s modified Eagle’s medium, and 2.25 ml of 1× Dulbecco’s modified Eagle’s medium. An aliquot of 1 ml was added to each well of a 12-well plate and allowed to gel at 37 °C for 60 min. The gel was then equilibrated overnight with 0.5–1.0 ml of complete medium, and the medium was aspirated from the gel. The aorta was cut into 10 pieces coronally, washed with culture medium, and then opened longitudinally to assure that all blood was removed. Each segment was then positioned lumen side down onto the gel (one segment/well) and placed in a humidified 5% CO2 incubator at 37 °C for 36 h. One ml of medium was applied per well. After 7–10 days, once visible outgrowth from the tissue was observed, the aortic segment and medium were removed. One ml of a 0.3% solution of collagenase I (Invitrogen) was then added to the attached cells and incubated at 37 °C for 30 min. The solution containing the cells was centrifuged, and the cells were reseeded onto a 100-mm collagen-coated plate containing 10 ml of complete medium.

When cells were confluent, the medium was removed, and the cells were washed with sterile phosphate-buffered saline (PBS). Four ml of 0.3% collagenase I was added to the cells and incubated at 37 °C for 30–40 min. The cells were then diluted with the medium, centrifuged, and washed with PBS containing 1% fetal bovine serum (FBS). A 30-µm pre-separation filter (MACS, Miltenyi Biotech GmbH) was washed three times with the same buffer. The cells were resuspended in 0.5 ml of PBS containing 1% FBS and passed through the pre-separation filter to remove clumps. The filter was washed twice. The cells were then centrifuged, and 0.3 ml of biotin-free diluent Fe receptor blocker (Accurate Chemical & Scientific Corp., Westbury, NY) was added to 105 cells and incubated on ice for 30 min. The cells were then washed twice with PBS containing 1% FBS. Rat anti-mouse CD105 and CD106 antibodies (10 µg/ml final concentration for both; both from Pharmingen) were added to the cells, and the volume was adjusted to 50 µl with RPMI 1640 medium. The cells were incubated on ice for 1 h, washed first with RPMI medium and then with PBS/FBS, and resuspended in an equal volume of PBS/FBS and MACS goat anti-rat IgG microbeads (100 µl of bead suspension/105 cells). The cells and beads were incubated at 4 °C for 30 min. During the incubation, the MiniMACS separation unit was attached to the MACS multistand, and the MACS separation column was placed in the MiniMACS separation unit. The column was washed twice with 0.5 ml of PBS and 1% FBS. The cells were then washed once with PBS and 1% FBS, resuspended in degassed PBS/FBS, and loaded onto the column. Cells that were positive for CD105 and CD106 were retained on the column, whereas cells that were negative for these two EC markers passed through the column. The column was then removed from the separator, and the cells were flushed using a plunger supplied with the column. Cells were centrifuged, resuspended in complete medium, and plated onto 100-mm collagen-coated plates in 10 ml of complete medium.

Confirmation of Homogeneity of ECs

When confluent, cells were released from the collagen matrix by incubation with 0.3% collagenase I and recounted. An aliquot of 1×105 cells was transferred to a 1.5-ml amber microcentrifuge tube and washed twice with dye loading buffer containing Hank’s balanced saline solution (Invitrogen), 20 mM Hepes (Sigma), and 1% bovine serum albumin (BSA) (Sigma). Cells were stained with 100 µl of 0.5 µM calcein (Molecular Probes, Inc., Eugene, OR) for 15 min at 37 °C in the dark and then washed twice with 1 ml of staining buffer containing PBS, 2% BSA, and 0.05% NaN3 (ACROS Organics, Pittsburgh, PA). The cells were stained with either rat anti-mouse CD105 or CD106 antibody (10 µg/ml) in staining buffer for 1 h on ice. They were then washed twice with staining buffer and labeled with 2 µg/ml Alexa 647-conjugated goat anti-rat antibody (Molecular Probes, Inc.) in 100 µl of staining buffer, followed by incubation on ice for 30 min in the dark. The cells were washed twice with staining buffer and resuspended at 2×105 cells/ml in cell buffer (cell assay reagents, Agilent Technologies, Palo Alto, CA). A cell assay LabChip (Agilent Technologies) was primed with priming solution (Agilent Technologies), after which 10 µl of the cell suspension (20,000 cells) was added to one of six channels. A focusing dye was applied to another chamber, which acted as a reference for the optical detection system. The ship was then placed in an Agilent Technologies Model 2100 bioanalyzer, which utilizes a vacuum to move cells, single file, through the microfluidic channels past a fluorescent detector. Fluorescent emission from the cells was detected at 510–540 and 674–696 nm. Typically, 500–1000 events/240 s are recorded. Fluorescent events were plotted against the fluorescent intensity (frequency

FIG. 2. Shown is the immunohistochemical staining of WT cells for EC markers: CD105 (a), CD106 (c), CD105 and CD106 (d), and EPCR (e). Negative controls were carried out in the absence of primary antibody to CD105 or CD106 (b) or EPCR (f). Magnification is ×100.
The TaqMan probe consists of an oligonucleotide with a 5′-reporter dye (6-carboxyfluorescein) and a 3′-quencher dye (6-carboxytetramethylrhodamine). The sequences of the primers or probes for the mouse are shown in addition to their gene accession numbers and amplicon sizes. HPRT, hypoxanthine phosphoribosyltransferase.

| Accession no. | Primer/probe Sequence (5′→3′) | Amplicon size |
|---------------|--------------------------------|---------------|
| L39017        | EPCR forward                   | AAAGGACTCTGAAGCATTGTTG | 83 |
|               | EPCR reverse                   | CCAAGTCTATGCTGTATATCCC | |
|               | EPCR probe (antisense)         | CCACTCTTCAGAAGCCTACAAAGCA | |
| NM_011345     | E-selectin forward             | GAAACCTCTCCTGGAACATCT | 108 |
|               | E-selectin reverse             | TGATTTGAGGCGTTGGACAGC | |
|               | E-selectin probe               | CATGGCTCTGCTGTGTACGATACTTTCGGAAGGAA | 77 |
| D8890         | Flt-1 (VEGFR-1) forward        | CTACCTTTACGGCCCAAGAGGA | 81 |
|               | Flt-1 (VEGFR-1) reverse        | GCCACTTGGCGCTTCTTATAC | |
| NM_010612     | KDR (VEGFR-2) forward          | CGCTCTCCCAAATAAGGTTGACAT | |
|               | KDR (VEGFR-2) reverse          | TGACCCTTACGGCCCACTTGG | |
| NM_010493     | ICAM forward                   | GAGGATGCGCGGAGAAGTT | 185 |
|               | ICAM reverse                   | AGTCCTCCCTGGCCAAAGCA | |
| M33960        | PAI-1 forward                  | GACACCCCTAGCTGTTCCATC | 218 |
|               | PAI-1 reverse                  | GAGGTTGCACTAACCACATGCA | |
|               | PAI-1 probe                    | TCTCTGCTAAGTCTCCTGGAAGACTGAG | |
| NM_008873     | uPA forward                    | CGTGTGCTCTGCCAGGTTTAG | |
|               | uPA reverse                    | ACCACATTAGGAAGAACGCCTCTTCTCCT | 105 |
| NM_011113     | uPAR forward                   | CGAGAGCAAGAAGAGGCTTTGA | |
|               | uPAR reverse                   | CTTGACGGCTCTGGCTATGGA | |
|               | uPAR probe                     | CTCTGGACGGCCAGGCCAGC | |
| BC029823      | VCAM forward                   | ACCAGGCGCTGAATTAGGACCA | 101 |
|               | VCAM reverse                   | CACCTCTTGGATCCTTGAATGGA | |
| S7052         | VEGF common forward            | AACGATGAGCAAGCTCCTGG | 236 |
|               | VEGF<sub>164</sub> reverse    | TGAGAGGCTCTGCTGGCAAGCA | |
|               | VEGF<sub>201</sub> reverse    | GCACAACTGCTCTTCCAG | 205 |
|               | VEGF<sub>201</sub> reverse    | GCTGTAAGGATCCTACCTCTCCTGATG | 251 |
| NM_013556     | HPRT forward                   | ACCTTGGCTGGAAGAGGAC | 189 |
|               | HPRT reverse                   | CAACCTTGGCTCTGGCTATGGA | |
|               | HPRT probe (antisense)         | CAACAAATGCTGGCTTGGTATCCACAC | |
| NM_008084     | GAPDH forward                  | TGACACACCACACCTGCTTGG | 177 |
|               | GAPDH reverse                  | GAGTGCGGGAAGATGCTTGC | |
|               | GAPDH probe                    | GAGAAGACTCTGAGTGGTACCCCTC | |

Expression and Quantitation of EC Marker Transcripts

**Extraction of RNA**—Total RNA was extracted from WT and PAI-1−/− murine ECs in culture using the QIAGEN RNeasy mini-extraction kit. RNA was then treated with DNase I according to the manufacturer’s instructions.

**Design and Construction of Primers/Probes for Real-time Quantitative Reverse Transcription (RT)-PCR**—PCR primers and probes for EPCR, E-selectin, intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), VEGF isomers (VEGF<sub>164</sub>, VEGF<sub>201</sub>, and VEGF<sub>201</sub>-untranslated region if multiple isoforms of a gene existed, i.e. VEGF). HPRT or GAPDH genes were used as housekeeping genes for purposes of data normalization. The specificity of the primer set was first tested by RT-PCR and melting curve analysis using the ABI PRISM<sup>™</sup> 7700 sequence detector. Subsequently, each PCR product was analyzed by gel electrophoresis; and if a single product of the predicted molecular size was observed, the corresponding probe was then synthesized. Primers were also analyzed in BLAST searches to ensure specificity for the gene of interest.

**RT-PCR**—RT and PCR were carried out using the LightCycler Fast-Start DNA Master hybridization probe kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. A coupled RT-PCR was performed using TaqMan chemistry in the Roche LightCycler instrument. The principles of TaqMan PCR have been reported (14). Each reaction contained 100 ng of total RNA, 2 μl of 10× Master mixture (dNTP mixture with dUTP instead of dTTP), 6 mM MgCl₂, 50 nM each primer, 100 nM probe, 16 units of RNase inhibitor.
(Promega, Madison, WI), 2 units of Multiscribe reverse transcriptase (Applied Biosystems Inc.), and diethyl pyrocarbonate-treated water to a final volume of 20 μl. Thermocycling conditions were as follows: 48 °C for 30 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 1 min, with fluorescent readings at the end of each cycle. The amount of mRNA detected was quantified against individual standard curves. All samples were run in triplicate.

Normalization of TaqMan PCR Results—The housekeeping genes HPRT and GAPDH were used to normalize input RNA for each reaction. The results were extrapolated from standard curves and are expressed as a ratio of the total RNA of the gene of interest to that of HPRT or GAPDH ± S.E.

Quantitation of VEGF in WT and PAI-1−/− EC-conditioned Media

RT-PCR results for VEGF transcripts were validated by detection of VEGF protein in cell culture media collected from plates and passed through a 0.22-μm filter prior to RNA extraction. The Quantikine® M enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) was used, and the assay was performed in triplicate. The results were compared with a standard curve utilizing murine recombinant VEGF (7.8–500 pg/ml).

Immunohistochemical Staining for Akt and Phosphorylated Akt

An aliquot of 2 × 10^5 ECs suspended in complete RPMI 1640 medium was plated onto collagen (1 mg/ml)-coated multiwell chambered cover-slips (Molecular Probes, Inc.). The cells were incubated overnight at 37 °C in a humidified 5% CO2 incubator. The next day, the medium was removed, and the cells were gently washed with complete medium without EC growth supplement. At various times (0, 24, 48, 72, and 96 h), the cells were rinsed twice with 1× PBS and then fixed with 4% paraformaldehyde in PBS for 10 min, followed by two rinses with PBS. The cells were treated with Peroxoblock (Zymed Laboratories Inc.) for 45 s to quench endogenous peroxidase activity and then permeabilized by treating cells with Tris-buffered saline (TBS) and 0.1% Triton for 30 min. Cells were washed and treated with normal swine serum (diluted 1:5 in TBS/BSA; Dako Corp., Carpinteria, CA) for 30 min to block nonspecific antibody interactions. The cells were then incubated with rabbit anti-phospho-Ser473 Akt primary antibody (diluted 1:100; Cell Signaling Technology, Beverly, MA) or primary antibody treated with a phospho-Ser473 Akt-blocking peptide (Cell Signaling Technology) as a control for specificity of the antibody/antigen interaction. The cells were incubated overnight at room temperature in a humidified chamber. The cells were then washed three times for 5 min and treated with swine anti-rabbit biotin F(ab)2 IgG (diluted 1:500 in TBS/BSA) for 30 min. The cells were washed three times for 5 min and incubated with horseradish peroxidase-conjugated streptavidin (diluted 1:50) for 30 min. After washing three times for 5 min, the chromogen Nova Red was used to visualize positive antibody interactions. The cells were then washed three times for 5 min. For counterstaining, the cells were rinsed six times with double-distilled water and stained with hematoxylin QS for 30 s and then rinsed six times with double-distilled water. The cells were allowed to dry overnight at room temperature and coverslipped with permanent mounting medium.

Synthesis and Purification of the SP5.2 Peptide

The SP5.2 peptide (NGYEIEWYSWVTHGMY) was synthesized using a standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) protocol and purified from crude product by high performance liquid chromatography on a Vydac C18 column using 22–50% water/acetonitrile (0.1% trifluoroacetic acid) for 40 min at a flow rate of 3 ml/min. The mass of the peptide (2035 Da) was verified by electrospray mass spectrometry using the micromass Quattro LC (University of Notre Dame Mass Spectrometry Facility).

**FIG. 3.** Quantitative RT-PCR of transcripts for EC markers from mRNA of confluent WT cells. The amount of mRNA detected was extrapolated from a standard curve, and the results are expressed as a ratio of the total RNA of the gene of interest to the total RNA of the HPRT housekeeping gene ± S.E.

**FIG. 4.** Quantitative RT-PCR of transcripts of VEGF-A isoforms and VEGFR-1 (Flt-1) in WT and PAI-1−/− ECs during cell proliferation. The amount of mRNA detected was extrapolated from a standard curve, and the results are expressed as a ratio of the total RNA of the gene of interest to the total RNA of the GAPDH housekeeping gene ± S.E. White bars, WT ECs; gray bars, PAI-1−/− ECs.
Western Blotting of Cell Extracts for Akt and Phosphorylated Akt

Cells at 0, 24, 48, 72, and 96 h during proliferation were washed with PBS and then harvested from the collagen-coated plates with collagenase. For some studies, the SPS.2 peptide (10 μM final concentration), which blocks VEGF/Pt−1 interaction and downstream effects (15), was added to the medium at 48 h, and cells were then collected 24 h later. The cells were lysed in buffer containing 1% Igepal CA-630, 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 250 mM NaF, 200 mM β-glycerophosphate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride for 20 min at 4 °C, and the debris was removed by centrifugation at 13,000 × g for 10 min. Protein concentration was determined by the micro-BCA protein assay (Pierce), and 50 μg of total protein was loaded onto a 10% SDS-polyacrylamide gel. The protein was then transferred to a polyvinylidene difluoride membrane (Osmonics, Inc., Minnetonka, MN) and washed with TBS at room temperature for 5 min. The membrane was then incubated in blocking buffer (TBS, 0.1% Tween 20, and 5% nonfat milk powder) for 1 h at room temperature and incubated with either rabbit anti-Akt primary antibody or rabbit anti-phospho-Ser473 Akt antibody, both of which were previously diluted 1:1000 times in buffer containing 1× TBS, 0.1% Tween 20, and 5% BSA. The membrane was incubated overnight at 4 °C with gentle agitation and washed three times for 5 min with TBS containing 0.1% Tween 20. It was then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad) diluted 1:2000 in blocking buffer for 1 h with gentle agitation. After this, the membrane was washed three times with TBS containing 0.1% Tween 20 for 5 min, and the proteins were detected using the Western Lightning chemiluminescence reagents (PerkinElmer Life Sciences). For densitometric analyses of Akt, Bioquant NOVA markers (Bio-Rad) were also used to determine the molecular mass of the Akt protein. For densitometric analyses of Akt, Bioquant NOVA software (Version 4.00.8, R&M Biometrics, Inc., Nashville, TN) was used to measure the density of the signals in the autoradiograms. Integrated optical densities were calculated using the green channel after background correction was applied.

Cell Proliferation Assays

Cell proliferation was measured by manual counts. Cells were suspended at 2 × 105 cells/ml in complete medium with EC growth factor supplement. An aliquot of 1 ml was added to collagen-coated 24-well plates. The cells were then incubated for 24 h at 37 °C in a humidified 5% CO2 incubator. After this time, the medium was removed, and fresh complete medium without EC growth factor supplement (±5 ng/ml human recombinant bFGF (BD Biosciences) or 10 ng/ml human recombinant VEGF (Antigenix America, Inc., Huntington Station, NY)) was added to the adherent cells. The plates were then incubated for 24, 48, 72, and 96 h at 37 °C in a humidified 5% CO2 incubator. Manual cell counts were performed at 24, 48, 72, and 96 h. For this, the medium was removed, and the wells were rinsed with PBS, fixed with 4% paraformaldehyde, and then stained with hematoxylin. The cells were viewed with a Nikon E600 microscope, and images were recorded. Cell counting was performed in triplicate/time/point/genotype (three fields/well) utilizing the Bioquant Nova software program. Data are expressed as average number of cells/field ± S.E. Two separate batches of EC preparations/genotype were analyzed.

RESULTS

Isolation of Aortic ECs and Confirmation of Homogeneity—Six aortic trees were processed per batch of EC preparation. After the initial plating of cells onto 100-mm plates, where one plate represents one aortic tree, typically 20 × 10⁶ cells were collected per six aortic trees prior to purification on the immunomagnetic beads. After purification, ~10% of the cells were recovered and were positive for CD105 and CD106. They were then replated onto 100-mm plates at an initial cell density of 0.5 × 10⁶ cells/plate.

Utilizing Agilent technology to confirm homogeneity of the cell population and antibodies to CD105 and CD106, >3000 fluorescent events were recorded. Fluorescent events were plotted against the fluorescent intensity and graphed as a frequency histogram (Fig. 1). A gate was then set on the calcein-positive cells (live cells), and the percent positive for the CD markers indicated that >95% were positive for CD105 (Fig. 1, a and b) and CD106 (Fig. 1, c and d). A negative control study (no primary antibody) was performed to confirm specificity (Fig. 1e).

The purity of the cells was further confirmed by manual immunohistochemistry with cells plated on collagen-coated Oncyte slides co-cultured with cells on the 100-mm plates. Greater than 95% homogeneity was confirmed utilizing antibodies to CD105 (Fig. 2a), CD106 (Fig. 2c), CD105 + CD106 (Fig. 2d), and EPCR (Fig. 2e). Control studies were performed in the absence of primary antibody (Fig. 2, b and f).

Quantitation of mRNA Transcripts for EC Markers from WT Mice—The primers and probes used for RT-PCR studies are listed in Table I. As expected for ECs, mRNAs for VCAM,
E-selectin, EPCR, ICAM, PAI-1, and VEGFR-1 (Flt-1) were all observed in RNA extracted from cultured WT ECs (Fig. 3). Values are expressed relative to the housekeeping gene HPRT and plotted in log fashion. Individual results were averaged and are expressed as the mean ± S.E.

Quantitation of mRNA for VEGF Isoforms and the VEGF Receptor Flt-1 in ECs from WT and PAI-1−/− Mice—mRNA transcripts of VEGF isoforms and Flt-1 were identified in cells from both WT and PAI-1−/− mice, prepared from three separate batches of cells/genotype. The mRNA levels of VEGF120, VEGF164, and VEGF186 were similar in cells from PAI-1−/− mice relative to WT cells (Fig. 4) at all time points studied. The levels decreased as a function of time as the cells became confluent. In contrast, the mRNA levels of Flt-1 increased as a function of time, and quantitative differences in transcript levels were observed in cells from WT and PAI-1−/− mice (Fig. 4). The mRNA levels of Flt-1 were significantly enhanced (p < 0.05) at all time points (except at t = 0) in PAI-1−/− cells relative to WT cells.

Quantitation of VEGF Protein in Media from Cultured ECs—Enzyme-linked immunosorbent assay of culture media from WT and PAI-1−/− ECs before RNA extraction identified VEGF in the media (Fig. 5), and its level increased as the cells approached confluency. No differences in the level of this protein in the culture media were observed in WT and PAI-1−/− ECs. Quantitation of mRNA for uPA and uPAR—mRNA transcripts of uPA and uPAR were identified in cells from both WT and PAI-1−/− mice, prepared from three separate batches of cells/genotype. The mRNA levels of uPA in PAI-1−/− cells was significantly enhanced relative to that in WT cells at 48 and 72 h during cell proliferation (Fig. 6a), whereas the mRNA levels of uPAR were similar during these time points (Fig. 6b).

Western Blot Analyses and Immunostaining of Phosphorylated Akt—Western blot and densitometric analyses of Akt and phosphorylated Akt in cell extracts of WT and PAI-1−/− ECs during proliferation indicated enhanced expression of Akt at 72 and 96 h in WT cells relative to PAI-1−/− cells (Fig. 7a). However, at both 72 and 96 h, the phosphorylated Akt/total Akt protein ratio was greater in PAI-1−/− cells relative to WT cells (0.09 for PAI-1−/− cells versus 0.06 for WT cells at 72 h and 1.53 for PAI-1−/− cells versus 0.12 for WT cells at 96 h). This indicates that, during proliferation, there is enhanced activation of this signaling pathway in PAI-1−/− ECs relative to WT cells. Immunohistochemical analysis of cells supported the results from Western analyses and indicated that phosphorylated Akt was enhanced in PAI-1−/− cells relative to WT cells; this was visually apparent at 72 h, but became more apparent at 96 h (Fig. 8). However, when cells were incubated with the VEGF/Flt-1-blocking peptide SP5.2, phosphorylation of Akt in PAI-1−/− ECs was significantly diminished compared with similarly treated WT cells (densitometric ratios for phosphorylated Akt/total Akt protein were 0.038 for PAI-1−/− cells and 0.11 for WT cells) (Fig. 7b).

WT and PAI-1−/− EC proliferation—Two separate cell preparations/genotype representing 12 mice/genotype were used to determine the effects of a PAI-1 deficiency on EC proliferation. With the exception of some of the data at 24 h with and without the addition of growth factors and at 48 h after the addition of bFGF, ECs from PAI-1-deficient mice exhibited a statistically significant enhanced growth rate relative to WT mice (Fig. 9). As an example, at 96 h after stimulation with exogenous VEGF, there were, on average, 235 ± 13.30 cells/field for PAI-1−/− cells versus 176 ± 12.68 cells/field for WT cells (p = 0.0096).

DISCUSSION

Angiogenesis involves the balanced orchestration of a complex network of cooperative interactions between cells, soluble factors, and extracellular matrix components that lead to the development of a mature vessel. There are five distinct steps involved in this process: 1) dissolution of surrounding basement membranes by proteases, 2) migration, 3) proliferation, 4) tube formation, and 5) maturation and stabilization of the vessel (16). A number of angiogenic inducers and inhibitors have been identified (16). The two most potent inducers, bFGF and VEGF-A, can stimulate independently an angiogenic cascade both in vitro and in vivo (17). Four FGFR receptors and three VEGF receptors, all tyrosine kinases, have been identified in ECs (16, 18). These ligand/receptor interactions lead to the activation of intracellular signaling cascades that facilitate alteration of the transcription of a number of genes involved in cell functions such as cell proliferation, apoptosis, and migration (19–24).

This study utilized ECs of low passage number from WT C57BL/6J mice and C57BL/6J mice deficient in PAI-1 to determine the effect of this deficiency on a critical component of the angiogenic process, cell proliferation. The homogeneity of the cells was confirmed by identification of EC markers and transcripts of EC-derived proteins.

VEGF isoform transcripts were identified in both WT and PAI-1−/− cells, and the levels diminished as the cells approached confluency. There were no significant differences in transcript levels in WT and PAI-1−/− cells. A number of in vivo studies have demonstrated a decrease in VEGF expression in the absence of PAI-1 (1, 4, 5). Although the results from the current in vitro study appear to contradict these in vivo observations, other soluble factors, cell types, and extracellular matrix proteins present in vivo may regulate the effect of PAI-1 on
VEGF expression. The transcript level of the VEGF receptor Flt-1 increased over time, consistent with the increase observed in the culture supernatant of VEGF protein in both WT and PAI-1/−/− cells, and differences in levels in the two genetically distinct cells were apparent. Flt-1 is believed to act as a sink for VEGF, potentially down-regulating its proliferative effect as cells approach a confluent state (25, 26). However, a recent study utilizing a peptide with high and specific affinity for Flt-1 demonstrated anti-angiogenic activity in vivo, including effects on EC proliferation (15). In the current study, no transcripts of VEGFR-2 (KDR), a receptor implicated in regulating VEGF effects on cell signaling (24) and therefore cell function, were observed at any time point in both WT and PAI-1/−/− ECs. Additionally, Akt phosphorylation was increased in PAI-1/−/− cells concomitant with an increase in the transcript levels of Flt-1. Other studies utilizing activated hepatic stellate cells have demonstrated a role for Flt-1 in Akt phosphorylation that is ERK/MAPK-dependent (27). Interestingly, in our study, incubation of cells with a VEGF/Flt-1-blocking peptide more effectively diminished phosphorylation of Akt in PAI-1/−/− cells relative to WT cells, indicating that phosphorylation of Akt in PAI-1/−/− ECs is more dependent on induction by VEGF/Flt-1 interaction than that in WT cells and may explain the observed enhanced mRNA levels of Flt-1 in PAI-1/−/− ECs. This could potentially indicate that phosphorylation of Akt in WT ECs is facilitated to a greater extent by induction by other growth factors or cytokines.

The mRNA levels of uPA were significantly increased (p ≤ 0.05) in PAI-1/−/− cells at 48 h and 72 h relative to WT cells. Studies in glioblastoma cells transfected with antisense uPA demonstrated diminished phosphorylation of Akt with downstream effects on cell functions (28). Because mRNA analyses of uPA in WT and PAI-1/−/− cells indicated an increase in transcript levels in PAI-1/−/− cells relative to WT cells, a PAI-1 deficiency may have an indirect effect on phosphorylation of Akt by regulating uPA levels.

Cell proliferation was significantly increased in PAI-1/−/−-derived cells with or without the addition of exogenous growth factors relative to WT cells. An imbalance of any of the events associated with the angiogenic response may have an impact on the development of a stable, well-organized vessel; and thus, this in vitro observation may be relevant to observed in vivo phenotypes where neovascularization is attenuated in some challenge models in PAI-1/−/− mice (1, 2, 8, 29). Additionally, differences in in vitro proliferation in these two genotypically distinct cells are not due to differences in expression of VEGF as determined from both mRNA and protein analyses. Unencumbered activation of the fibrinolytic system could potentially affect EC proliferation through activation of growth factors and cell signaling events involved in cell proliferation (30, 31). Additional studies in serum-free medium demonstrated a similar enhanced proliferation of PAI-1/−/− ECs relative to WT cells, indicating that serum components, i.e. plasminogen, are not involved in facilitating this difference (data not shown).

The results from this study demonstrate alterations in the rate of cell proliferation in PAI-1/−/− ECs that can have profound effects on vessel formation and stability. The enhanced growth rate observed in PAI-1/−/− ECs appears to be mediated by enhanced activation of the Akt signaling pathway in these cells.

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Fig. 9. WT and PAI-1/−/− EC proliferation with and without the addition of exogenous bFGF or VEGF. Samples were assayed in triplicate wells/time point/genotype/culture condition, and three fields were counted per well. Cell counts were performed utilizing the Biocount Nova software program. P values between WT and PAI-1/−/− mice at each time point under each growth condition were <0.05 except for at 24 h (with and without bFGF) and at 48 h in the presence of bFGF. Data are expressed as the average number of cells/field ± S.E. of two separate batches of cells (three wells/time point/batch).
Proliferation of PAI-1−/− Endothelial Cells

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