Both Protein Activation and Gene Expression Are Involved in Early Vascular Tube Formation *in Vitro*

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ABSTRACT

Purpose: **Gene expression and protein translation regulate and direct endothelial cell proliferation and differentiation. We initiated an unbiased global search for transcriptional changes occurring during endothelial cell vascular differentiation *in vitro*, focusing on genes not previously implicated in vascularization and angiogenesis.**

Experimental Design: **cDNA and protein from human umbilical vein endothelial cells forming vascular tubes on the basement membrane surrogate, Matrigel, were collected and subjected to a global unbiased search for alterations in expression of genes not previously linked to angiogenesis.**

Results: **Transcriptional inhibitors blocked vascular tube formation only when present within the first hour of incubation ($P < 0.05$). cDNA array analysis yielded 31 differentially regulated transcripts (of 5100 queried; false positive rate, 0.4%) from gene classes representing transcription, translational regulation, cell structure, and cell adhesion. mRNA levels of caldesmon, a cytoskeleton-associated protein not previously linked to angiogenesis, were markedly reduced during early tube formation. Caldesmon protein quantity was also markedly decreased as demonstrated by laser capture microdissection of tubule cells followed by immunoblotting. Strikingly, no significant changes in transcription of genes previously demonstrated to contribute to angiogenesis, invasion, or signal transduction contained on the array were observed. To investigate the possibility that posttranslational rather than transcriptional changes were involved in facilitating tube formation, we evaluated the activation status of two dominant signal pathways, RAS/mitogen-activated protein kinase and phosphatidylinositol 3-kinase/AKT. A net 3-fold reduction in phospho-AKT and a 4-fold reduction in phospho-extracellular signal-regulated kinase-1/2 occurred in a transcription-independent fashion.**

Conclusions: **These data suggest that both changes in gene expression and transcription-independent activation of signal transduction pathways may be involved in vascular tube formation. A combination of transcriptional and proteomic analysis has the potential to identify novel transcription-dependent and -independent molecular targets of angiogenesis.**

INTRODUCTION

Angiogenesis, the formation of new blood vessels from existing vasculature, occurs physiologically in processes such as wound healing, embryogenesis, and the ovulatory cycle, and in pathological states such as rheumatoid arthritis, diabetic retinopathy, and cancer progression. Angiogenesis occurs as a multistep process in which transcription and signal pathway activation are necessary for endothelial cell structural reorganization, acquisition of the invasive phenotype, proliferation, and cannulization of vascular sprouts (1–3). Despite knowledge of the basic steps involved in angiogenesis, further elucidation of its underlying molecular and cell signaling events is required both to facilitate wound healing in normal states and to develop effective interventions in pathophysiological conditions. We hypothesized that an unbiased global analysis of both transcriptional and posttranslational alterations occurring during endothelial cell remodeling into vascular tubules would identify novel mediators and processes. Dissection of these events can uncover molecular targets through which to regulate angiogenesis.

In vitro models of angiogenesis offer a controlled environment through which to test this hypothesis. The Matrigel matrix, like the *in vivo* basement membrane, furnishes a rich environment to promote tubulogenesis (4). It consists of a mixture of basement membrane proteins, proteoglycans, and enmeshed growth factors such as tumor growth factor-β, platelet-derived growth factor, insulin-like growth factor-1, and nerve growth factor (5). Electron microscopy demonstrates that the tubular structures formed by endothelial cells in this model are vascular-like structures containing lumens (4). This provides a model of *in vitro* differentiation for the molecular dissection of tubule formation.

Gene microarray and advanced protein technologies allow global, unbiased evaluation of a broad number of genes and protein expression and posttranslational modification simultaneously and, thus, can make the process of studying gene and protein expression more efficient, as well as providing the ability to establish unexpected links between transcriptional regulation and activation of signal transduction pathways. To provide proof of concept, we applied cDNA microarray and protein analyses in a pilot study to target a limited number of candidate genes and proteins for identification of potential targets that modulate endothelial cell differentiation into vascular

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tube structures in the Matrigel model. We now report that >30 genes/ESTs[2] not previously implicated in vascular differentiation were differentially expressed as a consequence of transcription at least 5-fold during the first hour of endothelial cell organization on Matrigel matrix. The expression of the limited number of known angiogenesis-related and cell-signaling genes present on the array was not altered, validating the power of the global unbiased approach. Laser capture microdissection was used for selective procurement of endothelial cells from the tubules to determine the activation state of two primary signaling pathways also expressed on the microarray. Although mRNA levels of ERK 1, ERK 2, and AKT did not change more than 2-fold with initiation of the tubulogenesis process, we observed a >3-fold down-regulation of activation of these signaling pathways as indicated by changes in phosphorylation of MAP kinases and AKT. These data suggest that changes in both transcription-dependent and transcription-independent pathways contribute to the initial phases of vascular tubulogenesis.

MATERIALS AND METHODS

Materials. Passage 1 HUVECs, endothelial growth medium, and bovine brain extract were obtained from Clonetics (San Diego, CA). Matrigel was from Collaborative Biomedical Products (Bedford, MA). [^{33}P]dCTP and [^{32}P]dCTP were purchased from ICN (Costa Mesa, CA), and actinomycin D was from Calbiochem (San Diego, CA). Bio-Spin 6 chromatography columns were obtained from Bio-Rad (Hercules, CA). I-Block and Western-Star chemiluminescent detection reagents were purchased from Tropix (Bedford, MA). An anticaldesmon mouse monoclonal antibody was from Serotec (Raleigh, NC). Anti-phospho-p44/p42 MAP kinase (ERK 1/2), anti-total ERK 1/2, anti-total Akt, anti-phospho-(Ser473)-Akt, and anti-phospho-(Thr308)-Akt rabbit polyclonal antibodies were obtained from Cell Signaling (Bedford, MA). An anti-α,β-tubulin mouse monoclonal antibody cocktail was purchased from Neo Markers (Union City, CA). TRIzol RNA isolation reagent and all other molecular biology-grade reagents were obtained from Life Technologies, Inc. (Gaithersburg, MD). GF200 cDNA microarrays were purchased from Research Genetics (Huntsville, AL). Polyacrylamide gels were from Novex (San Diego, CA).

Cell Culture and Tube Formation Assay. Tube assays and quantification were performed essentially as described previously (6), using a minimal volume of Matrigel (0.24 mg/cm^2) that both allowed HUVEC tubule formation and permitted vascular network visualization and quantification by image analysis. Briefly, HUVECs (passages 2–6) were cultured in endothelial growth medium containing 2% fetal bovine serum and bovine brain extract and plated (1.2×10^5 cells/ml) on Matrigel. Actinomycin D (2 μg/ml) was added as indicated. Cells were washed, fixed in methanol, and stained in DifQuik solution 2 before tube area measurement. No difference in the extent or quality of the HUVEC tube network was observed in cultures where actinomycin D was added after the 60-min time point (data not shown). Three replicate fields of triplicate wells were digitally photographed. Tube area was quantified using Metamorph software (Universal Imaging Corporation, West Chester, PA) as described previously (6).

Gene Expression Analysis. RNA was isolated using TRIzol reagent 15 min after plating (attachment, +15 min) and 1 h later. An extended DNase treatment was used to remove DNA fragments contained in the Matrigel, after which reverse-transcribed probes were generated in the presence of [^{33}P]dCTP and oligo(dT) primers from 1 μg of total RNA. GF200 cDNA microarray membranes were then prehybridized, hybridized with these probes, and washed according to the manufacturer's instructions. GF200 contains >5100 human genes or ESTs, approximately half of which are known. Phosphorimager array images were analyzed by P-SCAN software (7). The spots of each of the differentially expressed genes were then visually inspected on the array images to confirm changes. Duplicate profiles were done from each of two independent experiments using different lots of microarrays.

RT-PCR Analysis. Static RT-PCR was done with oligo-(dT)-primed cDNA made from 2 μg of total RNA as indicated. Gene-specific primer pairs used were as follows: caldesmon (sense, 5′-GCA-GAA-AAG-CAG-TGG-TGT-CAA-ATC-3′; antisense, 5′-CTC-AAA-CCT-TAG-TGG-GGG-AAG-TG-3′; 413 bp), and β_2-microglobulin (sense, 5′-CTT-GAG-GCT-ATC-CAG-CGT-3′; antisense, 5′-CCA-TGA-TGC-TGC-TTA-CAT-3′; 310 bp). After a 4-min initial denaturation at 94°C, amplification conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 68°C for 45 s for 30 cycles. Tracer quantities of [^{32}P]dCTP were added to the reaction to allow for quantification. The reaction mixture contained 60 mM Tris-SO_4 (pH 8.9), 18 mM ammonium sulfate, 2 mM $MgCl_2$, 0.2 μM oligonucleotide primers, deoxynucleotide triphosphates (200 μM dATP, dGTP, and dTTP; 20 μM dCTP), 1.25 μCi of [^{32}P]dCTP, 1.25 units of Platinum *Taq* DNA polymerase, and 1 μl of cDNA reaction mixture. Negative controls, consisting of a sample without reverse transcriptase and one without template, were included in each experiment. PCR products were electrophoresed on 6% polyacrylamide gels, stained with ethidium bromide, photographed, and then exposed to a phosphorimager screen. The intensities of the bands were quantified by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Results were normalized to β_2-microglobulin expression and the average change in expression reported. The experiment was repeated two times.

Laser Capture Microdissection and Immunoblot Analysis. Tube cultures were fixed at attachment +1 h, or attachment +17 h, after which they were dehydrated in graduated ethanol baths followed by a final treatment in xylene. Approximately 500 HUVECs were microdissected from tube cultures by use of a Pixcell 200 laser capture microdissection system essentially as described previously (8). Single HUVECs were microdissected from attachment cultures, and tubule cells were preferentially microdissected from tubule cultures. Matrigel was microdissected from cell-free areas of the attachment cultures to provide a negative control for Matrigel protein contamination. The microdissected proteins (Matrigel or cells) were lysed directly on the laser capture microdissection cap in 20 μl of a 1:1 solution of Tris-

[2] The abbreviations used are: EST, expressed sequence tag; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; HUVEC, human umbilical vein endothelial cell; RT-PCR, reverse transcription-PCR; COMT, catechol-*O*-methyltransferase.

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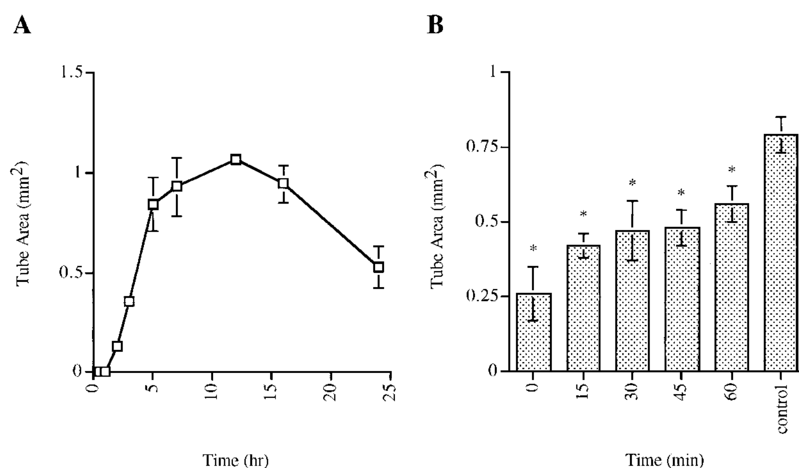

Fig. 1 HUVEC tube formation on Matrigel. *A*, time course. HUVECs were cultured on Matrigel for the indicated times as described. Tubule area was quantified from three fields of each sample, performed in triplicate. Data are expressed as mean ± SD (*bars*) of a representative experiment ($n = 3$). *B*, tubulogenesis requires gene transcription. HUVECs were cultured on Matrigel for a total of 16 h. The transcriptional inhibitor actinomycin D (2 μg/ml) was added at the indicated times in culture with the 16-h time point receiving 0.1% DMSO vehicle. Tubule area was quantified as described. Data are expressed as mean ± SD (*bars*) of a representative experiment ($n = 3$). *, $P \leq 0.05$ compared with control.

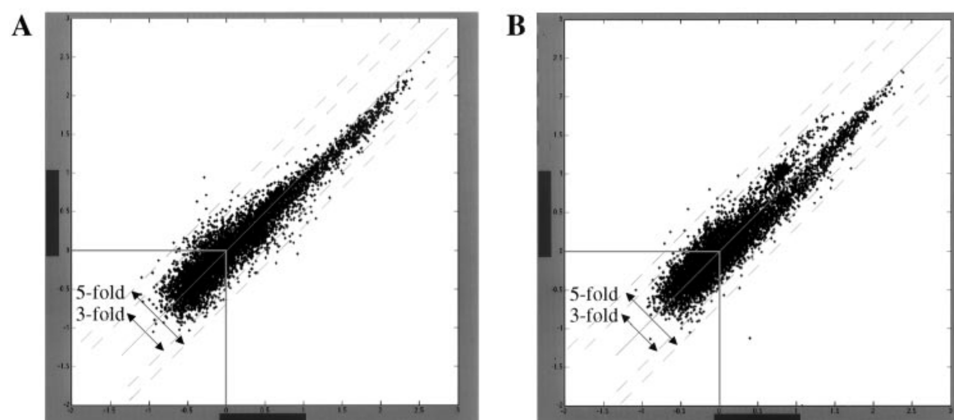

Fig. 2 Scatter plot control analyses of intra- (*A*) and intersample (*B*) variability. The plots consist of log-transformed expression data for probes derived from two aliquots of the same cDNA (intra; *A*) and those derived from cDNA isolated in independent experiments (inter; *B*). Each point represents the normalized expression level of an individual gene within both cDNA aliquots. Points above zero are expression above background (3200 genes). The predicted mean is represented by the *solid line*, whereas the *dashed lines* represent 3-fold (2.2 SD) and 5-fold (3.2 SD) changes in gene expression as indicated.

buffered saline containing 1% Triton X-100 lysis buffer mixed with standard 2× reducing sample buffer and incubated at 70°C for 2 h. A modified Western blot analysis was performed essentially as described previously (9). Briefly, 2-μg aliquots of the protein lysates were electrophoresed through 4–20% gradient polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes. The membranes were incubated with I-Block for 30 min at 25°C and then with primary antibody as indicated (anticaldesmon, 1:1600 dilution; anti-phospho- or anti-total p42/p44 ERK 1/2, 1:1000 dilution; anti-total Akt, 1:1000 dilution; a combination of anti-phospho-(Ser473)-Akt and anti-phospho-(Thr308)-Akt, 1:500 dilution of each; or anti-α,β-tubulin cocktail, 1:1000 dilution). A highly sensitive detection system, Western-Star chemiluminescent detection reagent, was used to detect protein presence from the 2-μg initial protein load background. The images were scanned, and the intensities of the bands were determined using ImageQuant software. Each experiment was repeated at least two times. The average change in band intensities normalized against tubulin is reported.

RESULTS

Gene Expression Profiles during Early Tubulogenesis. Endothelial cell tube formation on Matrigel took 6–12 h for completion (Fig. 1). Inclusion of the transcriptional inhibitor actinomycin D significantly but incompletely inhibited tubule network formation, but only when added within the first hour of culture ($P \leq 0.05$, Student's *t* test; Prism Software, San Diego, CA), indicating a contribution of new gene expression for HUVEC differentiation into tubes. No effect of transcriptional blockade with actinomycin D on extent or complexity of the tubules formed was observed when actinomycin D was added to and maintained in culture after the first 60-min period. Nylon cDNA microarrays were used to evaluate gene expression changes during the first hour of incubation of HUVECs on Matrigel. Parallel arrays were hybridized with probes made

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Table 1 Genes differentially expressed during tubulogenesis on Matrigel

	Category	GenBank accession no.	Fold change at +1 h[a]
Genes with increased expression at 1 h			
Catechol-*O*-methyltransferase	Metabolism	R44202	32
Lymphocyte phosphatase-associated phosphoprotein	Unknown	AA481547	32
EST	Unknown	R36958	18
Multiple exostosis-like protein	Unknown	H19522	12
von Willebrand factor precursor	Coagulation	AA487787	11
Nicotinamide *N*-methyltransferase	Metabolism	T72235	11
EST	Unknown	AA030013	11
Latent transforming growth factor-β binding protein-4	Extracellular matrix	R87406	10
EST	Unknown	R36415	10
EST	Unknown	R09873	10
EST	Unknown	AA485653	9
Syndecan 4	Adhesion	AA148736	8
V-*ral* simian leukemia viral oncogene homolog B	Cell signaling	W39343	8
Diacylglycerol kinase γ	Cell signaling	H05774	7
Splice factor transformer 2-β	Splice regulation	H11792	7
Nuclear chloride ion channel protein	Ion channel	AA486518	6
EST	Unknown	N63646	6
Genes with decreased expression at 1 h			
EST	Unknown	AA281667	1/40[b]
Inhibitor of DNA binding 1	Transcription regulation	AA457158	1/24
EST	Unknown	R62633	1/20
Caldesmon	Cell structure	AA076063	1/18
dUTP pyrophosphatase	Proliferation	AA489219	1/10
Coatomer protein (HEPCOP)	Protein transport	R40212	1/10
Programmed cell death 2	Apoptosis	AA521466	1/9
EST	Unknown	AA447531	1/6
KIAA0121 protein	Unknown	AA504600	1/6
Apolipoprotein E	Lipid	AA478589	1/6
EST	Unknown	AA425757	1/6
Eukaryotic initiation factor-2-associated p67 homolog	Translation regulation	R51346	1/6
β-Actin	Cell structure	R44290	1/5
EST	Unknown	AA019591	1/5

[a] Average fold change of two duplicate microarray analyses.
[b] Also presented as −40 fold.

from aliquots of the same control mRNA for intrasample variability and from different RNA isolations for intersample variability. Similarly, different lots of arrays were tested. P-SCAN software was used to generate scatter plots of expression intensities (Fig. 2; Ref. 7). Intrasample variability studies indicated that 100 of the 3200 points expressed above membrane background were observed at a ≥3-fold change in gene expression on only one of the paired arrays and were false positive (false positive rate, 3.1%). At a 5-fold level of stringency, fewer points were differentially expressed between the controls, yielding a false positive rate of only 0.4% of expressed genes. Control samples from two independent tubulogenesis assays were used to probe microarrays from the same lot for the intersample variability studies, showing 17 (2%) and 3 (0.3%) false positives at 3- and 5-fold stringency, respectively. We therefore chose to operate at the 5-fold level of stringency of differential expression. This approach essentially allowed us to do a subtractive analysis of gene expression occurring during the first hour of tube formation.

Differential Expression of Caldesmon Transcript and Protein. By these criteria, 31 transcripts were expressed differentially (false positive rate, 0.4% of genes queried) within the first hour after attachment (Table 1). Multiple gene classes were represented, including transcription, translation regulation, cell structure, and cell adhesion. The expression of caldesmon, a cytoskeletal protein that binds to actin, myosin, tropomyosin, and calmodulin (10–15), not previously linked to endothelial cell function or differentiation was markedly and reproducibly down-regulated (mean, 18-fold; Fig. 3). RT-PCR analysis reproducibly confirmed this reduction in gene expression (12-fold). Caldesmon protein expression was investigated in the differentiating endothelial cells by use of laser capture microdissection of HUVEC tubules followed by immunoblot analysis. Microdissection of single attached cells provided the protein controls; HUVEC tubules and network nodal points were microdissected for the differentiated cell lysates (example microdissection is shown in Fig. 3*B*). Caldesmon protein levels were essentially unchanged at +1 h compared with attached single cells (data not shown). It is possible then that caldesmon gene expression is not translated into altered protein quantity or that the effect on protein quantity is delayed compared with the measured expression changes. To determine whether the change in caldesmon transcription would be manifested as changes in caldesmon protein quantity at a later time, HUVEC tubules were microdissected for immunoblot analysis at +17 h when tubule formation was complete. Densitometric analysis revealed an average 3.6-fold decrease in caldesmon protein levels, indicating that the transcriptional changes were translated into altered

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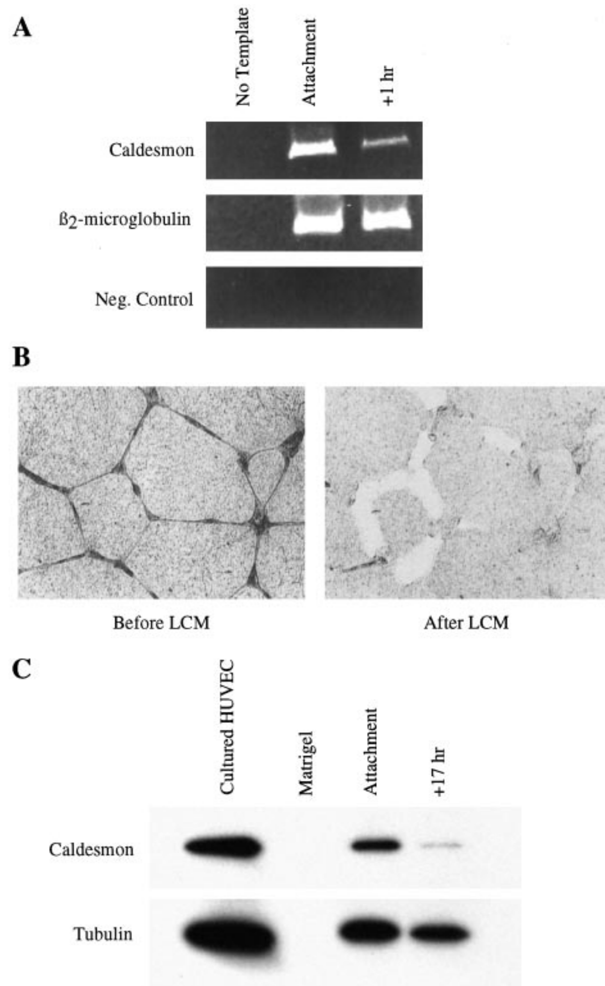

Fig. 3 Analysis of caldesmon expression during tubulogenesis on Matrigel. *A*, RT-PCR analysis confirms a decrease in caldesmon gene expression. Total RNA was isolated from HUVECs as described and subjected to RT-PCR analysis. β_2-Microglobulin expression was included as an internal control. A negative control consisting of a reverse transcription reaction in the absence of reverse transcriptase was included to monitor genomic DNA contamination. *B*, laser capture microdissection (*LCM*) of +17 h tubules. Samples were prepared as described. A pure population of differentiated endothelial cells was obtained. *C*, Western analysis reveals a decrease in caldesmon protein expression. HUVECs were isolated by laser capture microdissection and subjected to Western analysis for caldesmon expression. Blots were stripped and reprobed for tubulin expression as a loading control. Lysates from monolayer cultured HUVECs (P5) were included as a positive control. Microdissected Matrigel lysate was included as a monitor of Matrigel contamination in the HUVEC tubule lysates. These data are representative of at least two experiments.

Table 2 Angiogenesis-related genes are not significantly changed during tubulogenesis

	Fold change at +1 h[a]
Genes with increased expression at +1 h	
Endothelial receptor kinase (TIE1)	2
Endothelin receptor type B	2
Epidermal growth factor receptor	2
Fibroblast growth factor receptor 2	2
Vascular cell adhesion molecule-1	2
Genes with no change in gene expression	
Matrix metalloproteinase-2	NC[b]
Platelet-derived endothelial growth factor receptor β	NC
Platelet/endothelial cell adhesion molecule	NC
Transforming growth factor β 3	NC
Vascular endothelial growth factor receptor (Flt1)	NC
Vascular endothelial growth factor receptor (KDR)	NC
Genes with decreased expression at +1 h	
Tissue inhibitor of metalloproteinase-2	−2
Vascular endothelial (VE) cadherin	−2
Hematopoietic progenitor cell antigen (CD34)	−2

[a] Average fold change of two duplicate microarray analyses.
[b] NC, no change in gene expression.

Table 3 Expression profile of cell signaling genes

	Fold change at +1 h[a]
Genes with increased expression at +1 h	
Phosphatidylinositol 3-kinase γ	2
Extracellular signal-regulated kinase 3 (ERK3)	2
Focal adhesion kinase	2
v-*abl* proto-oncogene homolog	2
Signal transducer and activator of transcription 1 (STAT1)	2
Genes with no change in gene expression	
K-*ras* oncogene	NC[b]
MAP kinase kinase 5	NC
Dual-specificity MAP kinase kinase 3	NC
v-*akt* viral oncogene homolog 1 (AKT1)	NC
v-*akt* viral oncogene homolog 2 (AKT2)	NC
Phosphytidylinositol 3-kinase α	NC
Nuclear factor of κ light polypeptide gene enhancer in B-cells 1 (NFKB1)	NC
Stress-activated protein kinase 1b	NC
Genes with decreased expression at +1 h	
ERK 1	−2
c-*jun* proto-oncogene	−2
Phospholipase C γ 2	−2

[a] Average fold change of two duplicate microarray analyses.
[b] NC, no change in gene expression.

protein levels. These studies suggest that a global, unbiased analysis of transcriptional changes has the potential to identify novel contributors to the process of neovascularization.

Posttranslational Modification Occurs during Early Tubulogenesis. The GF200 array contains representative angiogenesis, invasion, and signal pathway-related genes. No significant change in expression (≥3-fold; false positive rate, 3%) was observed for these classes of genes during the first hour of HUVEC differentiation into tubules (Tables 1–3). This implies that transcription-independent events contribute to early tubulogenesis. We evaluated select proteins, using modified immunoblot techniques to provide proof of concept that analysis of protein levels or posttranslational modifications would identify pathways contributing to tubulogenesis. Attached single HUVECs, Matrigel protein control, and HUVEC tubules and cellular network nodes were microdissected, and cell lysates and proteins were subjected to immunoblot. Akt and p44/p42 MAP kinase (ERK 1/2) have been shown to be involved in cell survival and proliferation pathways, respectively, and are rep-

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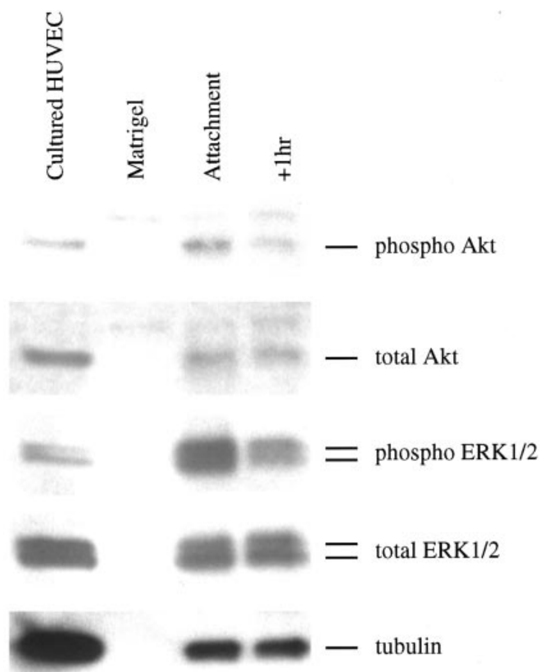

Fig. 4 Phosphorylation of signaling proteins Akt and ERK 1 and 2 is reduced early in tubulogenesis. HUVECs were isolated as described and analyzed by Western analysis for total and phosphorylated Akt and ERK 1/2 expression. Blots were stripped and reprobed for tubulin expression as a loading control. Lysates from monolayer cultured HUVECs (P5) were included as a positive control. Matrigel-only lysate was included to monitor for Matrigel contamination in the HUVEC tubule lysates. Data shown are representative of at least two experiments.

resented on the cDNA microarray (16, 17). RT-PCR analysis of these genes confirmed lack of significant change in their expression at +1 h, consistent with the array results (data not shown). These proteins are activated by phosphorylation that can be evaluated by specific antiphosphoprotein antibodies. Phosphoprotein blotting revealed reduced phospho-Akt and reduced phosphorylated ERK 1 and ERK 2 at the +1 h time point, corrected for loading (Fig. 4). There was a 2.8- and a 3.9-fold average reduction in the phosphorylation of Akt and ERK 1 and 2, respectively. The Matrigel control lanes did not contain immunoreactive bands, indicating that the phospho-AKT and ERK were from cells rather than from a Matrigel contaminant. The higher molecular weight band found in the Akt immunoblots was a nonspecific contaminant from the Matrigel, as it did not appear in the cultured HUVEC samples.

Because inhibition of the MAPK pathway has previously been reported to reduce angiogenic activity *in vitro* (18, 19), the effect of the ERK inhibitor U0106 was tested. Commensurate with the findings that the pathway activation is down-regulated rather than stimulated, U0106 had no effect on the ability of HUVECs to form tubes on Matrigel (data not shown). These data suggest a role for the activation state of these signaling proteins in early tubulogenesis without concomitant changes in their gene expression.

DISCUSSION

It is not fully understood how transcription and signal pathway activation may link in angiogenesis. Dissection of the molecular and proteomic events underlying endothelial activation and vascular tube formation is needed to advance our understanding of the mechanisms regulating this process as well as for the development of molecular targeted antiangiogenic therapies. We hypothesized that although gene transcription may be involved in vascular remodeling, regulation of transcription-independent signal transduction pathways is also a dominant event during vascularization. An unbiased global pilot cDNA microarray analysis was carried out to identify transcriptional changes not previously linked to angiogenesis, to be followed by focused analysis of transcription-independent signaling events. Transcription blockade during the first hour of endothelial cell differentiation into vascular tube-like structures decreased but did not abrogate tube formation. cDNA microarray analysis using statistically validated experimental approaches revealed altered transcription of a number of unexpected and interesting genes during tube formation. Expression of caldesmon, a protein involved in cytoskeletal reorganization but not yet implicated in angiogenesis, was markedly down-regulated in microarray expression, a finding confirmed by RT-PCR. The decreases in transcription and mRNA levels were accompanied by a delayed but significant reduction in the levels of the caldesmon protein product. No significant changes in transcription were observed in expression of the spectrum of genes contained on the array previously demonstrated to contribute to angiogenesis, invasion, or signal transduction, suggesting that other regulatory mechanisms contribute to tube formation. Both the phosphatidylinositol 3′-kinase/AKT and RAS/MAP kinase pathways are critical regulators of migration, proliferation, and survival (Ref. 20 and the references therein). Phosphorylation of AKT and ERK 1/2 was selected as an indicator of activation of the pathways regulated during vascular tube formation. Microdissection followed by immunoblot analysis of attached single HUVECs and those organizing into vascular tubes demonstrated that the phosphorylation states of three signaling proteins, ERK 1, ERK 2, and Akt, were reduced in the absence of significant changes in their transcript levels or alterations in their total protein quantities. This counterintuitive finding may lead to new insights into the pathway events in cell differentiation and activation. The present study indicates the need to expand proteomic characterization of the angiogenic process.

An objective of the present study was to identify genes not previously associated with angiogenesis, such as caldesmon, through a global unbiased search. Caldesmon is a regulator of cell contraction in smooth muscle and nonmuscle cells, working through control of actin remodeling (10–15, 21). Overexpression of caldesmon in human fibroblasts inhibits cell contractility and interferes with Rho A-mediated formation of stress fibers and focal adhesions (13). Both increasing cellular calcium concentrations and caldesmon phosphorylation cause dissociation of caldesmon from actin, allowing remodeling to occur (10). We showed previously that HUVEC spreading on basement membrane type IV collagen produced a dynamic increase in intracellular calcium (22). Furthermore, the increase in intracellular

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calcium was shown to drive actin remodeling in HUVECs, where augmenting calcium influx with thapsigargin or ionomycin yielded faster and more intense actin rearrangement into stress fibers (23). These results, coupled with our findings on the gene microarray and protein blot, suggest a potential role for caldesmon in regulating endothelial cell spreading and elongation, two components of vascular development (13, 22–24). Other genes were found to be altered in expression in this pilot search, such as the down-regulated Id-1 and up-regulated COMT. Although provocative points for study, as described below, the altered expression of these genes and its magnitude could not be consistently confirmed. Expression of Id-1 has recently been shown to be reduced in other biological behaviors in which growth arrest is associated with remodeling, such as mammary gland involution and postischemic regenerating kidney (25, 26). In addition, increased expression of Id-1 has been described in homocysteine-injured endothelium and in angiogenesis (27–29). Expression of COMT was up-regulated. COMT is the enzyme that converts estradiol to 2-methoxyestradiol, an antiangiogenic compound, when used pharmacologically (30, 31).

Angiogenesis-related genes studied on the GF200 array comprise predominately growth factor receptors, signal transduction molecules, and invasion-related molecules. Expression changes in these gene categories were infrequent and of relatively small magnitude during the first hour of tube formation. These data may be interpreted several different ways. First, the small changes in gene expression observed (up to 2-fold) may be adequate for triggering and maintaining tube development. Alternatively, (*a*) gene expression changes in these classes of genes may not be required for tubulogenesis in this model, (*b*) genes regulated by transcriptional changes during tubulogenesis are not present on this 5000-gene array, (*c*) translational efficiency is altered during tubulogenesis, and/or (*d*) the basal RNA levels of these genes are sufficient for the levels of protein expression required for tubulogenesis. The last interpretation suggests that changes in gene expression may not be required but that activation of already expressed signaling proteins encoded by these genes is important for tubulogenesis. The lack of changes in total protein quantity of the index signaling proteins supports this notion.

To address the possibility that the posttranslational modification is critical to tubulogenesis, we characterized the activation status of two parallel but independent signaling pathways in angiogenesis. Akt is an integral part of cell survival and permeability pathways, whereas p44/42 MAP kinase (ERK 1/2) is associated with proliferation and migration (15, 18, 32–34). Both proteins have been shown to be activated during vascular endothelial growth factor- and fibroblast growth factor-stimulated angiogenesis (35). The Matrigel tubulogenesis model requires differentiation but not proliferation; therefore, a reduction in phosphorylation and thus inactivation of ERK 1 and ERK 2 may be expected (5). HUVECs in this model have been shown to be sensitive to regulation of invasion pathways, but do not have marked proliferative activity (17).[3] Thus, the down-regulation of ERK activation may be explained by its predominant role in proliferation. A recent study demonstrated a similar finding in that down-regulation of the ERK pathway pharmacologically or by inhibition of Ras activation had a minimal effect on tubulogenesis on a collagen gel background (36).

The decrease in Akt phosphorylation is harder to explain but may be required for the process of differentiation to occur. To our knowledge, the activation state of Akt has not been characterized in a differentiation model such as the one described here. The unexpected reduction in activation of these two signaling pathways also may be selectively modified as a result of the interaction of the endothelium with the local Matrigel microenvironment. How the local microenvironment drives gene expression and pathway activation in cancer development, dissemination, and angiogenesis is a nascent area for investigation (20). The observed marked decrease in signaling through the phosphatidylinositol 3′-kinase/AKT and RAS/MAP kinase pathways in the absence of significant changes in gene expression or quantities of these proteins suggests an important role of protein activation/deactivation in tubulogenesis.

The development of new technologies such as gene microarrays and laser capture microdissection has allowed us to initiate examination of differentiating endothelial cells at both the gene and protein levels. Taken together, these data point to an integrated use of assessment of gene expression, protein levels, and posttranslational modification to understand the mechanisms regulating endothelial differentiation into vascular tube-like structures *in vitro*. We have identified potential novel molecular targets that, after validation, may be appropriate for the development of antiangiogenic therapies. This work provides insight into the molecular mechanisms involved in angiogenesis and contributes to our understanding of the regulation of the neovascularization process. This pathway-profiling approach may direct further molecular targeting in angiogenesis therapeutics.

[3] E. C. Kohn and A. D. Grove, unpublished observations.

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