Comparison of Gene Expression in Bovine Aortic Endothelium In Vivo Versus In Vitro Differences in Growth Regulatory Molecules

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Many studies of the endothelium have assumed equivalence between cultured confluent cells and an endothelial lining in vivo. We compared gene expression of bovine aortic endothelial cells (BAECs) in culture versus freshly isolated cells from bovine aortas. Our technique of harvesting in vivo tissue yielded cells that were endothelial by the criteria of their containing von Willebrand factor (vWF) and lacking smooth muscle α-actin, by both immunocytochemistry and mRNA analyses. We found that several genes are overexpressed when BAECs are placed into culture, including basic fibroblast growth factor, platelet-derived growth factor B-chain, and thrombospondin. On the other hand, message for vWF is highly expressed in vivo and at lower levels in confluent cultures. The transcripts for transforming growth factor-β, plakoglobin, and flg (fms-like gene, FGF receptor-1) are comparable in vitro and in vivo. These results demonstrate that significant changes in gene expression occur in the transition from in vivo conditions to tissue culture of endothelial cells. Studies of in vitro endothelium may poorly reflect a quiescent endothelial lining in vivo but may be more similar to cells responding to injury or angiogenic stimuli.

Arteriosclerosis and Thrombosis 1993;13:985-993

KEY WORDS • aortic endothelium • gene expression • tissue culture

Study of endothelial biochemistry became possible in the early 1970s with the availability of reproducible culture systems. Until that time there were no practical means of isolating quantities of endothelial cells sufficient for biochemical assays. From then until the present, progress in endothelial biochemistry has largely been based on studies in vitro, which were later confirmed by data in vivo. The assumption that patterns of gene expression in cultured cells accurately represent in vivo patterns is particularly worrisome for molecules involved in growth control, since it is difficult to perform comparable experiments using defined media and added growth factors in vivo and in culture. Concern for the validity of in vitro findings may also be increased by changes that we know occur as passage number increases. These studies have shown that endothelial cell properties, such as the percentage of cycling cells, rate of protein release, and transcript levels of particular genes alter as a function of time in culture. The only transcript that has been studied both in vivo and in cultures of bovine endothelium is the sis gene mRNA, but in those experiments the freshly prepared in vivo tissue was composed of between 35% and 50% smooth muscle cells by histological examination.

We find that several genes differ in their expression between in vivo samples and cultured bovine aortic endothelial cells (BAECs) and suggest that the in vitro endothelium is a poor model of the quiescent endothelium in vivo on the basis of transcript levels of several growth regulatory molecules.

Methods

Cell Culture

BAECs were maintained in Waymouth's complete medium supplemented with 10% fetal bovine serum (FBS) and 100 U/mL each penicillin and streptomycin (GIBCO, Grand Island, NY). Endothelial cells were isolated from bovine aortas as previously described. Briefly, sections of bovine aortas obtained from freshly slaughtered cows were gently scraped with a cell scraper. This cell suspension was dilutedly plated in Waymouth's medium containing 20% FBS into 24-well plates. Wells containing monolayered cells with a "cobblestone" morphology were pooled with others from the same aorta and grown in flasks. The resultant cultures were tested for Mycoplasma contamination and screened for endothelial markers, von Willebrand factor (vWF), and uptake of acetylated low-density lipoprotein, as well as for the absence of smooth muscle α-actin. Resultant cell lines were expanded and frozen, and experiments were done with cells of low passage and a "healthy" morphology. Cultures were fed every week with medium containing 10% fetal calf serum until confluence, at which time they were fed every 2 weeks or were passaged by detaching with 0.05% trypsin. Cells were maintained in a humidified incubator at 37°C with 5% CO₂. For experiments, cultures of healthy morphology between passages 4 and 10 were used. Cells were

Received November 11, 1992; revision accepted March 29, 1993.
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plated at a density of 20,000 cells/cm² in 100-mm plates and harvested either after confluence (usually about 1 week) or after 48 hours for sparsely growing cells. For experiments in which cells were grown on collagen, the growth area of chambered glass slides was covered with Vitrogen 100 (Celtrix Laboratories, Palo Alto, Calif), a solution of purified bovine dermal collagen. Isotonic Vitrogen 100 was added to growth plates and allowed to gel at 37°C. Plates were then rinsed with phosphate-buffered saline (PBS) and cells were plated as usual. Rat smooth muscle cells¹ and human umbilical vein endothelium² were cultured as previously described.

**Antibody Studies**

The neutralizing antibody to basic fibroblast growth factor (bFGF) was made in rabbits and purified on protein G, as described in detail elsewhere.³ This antibody was added daily to cultures at a concentration of 10 μg/mL.

**RNA Isolation**

**In vitro samples.** Total cellular RNA was isolated from cultures of BAECs by methods that have been previously described.⁴ In vivo samples. Aortas from freshly slaughtered cows were processed immediately after removal from the animal. The aortas were cut longitudinally near the intercostals and rinsed with sterile PBS. A solution of 4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 mmol/L 2-mercaptoethanol was added in small quantity onto the luminal surface, and endothelial cells were scraped off gently with a plastic cell scraper, taking care to scrape any given surface only once. The remainder of the procedure was identical to that used for in vitro experiments. We obtained three independent pools of in vivo RNA on separate occasions and used all three isolates in our Northern blot analyses. In addition, cell samples were also taken for immunocytochemistry. In this case, an aorta was cut and rinsed, and cells were scraped with a cell scraper and immediately smeared across a gelatin-coated glass slide. The cells were allowed to dry quickly and were then fixed in 4% paraformaldehyde for 5 minutes. They were placed in PBS until staining on the following day.

**En face preparations.** Aortas were obtained from freshly slaughtered cows and rinsed with PBS. Small pieces were immediately fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.3, and transferred to PBS. The pieces were cut open longitudinally through the intercostals, and the outer media was removed with a scalpel. The pieces were pinned out on Teflon cards for staining as described below. After immunocytochemistry, the tissue was stored in 4% paraformaldehyde until Häutchen preparations were made of the endothelium as previously described.⁵

**Immunocytochemistry.** Paraformaldehyde-fixed samples were rinsed in PBS and nonspecific binding was blocked by incubation with 2% normal goat serum. A rabbit antibody (immunoglobulin G [IgG]) against human factor VIII–related antigen (DAKO Corp, Carpinteria, Calif), was used at a 1:1000 dilution, followed by a biotinylated goat anti-rabbit IgG at 1:400 dilution (Vector Laboratories, Burlingame, Calif) and peroxidase-labeled avidin–peroxidase conjugate (ABC Elite, Vector Labs). All incubations were at room temperature for 30 minutes, and antibodies were di-luted in 1% bovine serum albumin in PBS. Antibody was visualized by incubation with the substrate 3,3'-diaminobenzidine (Sigma Chemical Co, St Louis, Mo). Immunochemistry for bFGF was done in the same manner as described above with a mouse monoclonal anti-bFGF antibody (kindly provided by Dr Thomas Reilly, DuPont Merck, Wilmington, Del) at a 1:800 dilution and a horse anti-mouse secondary antibody at 1:400 dilution (Vector Labs).

**Autoradiography and cell proliferation.** Cells were labeled with 1 μCi/mL [³H]thymidine (New England Nuclear) for 30 minutes and harvested at various time points. Cultures were rinsed with PBS and fixed with 4% paraformaldehyde. They were dehydrated and mounted on glass slides for dipping in a 1:1 vol/vol solution of NTB2 (Kodak) emulsion/distilled water. The slides were exposed for 2 weeks, developed, and stained with hematoxylin. Proliferation rates were determined by the ratio of [³H]thymidine-labeled cells to total cells, expressed as a percentage.

**RNA and DNA determination.** Samples for RNA and DNA content analysis were collected by scraping into PBS. The cell lysates were sheared with an 18G needle and immediately frozen. Equal volumes of cell lysates were used for the following reactions, a total RNA extraction, and further isolation of mRNA. The methods for DNA and RNA determination were carried out as previously described.⁶ Briefly, cell lysates were incubated with 0.6 mol/L perchloric acid (PCA) and centrifuged, and the pellet was then washed with 0.2 mol/L PCA and 0.3 mol/L KOH and incubated for 60 minutes at 37°C. The sample was then mixed with 1.2 mol/L PCA and centrifuged. The supernatant was added to 0.6 mol/L PCA and 6 mL water and the pellet saved for subsequent DNA determination. The RNA sample was then quantified by reading at 260 nm against 0.1 mol/L PCA as a blank. For DNA determination, the pellet was resuspended in 0.5 mol/L PCA and heated at 75°C for 60 minutes. The sample was then centrifuged and the supernatant mixed with a color reagent of 1.5% diphenylamine in glacial acetic acid with 1.5% H₂SO₄ and 0.5% acetaldehyde (from 2% stock). Known amounts of standard DNA were also incubated with the color reagent for 16 hours at room temperature. Samples were read at 600 nm in a spectrophotometer. Total DNA was also isolated from the same amount of starting material in the manner described above. This RNA was subjected to mRNA purification by repeated runs over an oligodeoxythymidine column according to the manufacturer's instructions (Promega, Madison, Wis). The amounts of RNA and mRNA were normalized to the DNA content of the sample.

**Northern blots.** Twenty micrograms of total RNA per lane was electrophoresed on a 1.2% agarose gel submerged in 2.2 mol/L formaldehyde/10 mmol/L sodium phosphate, pH 8.2. RNA was transferred overnight to a nylon membrane (Hybond, Amersham) in solution of 1.5 mol/L NaCl and 0.15 mol/L sodium citrate, and the membrane was heated at 80°C for 2 hours. Blots were hybridized with Amersham Fast Hybe Buffer at 65°C for 2 hours and washed in a solution of 0.045 mol/L NaCl, 0.045 mol/L sodium citrate, and 0.1% sodium dodecyl sulfate (pH 7.0). cDNA probes were labeled with deoxyoxymidine S⁻¹(3²P)orthophosphate (New England Nuclear) and added to the hybridization solution at 1x10⁶ cpm/mL. The Northern blots of in vivo and in vitro
RNA were run from three independent pools of RNA from each source obtained on separate occasions. Glyceraldehyde 3-phosphate dehydrogenase, /3-tubulin, and actin were used as controls for loading.

cDNA probes. The probes used in this study were as follows: the bFGF probe was pJJ11-1, a bovine cDNA that is a 1.4-kb insert in pBR322; actin was probed with pBA-1, a 1.3-kb insert in pBR322; the platelet-derived growth factor (PDGF) B-chain probe was a 2.1-kb Sac I/Sac II human cDNA fragment of pSM-15; vWF was a Pst I/EcoRI fragment at the 5' end of the gene; thrombospondin was a 1.3-kb clone in the 5' region of the gene; transforming growth factor (TGF) -/3 was a 2.1-kb EcoRI human cDNA fragment; plakoglobin was a human cDNA clone; flg was a BamHI fragment cloned into pGem 1; glyceraldehyde 3-phosphate dehydrogenase was a 1.2-kb human insert into the Pst I site of pBR322; and /3-tubulin was a chicken cDNA.

Results

We obtained aortas of freshly slaughtered cows as a source for endothelium representing the phenotype of cells in vivo. Our in vitro samples were collected from confluent cultures of BAEC lines. The simplest question to ask with regard to these two cell sources is the relative amounts of RNA levels in vivo versus in vitro. Endothelium in vivo has an extremely low frequency of DNA synthesis when compared with in vitro cultures, and thus, we might predict that mRNA transcript levels in vivo versus in vitro samples were collected from confluent BAEC cultures, except when stated otherwise.

In vivo samples were smeared onto a gelatin-coated slide and stained for the presence of vWF to identify the cellular makeup of our samples. The results were compared with vWF staining of a confluent BAEC culture. As shown in Fig 2, all of the cells examined from the scraped aortic sample stained strongly for vWF (panel a), indicating that our method had obtained endothelium from the aorta. In culture as well (panel b), cells were positive for vWF, although the staining decreased when cells had been confluent for several days, as in the culture shown. Conversely, there were only background amounts of staining when both scraped samples and BAEC cultures were stained with an antibody specific for smooth muscle a-actin (data not shown).

This immunocytochemistry finding was consistent with Northern blots for both vWF and smooth muscle a-actin in our samples. At first, the comparison of vWF message in culture (Fig 2c, lane 1) and in freshly scraped tissue (lane 2) was somewhat surprising. Although present at high levels in the fresh tissue, vWF message was not apparent in postconfluent BAECs, thus conflicting with the immunostaining results. However, since the mature protein is stored in the Weibel-Palade bodies, we looked at subconfluent cultures to see whether vWF mRNA was detectable during growth of the cells (Fig 2, lanes S and C). Sparserly plated replicating cells expressed higher mRNA levels for vWF than did the quiescent monolayer. This explains the presence of the protein in postconfluent cultures. Similar downregulation of vWF message has also been reported in human umbilical vein endothelium as a function of time in passage, where primary cultures express the message strongly and it is no longer detectable by passage 21 in tissue culture.

The most likely contaminant in our freshly scraped aortic preparations is smooth muscle cells underlying the endothelium. As a second test for such contaminants in the samples, we looked for expression of smooth muscle-specific a-actin using a probe that recognizes all forms of actin. Fig 3 demonstrates that BAEC cultures (lane 1) as well as cells from fresh tissue (lane 2) express a band that represents the predicted cytoplasmic B- and/or y-actin mRNAs at 2.1 kb, whereas rat smooth muscle cell cultures additionally
express α-smooth muscle actin mRNA at 1.7 kb (lanes 3 and 4), typical of cultured rat smooth muscle cells. However, the samples contain equal amounts of message for β-tubulin, although the predominant message in bovine tissue is 3.5 kb and those in rat tissue are 2.5 and 1.8 kb. Thus, both immunocytochemistry and Northern analysis demonstrate that our fresh tissue samples are representative of the endothelium.

The second group of genes included those for molecules that have been implicated in growth control. Speir et al have reported increased FGF-like activity on 3T3 cells from extracts of cultured BAECs when compared with freshly harvested tissue. Additionally, data from our laboratory have demonstrated that various lines of cultured BAECs express varying amounts of message for bFGF (J. Medina, PhD, et al, unpublished observations, 1993). A comparison of bFGF mRNA from freshly isolated bovine aortic endothelium and cultured BAECs (Fig 4a) showed that the amount of bFGF message in fresh tissue was undetectable (lane 1), although it was present in BAEC cultures (lane 2). We failed to see message in fresh endothelial samples even when the blots were exposed for as long as 3 weeks. The presence of bFGF mRNA appears sometime during introduction and passage in culture. It is of interest to note that it is not necessary to add any heparin-binding growth factors to BAEC cultures, and we routinely passage and grow our bovine cells without exogenous FGF. We also examined the level of one of the FGF receptors, flg, in these cell samples (Fig 4b) and found that the transcript is present in fresh endothelium (lane 1), cultured BAECs (lane 2), and human umbilical vein endothelial cultures (lane 3). In addition, we probed the same blots with an acidic FGF cDNA but were unable to detect any message in fresh tissue or our cultured cell lines (data not shown).

Although Northern analysis for bFGF seemed to suggest that very little message is present in vivo, the transcript levels of a particular gene do not always correlate with the amount of cellular protein, for exam-
We looked, therefore, for the presence of bFGF protein in both cell sources by immunocytochemistry. Using aortic pieces from freshly slaughtered cows, we prepared en face preparations, which resulted in separation of the endothelial layer from the rest of the vessel. These en face slides and confluent cultures of endothelium were stained with either an antibody to bFGF or normal mouse IgG. Fig 5a shows that the aortic pieces were immunopositive for bFGF despite the lack of detectable mRNA. The cultured BAECs were strongly positive (Fig 5c), and in both cases the staining was predominantly nuclear. The presence of bFGF protein in vivo suggests one of the following possibilities: bFGF is a very long-lived protein in vivo, the in vivo mRNA is undetectable because of rapid degradation, or the transcript is upregulated only when stimulated by factors that we have not examined.

The second growth factor that we examined was platelet-derived growth factor (PDGF). PDGF is not thought to be an autocrine regulator of the endothelium because of the absence of receptors to this ligand except in the microvessel endothelium. However, PDGF has been postulated to act in a paracrine manner in vivo on smooth muscle cells and fibroblasts, since these cell types have receptors and respond biologically to the molecule. We found that PDGF-B mRNA was upregulated in cultured BAECs (Fig 6a, lane 1) when compared with freshly isolated samples (lane 2). An \( \approx 3.5 \)-kb message of PDGF-B was detected in vivo at a significantly lower level than in culture. This is consistent with the earlier report of PDGF levels in BAEC preparations that were obtained from freshly slaughtered cows as well, but which contained smooth muscle cell contaminants.
Another growth regulator of many cell types in the vessel wall is TGFβ. Fig 6b shows that BAECs from both cultures (lane 1) and fresh vessels (lane 2) contained comparable levels of message. The two species detected are ≈2.5 and 4.2 kb. The typical message size found for human TGFβ is 2.5 kb, and the splice junctions of the gene are preserved in the bovine gene.36 A similar TGFβ-related 4.0-kb mRNA has also been reported in rat tissue.37

The third set of genes we studied encodes for products associated with morphological changes in the endothelium. The extracellular matrix protein thrombospondin has been reported to induce adhesion and spreading of BAECs38 while inhibiting cell growth.38-39 The in vivo importance of this protein with regard to the endothelium is at present unclear, although it has been suggested that these changes are critical during the process of angiogenesis. Our studies show that thrombospondin mRNA was greatly upregulated in cultures of BAECs (Fig 7a, lane 1) when compared with fresh specimen samples (lane 2), in which the ≈6.0-kb message was undetectable.

Second, it was of interest to test the effects of morphological change on mRNA transcript levels by growing the cultures under conditions in which the phenotype was not a typical cobblestone monolayer. Many reports of endothelial remodeling have involved culturing cells on basement membrane components such as collagen, laminin, and fibronectin.43-47 When our BAEC cultures were grown on a collagen substrate (Fig 9b), they did undergo morphological change compared with a control culture (Fig 9a). The expression of bFGF, PDGF B, and thrombospondin mRNA of the mass culture grown on a collagen substratum is identical to that of our cultures grown in a monolayer (data not shown).

**Discussion**

Cultured endothelial cells are often thought of as an ideal model system for growth control. These cells, at least those from bovine or porcine sources, can be grown in plasma-derived serum without added growth factors48-49 and will become quiescent once a confluent monolayer is established. Culture conditions used to maintain human endothelial cells, on the other hand, involve exogenous growth factors, leading to concomitant increases in turnover rates at confluence. The definition of quiescence, however, is somewhat arbitrary. While the cell number in a BAEC culture remains consistent at about 150,000 cells/cm², turnover persists at relatively high levels of 5%,7 similar to that of the focal areas of cells in the aorta that are undergoing high rates of turnover.50

Under these conditions, it is reasonable to ask whether the pattern of gene expression in vitro, even in quiescent cells, is an accurate approximation of patterns seen in truly quiescent endothelium in vivo. This question is especially important for genes whose expression or overexpression has been described as critical for endothelial regeneration or angiogenesis, since modu-
The high levels of expression of bFGF and PDGF-B may be of special interest if the culture system does mimic areas of high turnover, response to injury, or angiogenesis in vivo. We have very limited information regarding the expression of these genes in the endothelium in vivo in response to morphological remodeling. Lindner et al. have reported increased levels of bFGF protein in proliferating endothelium in the rat balloon-injury model, where immunostaining revealed that bFGF levels were correlated with regeneration. A similar pattern has also been found by Schulze-Osthoff et al., who reported little bFGF immunoreactivity in the endothelium in normal tissue in vivo but greatly enhanced staining in the endothelium that was associated with inflammation and tumor tissues. In addition, Wilcox et al. have detected elevated PDGF-B mRNA by in situ hybridization in the endothelium of vasa vasorum in human atherosclerotic plaques. Beyond that, expression of these growth regulatory molecules has not been specifically examined in vivo in the endothelium in response to morphogenic stimuli. Our data and these in vivo results are consistent with the idea that the cultured endothelium may be more similar to remodeling endothelium in regard to expression of these transcripts.

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addition of anti-bFGF to our cells led to loss of cells from the monolayer, leaving patches on the culture dish. However, mRNA levels of bFGF, PDGF, or thrombospondin did not decrease during this period. This does not rule out the possibility that bFGF acts in an autocrine manner to upregulate these transcripts, since we did not treat our cultures with anti-FGF from primary culture. The role of PDGF is harder to hypothesize; however, there have been speculations that this growth factor may have an important role in the recruitment of smooth muscle cells at sites of endothelial injury. It is of interest in this regard to note that cultured endothelial cells secrete PDGF-BB in a vectorial fashion, ie, toward the underlying vessel wall rather than the lumen. Moreover, PDGF-BB seems to play a specific role in stimulating smooth muscle cell chemotaxis rather than proliferation. Again, it would be intriguing to know whether PDGF-B expression is selectively turned on at sites of vascular remodeling in vivo.

Acknowledgments

This work was supported by National Institutes of Health grants HL-18654 and HL-07312. The authors would like to thank Dr John Medina, Isa Werny, and Trudy Bartosek for helpful discussion in preparation of this work as well as assistance in obtaining tissue samples for analysis.

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Differences in growth regulatory molecules.
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doi: 10.1161/01.ATV.13.7.985
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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