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Expression of the *sis* gene by endothelial cells in culture and *in vivo*

(platelet-derived growth factor/growth factors/bovine aorta/human umbilical vein/endothelium)

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ABSTRACT **Recognition that the *sis* gene codes for a protein homologous with at least one of the two chains of platelet-derived growth factor has made it possible to directly assess transcriptional expression of platelet-derived growth factor both in cultured cells and in tissue obtained *in vivo*. We have found that a 3.7-kilobase RNA homologous to the *sis* gene is expressed at moderate levels in cultured human and bovine endothelial cells, at low levels in *in vivo* endothelium from human umbilical vein, and at very low levels in bovine aortic endothelium *in vivo*. This RNA migrates at the same rate as the previously reported *sis* band in the HUT 102 human T-cell lymphoma line. This band is not found in RNA extracted from freshly obtained bovine aortic media or from human foreskin fibroblasts or cultured fetal human aortic smooth muscle cells. Our *in vitro* results suggest that the *sis* gene is responsible for at least part of the platelet-derived growth factor-like mitogenic activity secreted by cultured endothelial cells and indicate that the *sis* gene is readily activated in endothelial cells during the transition from *in vivo* conditions to *in vitro* growth as a monolayer on plastic. Expression of the *sis* gene by endothelium *in vivo* raises the possibility that platelet-derived growth factor has a role in the development of the vascular system in the young animal and in the maintenance of the normal vascular system in the adult.**

Although the production of peptide growth factors by tissues *in vivo* has long been suspected to be of biological importance (1), the *in vivo* detection of growth factor production has been limited by the sensitivity of available assay systems. Most known growth factors may be present in amounts too low to be directly detected *in vivo* by current techniques. Cultured endothelial cells secrete a significant amount of mitogenic activity as assayed on fibroblasts and smooth muscle cells (SMC) (2). Most of this mitogenic activity has been attributed to a growth factor or factors unique and specific to endothelial cells, which is biochemically distinct from platelet-derived growth factor (PDGF) and several other characterized growth factors (3). Approximately 25% of the mitogenic activity in endothelial cell conditioned media, though, has been specifically attributed to a PDGF-like protein (4, 5), as determined by ^{125}I-labeled PDGF radioreceptor competition assay and by inhibition of the competitor activity by antiserum against human PDGF.

The discovery that the peptide sequence of the simian sarcoma virus transforming protein (p28^{sis}) predicted from the nucleotide sequence of the simian sarcoma virus has considerable homology to the known sequence for at least one of the two chains of PDGF (6, 7) and more recent data that the coding sequence of the human c-*sis* gene predicts a peptide highly homologous to this same chain of PDGF (8) make it likely that the *sis* gene codes for at least one of the two chains of PDGF derived from human platelets. Whether the *sis* gene encodes the second chain of platelet PDGF remains to be determined. This identification of PDGF at both the protein and genomic level now makes possible studies of mechanisms of control for one of the endothelial cell-derived mitogens. We have done hybridizations to RNA transferred from agarose to nitrocellulose, using a cloned genomic probe to detect whether the *sis* gene is transcribed by endothelial cells either in culture or *in vivo*, and we have found a single RNA species to be present under both conditions.

MATERIALS AND METHODS

Cells. Human umbilical vein endothelial (HUVE) cells for growth in culture were obtained from fresh intact umbilical cords. The umbilical vein was filled with approximately 10 ml of collagenase at 1 mg/ml (20), incubated at 37°C for 15–20 minutes, and massaged, and the resultant freely suspended cells were plated and grown for 1 week in 10% human serum without passage (9). Bovine aortic endothelial (BAE) cells for growth in culture were removed from adult bovine aortas by scraping lightly with a scalpel, immediately plated, and grown in 10% adult bovine whole blood serum (10). Cultures were held at confluency for 7 days prior to extracting RNA. Fetal human aortic SMC were obtained by explant and grown in 20% fetal calf serum (11). The HUT 102 T-cell lymphoma line was grown as described (21). Human foreskin fibroblasts were grown in 10% fetal calf serum. HUVE cells to be used in RNA assays immediately after their removal from fresh tissue (which we will call *in vivo* HUVE cells) were obtained by two techniques. Fresh umbilical cords held at 4°C in normal saline were processed within 20–30 min after delivery. Cells were removed either by enzymatic digestion, as described above, but using trypsin at 0.5 mg/ml with EDTA (GIBCO) at 0.2 mg/ml instead of collagenase, or by light scraping with a scalpel, and RNA was extracted immediately. BAE cells to be used in RNA assays immediately after their removal from fresh tissue (which we will call *in vivo* BAE cells) were obtained from fresh adult aortas within 15–20 min of death by scraping with a scalpel, as described above for cultured cells, only with greater pressure to ensure high yields, and RNA was extracted immediately. These preparations do include some from the aortic intima and inner aortic media as determined by the morphology of the cultured cells. Adult bovine aortic media was prepared from freshly obtained aorta by thoroughly scraping the endothelium, then dissecting away the adventitia. Representative areas of a vessel scraped for *in vivo* endothelial RNA were fixed with Formalin and embedded in paraffin for preparation of hematoxylin/eosin-stained sections.

RNA Extraction, Gels, and Transfer to Nitrocellulose. RNA was extracted by the method of Chirgwin *et al.* (12), including pelleting through cesium chloride. Poly(A)$^+$ RNA was selected by passage over oligo(dT)-cellulose (Collaborative

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Abbreviations: PDGF, platelet-derived growth factor; SMC, smooth muscle cell(s); BAE, bovine aortic endothelial; HUVE, human umbilical vein endothelial; kb, kilobase(s).

Research) per the supplier's instructions. RNA was electrophoresed in formaldehyde gels (13), transferred to nitrocellulose, and hybridized in 50% (vol/vol) formamide/0.75 M NaCl/0.075 M sodium citrate, pH 7.0/25 mM $NaPO_4$/1× Denhardt's solution/250 μg of salmon sperm DNA per ml/10% dextran sulfate at 42°C with ^{32}P-labeled nick-translated DNA probes (14). The c-*sis* probe used was a 1.6-kilobase (kb) *Bam*HI genomic fragment (generously provided by R. Gallo) which includes coding sequences presently described as the fifth and sixth exons of the human c-*sis* gene (8). Blots were washed at 68°C in 0.045 M NaCl/0.0045 M sodium citrate, pH 7.0/0.1% sodium dodecyl sulfate. Size markers of *Hin*dIII-digested λ phage DNA were denatured and electrophoresed identically to RNA samples.

Relative Intensity of Hybridization Signals. The relative intensity of the major *sis* hybridization band per microgram of total cytoplasmic RNA was estimated for each sample. For this purpose a single RNA preparation from cultured BAE cells was used as a common internal standard in all hybridizations. Each blot was then exposed for multiple lengths of time ranging from 1 day to 3 weeks. For each sample run on a single blot, an exposure giving a signal of moderate intensity was paired with a BAE *sis* signal of approximately equal intensity. The relative intensities of these approximately equal paired signals were estimated as the inverse of the ratio of their respective exposure times. Corrections were applied for (*i*) the decay half-life of ^{32}P and (*ii*) the amount of RNA loaded per track. An additional correction factor was used for RNA samples selected on an oligo(dT)-cellulose column. Since in our hands selection of message once on an oligo(dT)-cellulose column enhances the strength of the signal obtained by hybridization with an actin cDNA probe approximately 8-fold (data not shown), this correction factor was applied where appropriate. In the instance of HUT 102 cells, RNA was selected twice over an oligo(dT)-cellulose column and a correction factor of 25 was used, based on the assumption that at most 4% of total cellular RNA is poly(A)$^+$ RNA.

RESULTS

Expression of the *sis* gene was detected in both types of endothelial cells studied, although at greatly varying concentrations (see Fig. 1). HUVE cells showed a single strong band at 3.7 kb which was about 10 times more intense (per μg of RNA) in cultured cells than it was in *in vivo* umbilical vein endothelium obtained by trypsinization (see Table 1). The same relative signal intensity was observed irrespective of whether the endothelium was removed by scraping or by trypsinization. Cultured BAE cells gave a single strong high molecular weight band, which appeared to be approximately 100 base pairs smaller (i.e., 3.6 kb) than the corresponding band (3.7 kb) in HUVE cells. The first RNA preparation of bovine endothelium scraped from four fresh aortas was negative; a second preparation, obtained from eight aortas, showed a weak band after long exposure, migrating at the same molecular weight as the prominent band in cultured BAE cells. HUT 102 cells showed a major band migrating with the single HUVE cell *sis* band at 3.7 kb. Westin *et al.* (15) gave an estimate of 4.2 kb for the HUT 102 *sis* RNA; this apparent discrepancy in size is likely due to differences in method, including reference markers and gel systems used [for example, we used formaldehyde denaturing agarose gels, whereas Westin *et al.* (15) used methylmercury denaturing gels]. The HUT 102 cells also showed two distinct lower molecular weight bands, at approximately 2.6 kb and 1.3 kb. Low molecular weight forms have been previously described in RNA from tumor cells (16). Although we did not observe secondary bands in any of the endothelial cell RNA preparations, cultured fetal human aortic SMC did show a 1.3-kb band similar to HUT 102 cells. In addition, a diffuse

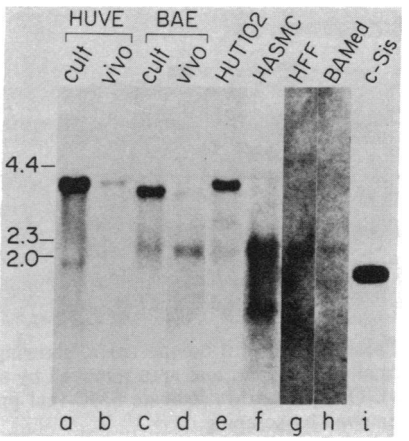

FIG. 1. Expression of the *sis* gene. RNA samples were electrophoresed in agarose, transferred to nitrocellulose, and hybridized with a c-*sis* probe. Lanes: a, 14 μg of *in vitro* HUVE cell total RNA, exposed for 2 days; b, 14 μg of *in vivo* HUVE cell total RNA, exposed for 2 days; c, 8 μg of *in vitro* BAE cell total RNA, exposed for 2 days; d, 5 μg of *in vivo* bovine aortic endothelium poly(A)$^{+*}$ RNA, exposed for 25 days; e, 10 μg of HUT 102 poly(A)$^{+\dagger}$ RNA, exposed for 2 days; f, 20 μg of *in vitro* fetal human aortic SMC poly(A)$^{+*}$ RNA exposed for 25 days; g, 5 μg of human foreskin fibroblast poly(A)$^{+*}$ RNA, exposed for 21 days; h, 5 μg of poly(A)$^{+*}$ RNA, extracted from freshly obtained bovine aortic media, exposed for 21 days; i, c-*sis* plasmid reconstruction. On the left are positions of size markers, given in kb.

* Poly(A)$^+$ RNA selected once over oligo(dT)-cellulose.

† Poly(A)$^+$ RNA selected twice over oligo(dT)-cellulose.

area of hybridization was generally seen between 2.0 and 2.3 kb both in specimens showing a strong 3.7-kb band and in those showing none. We have tentatively interpreted this to be due to weak hybridization with rRNA bands. No 3.7-kb band could be detected in poly(A)$^+$ RNA preparations from cultured human foreskin fibroblasts, cultured fetal human aortic SMC, or freshly obtained adult bovine aortic media.

Cells obtained by limited enzymatic digestion of fresh umbilical vein have been shown to contain only rare SMC as judged by the morphology of resultant cultures (9). Since we have only rarely seen cells with the appearance of SMC in our cultures, we believe that the human umbilical vein cells expressing the *sis* gene are indeed endothelial cells. On the other hand, to get maximum yields when scraping bovine aorta for *in vivo* RNA assay (not for *in vitro* assay), we scraped firmly enough to remove not just endothelial cells but also immediately underlying SMC. As shown in Fig. 2,

Table 1. Relative intensities of *sis* signals

RNA source	Relative signal intensity
HUVE cells cultured	100
HUVE cells *in vivo*	10
BAE cells cultured	25
BAE cells *in vivo**	0.3
HUT 102†	2
Human aortic SMC*	NSD
Human foreskin fibroblasts*	NSD
Bovine aortic media*	NSD

Intensities of the major 3.6- to 3.7-kb band were visually determined and standardized per microgram of total cellular RNA. When poly(A)$^+$ RNA was used, the indicated additional corrections were made (as described in *Materials and Methods*). NSD, no signal detected.

*Poly(A)$^+$ RNA selected once over oligo(dT)-cellulose; correction factor = 8.

†Poly(A)$^+$ RNA selected twice over oligo(dT)-cellulose; correction factor = 25.

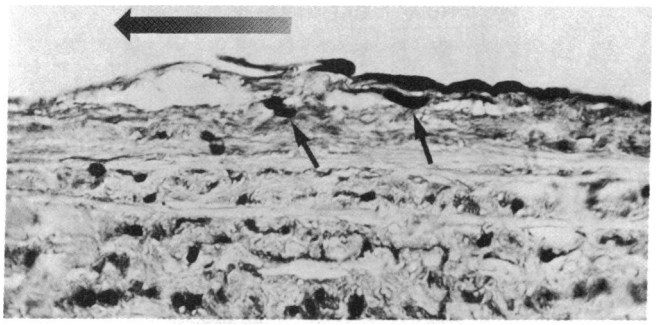

FIG. 2. Cross-section of a bovine aorta, showing the junction between normal endothelium and area removed by scraping (horizontal arrow). Oblique arrows indicate SMC that probably would have been removed by scraping.

endothelium together with some underlying SMC extending down to the first major band of elastic lamina was removed. Examination of multiple histologic sections of the scraped vessel indicates that the SMC contamination of these endothelial cell preparations may be between 35% and 50% of the total cell number (data not shown). However, it seems unlikely that the *sis* RNA could be produced by these SMC immediately underlying the endothelium, since we have not been able to detect a *sis* message in freshly removed adult bovine aortic media (see Fig. 1, lane f, and Table 1). Because our *in vivo* BAE cell sample may have been diluted by a significant proportion of SMC, our estimate for *in vivo* bovine endothelial *sis* signal intensity given in Table 1 could be lower than the true signal intensity per microgram of endothelial cell RNA.

DISCUSSION

Our results indicate that both human and bovine large vessel endothelial cells can express the c-*sis* gene at readily detectable levels and produce a single, approximately 3.7-kb, RNA band. This differs from the T-cell lymphoma line HUT 102 and mesenchymal tumor cell lines (16), in which additional lower molecular weight RNA species are also produced. These two endothelial cell types have been shown to secrete significant amounts of a PDGF-like protein *in vitro* (4, 5). Our results indicate that at least some of this PDGF-like mitogenic activity is coded for by the *sis* gene and consists of PDGF-I (7). Until the gene coding for PDGF-II is identified and the potentially different types of PDGF secreted by endothelial cells are determined, it will not be clear whether the *sis* gene is responsible for all or only part of the endothelial cell PDGF activity. Our data are strongly suggestive that endothelial cells *in vivo* also express the *sis* gene unless gene activation occurs during the time of collection or, in the case of human umbilical vein cells, during labor and delivery.

The possible biological role of a PDGF-like protein secreted from endothelial cells remains speculative. Our data show that in large vessel endothelial cells the *sis* gene is activated in response to cell culture conditions. Detection of moderate levels of *sis* expression in human umbilical vein endothelium *in vivo* raises the possibility that the *sis* gene product could have a role in the normal growth and development of vessel wall. Persistent low level expression *in vivo* by bovine aortic endothelium suggests the *sis* gene could have a continuing role in maintenance of adult vessel wall as well. Since cultured large vessel endothelial cells lack PDGF receptors, while cultured aortic SMC and fibroblasts express receptors (17), a likely target for the *sis* gene product *in vivo* is SMC and, under certain circumstances, possibly fibroblasts. Assuming the *sis* RNA is both translated and secreted, an obviously important point will be the direction of secretion, whether luminal or through the basement membrane. Luminal secretion raises the possibility of interaction with platelets, other blood components, or even a systemic effect, whereas secretion through the basement membrane suggests a direct effect on SMC. If small vessel and capillary endothelial cells are likewise able to synthesize the *sis* gene product, this suggests that PDGF could have a role in the recruitment and organization of SMC and fibroblasts during the neovascular response, since PDGF is both chemotactic (18, 19) and mitogenic. Finally, production of the *sis* gene product under specific circumstances could be important in the pathobiology of the vessel wall, such as during the SMC migration and proliferation of atherosclerosis.

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