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Erythropoietin receptor mRNA expression in human endothelial cells

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ABSTRACT **A previous report demonstrated that endothelial cells have erythropoietin receptors and respond to this hormone with enhanced proliferation. The present study demonstrates the existence of mRNA for erythropoietin receptor in human umbilical vein endothelial cells. We have reverse transcribed mRNA of endothelial cells and then used different PCR primers to amplify erythropoietin receptor target cDNA between exons 5 and 6 as well as 3–5 in addition to an internal standard DNA fragment. Correspondence of size as well as location of restriction endonuclease scission (*Ava* II) was used in comparing the amplified fragments of human endothelial cell erythropoietin receptor to those of two human erythroleukemia cell lines, OCIM1 and K562. No α- or γ-globin mRNA was detected in endothelial cells but was readily demonstrable in OCIM1 cells. In addition, to determine whether the expression of human erythropoietin receptor on endothelial cells occurs *in vivo*, sections of umbilical cord and placenta were immunostained with antibodies against the extracellular portion of the receptor; the results showed strong positive staining of the vascular endothelium.**

Erythropoietin (Epo) is a 30,400-Da polypeptide known to be the principal hormone regulating the proliferation, differentiation, and survival of erythroid cells (1). Its cell surface receptors, which belong to the hematopoietic (cytokine) receptor superfamily (2), are considered highly specific for cells of the erythroid lineage. We have previously reported a proliferative effect of recombinant human erythropoietin (rhEpo) on cultured human umbilical vein endothelial cells (HUVECs) and bovine adrenal capillary endothelial cells (3) and Carlini *et al.* (4) have described a similar mitogenic effect on bovine pulmonary artery endothelial cells. In this study we used PCR techniques to detect and quantitate human Epo receptor (hEpoR) gene transcripts in HUVECs and compared the abundance of this mRNA with that expressed in the erythroleukemia cell lines OCIM1 and K562. K562 cells express only 4–6 hEpoRs per cell on average (5), whereas OCIM1 cells exhibit about 3000 of these receptors per cell (6). As a negative control we used HeLa cells, which do not express hEpoR. In addition, we utilized a monoclonal antibody to the extracytoplasmic portion of hEpoR (37) which has recently become available to immunostain the endothelial lining of human blood vessels and demonstrated strong reactivity of the vascular endothelium.

MATERIALS AND METHODS

HUVEC. HUVECs were obtained from umbilical cords from cesarean sections. The cells were cultured by standard methods in the presence of heparin and endothelial-cell growth supplement (7). They were characterized by their homogeneous and typical cobblestone morphology, factor VIII antigen positivity, and the presence of Weibel–Palade bodies on electron microscopy. HUVECs were used for these studies after three to five passages.

For clonal culture to exclude contamination with hematopoietic stem cells, HUVECs (1–5 × 10^5 cells per ml) were plated in methylcellulose cultures as described (8), with the modification that half of the fetal bovine serum was replaced with human umbilical cord blood serum. Hematopoietic growth factors were added as follows: rhEpo, 2 units/ml; stem-cell factor, 10 μg/ml; granulocyte/macrophage-colony-stimulating factor, 200 units/ml; interleukin 3, 200 units/ml; endothelial-cell growth factor, 20 μg/ml.

Preparation of mRNA. After 1–5 × 10^7 cells were harvested and washed twice with phosphate-buffered saline, RNA was extracted with guanidinium thiocyanate and lauryl sarcosinate (9). mRNA was adsorbed onto oligo(dT)-cellulose columns (Pharmacia) and, after the columns were washed with high- and low-salt solutions, was eluted with 10 mM Tris·HCl buffer containing 1 mM EDTA (pH 7.4) at 65°C. Total amount and concentration of mRNA were determined spectrophotometrically and confirmed by agarose gel electrophoresis.

Reverse Transcription of RNA and Amplification of cDNA. An aliquot of mRNA (0.5–1 μg) was used for reverse transcription according to Gubler and Hoffmann (10). Synthesis of the first-strand cDNA began at the 3′ end of poly(A)$^+$ mRNA by using $(dT)_{15}$ primer in 20 μl of 50 mM Tris·HCl, pH 8.5/8 mM $MgCl_2$/30 mM KCl/0.01 mM dithiothreitol containing 8 units of RNase inhibitor (Boehringer Mannheim), 1 μM each dNTP, and 40 units of avian myeloblastoma virus reverse transcriptase (Boehringer Mannheim). The reaction was carried out at 42°C for 60 min in a DNA thermal cycler (Perkin–Elmer). After the reaction was stopped, 5–10 μl of sample was used for PCR amplification. Synthetic oligonucleotide primers from exon 5 (sense, 5′-GCA-CCG-AGT-GTG-TGC-TGA-CGA-A-3′) and exon 6 (antisense, 5′-GGT-CAG-CAG-CAC-CAG-CAT-GAC-3′) were used to amplify hEpoR target cDNA, giving rise to a 197-bp fragment (38). Results from endothelial cells were compared with results from OCIM1 cells (positive control) and HeLa cells (negative control). To determine the specificity of the reaction, the product of the PCR amplification was subjected to digestion with the restriction endonuclease *Ava* II to obtain the expected 57-bp and 140-bp fragments.

Quantitative PCR. To quantify hEpoR mRNA, mRNA was reverse transcribed (see above) and multiple reaction mixtures were made with the resultant cDNA. Serial dilutions of

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Abbreviations: (r)hEpo, (recombinant) human erythropoietin; hEpoR, human Epo receptor; HUVEC, human umbilical vein endothelial cell; vWF, von Willebrand factor.

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an internal standard were added to each reaction mixture and coamplified (11). The internal standard was constructed by PCR of hEpoR cDNA using the primer set 5′-GCGTCCCT-CTAGAGTTCGCGCT-3′ and 5′-TGGCTCATCCGCTAGG-CGTCAG-3′ and includes a 378-bp fragment extending from exon 3 to exon 5 of hEpoR (35). PCR amplification using *Taq* DNA polymerase Stoffel fragment (Perkin–Elmer) was performed in 10 mM Tris·HCl, pH 8.3/10 mM KCl/3 mM $MgCl_2$/0.2 mM dNTPs; 0.25–0.5 μg of mRNA was used for each PCR. Standard DNA and hEpoR primers were used at concentrations varying from 20 to 50 ng for HUVEC and OCIM1 mRNA. [α-^{32}P]dCTP (2.5–3.0 μCi; 1 μCi = 37 kBq) was added. The thermal cycler program was begun with a melting step, 3 min at 94°C, followed by 30–35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min. Final extension was at 72°C for 7 min. Products of the reaction were analyzed by electrophoresis in 2–2.4% agarose gels (SeaKem ME/NuSieve GTG, 1:2; FMC). The bands representing hEpoR and the standard fragment were cut out for scintillation counting. hEpoR was quantified based on the amount of standard added and on radioactivity of the standard and of the hEpoR cDNA-specific band.

Immunohistochemistry. Representative sections from fresh human placentas and umbilical cords were fixed for 6–18 hr in 96% absolute ethanol/1% glacial acetic acid/3% distilled water. The tissue was processed manually and embedded in paraffin. Sections of 5 μm were cut, heated at 55°C for 1 hr, deparaffinized in Clear-Rite-3 (Richard Allen, Richland, MI), and rehydrated in graded ethanol solutions to water. Endogenous peroxidase activity was quenched by freshly prepared 3% hydrogen peroxide for 30 min, and the slides were rinsed and placed in phosphate-buffered saline (pH 7.3). Monoclonal anti-human erythropoietin receptor antibody (mh2er/16.5.1, mouse IgG1, affinity purified on protein A) (Genetics Institute, Cambridge, MA) was added at a dilution of 1:25 and incubated for 75 min. Biotinylated anti-mouse IgG was used as secondary antibody and was recognized by streptavidin-peroxidase (labeled streptavidin-biotin kit, K-681; Dako); peroxidase activity was analyzed with 3-amino-9-ethylcarbazole (Vector Laboratories) as chromogen. The sections were counterstained with Harris hematoxylin. Similar tissue sections were stained for von Willebrand factor (vWF) with the standard Dako kit.

RESULTS AND DISCUSSION

Since umbilical cord blood is rich in hematopoietic stem cells, we took several safeguards to assure that the target cells were indeed endothelial cells and not a small number of contaminating hematopoietic progenitor cells. We extensively washed the umbilical cords free of contaminating blood, removed repeatedly all nonadherent cells, and saw no evidence of emperipolesis. More than 95% of the cells were positive for vWF. To avoid ingrowth of fibroblasts, we used HUVECs only at passages 3–5 from individual donors. We observed no proliferative effect of rhEpo on umbilical cord-derived fibroblasts or smooth muscle cells. Bovine adrenal capillary endothelial cells respond to rhEpo similarly to HUVECs even after 11–18 passages (3). HUVECs plated in a methylcellulose stem-cell culture system in the presence of endothelial-cell growth factor produced a homogeneous endothelial layer with typical cobblestone morphology. Addition of hematopoietic growth factors failed to give rise to any hematopoietic colonies over a period of 1–4 weeks but did stimulate "capillary tube" formation as described in other angiogenic assays.

To demonstrate the presence of hEpoR mRNA in HUVECs, RNA PCR was performed with avian myeloblastosis virus reverse transcriptase and $(dT)_{15}$ primer. Second-strand cDNA synthesis and amplification of cDNA were performed with primers from exon 5 (sense) and exon 6 (antisense) of hEpoR mRNA (Fig. 1). The amplified fragment was subjected to digestion with *Ava* II, which splits the 197-bp target into 140- and 57-bp fragments. HUVECs yielded the expected fragment sizes, which were identical to those of OCIM1 cells, confirmation that the amplification product represented hEpoR mRNA. mRNA extracted from HeLa cells did not exhibit any amplification product in the 197- to 57-bp range (Fig. 1*A*). Amplification of a different target cDNA of HUVECs corresponding to a 265-bp fragment extending from exon 1 to exon 3 of hEpoR mRNA showed results identical to those obtained with OCIM1, as well as with K562 cells (Fig. 1 *B* and *C*). HeLa cells exhibited no evidence of this mRNA. The abundance of hEpoR mRNA evaluated by comparison with that of coamplified β-actin cDNA revealed roughly similar levels in HUVECs and K562 cells which were distinctly lower than those of OCIM1 cells. These studies were complemented by quantification of hEpoR mRNA (Fig. 2)

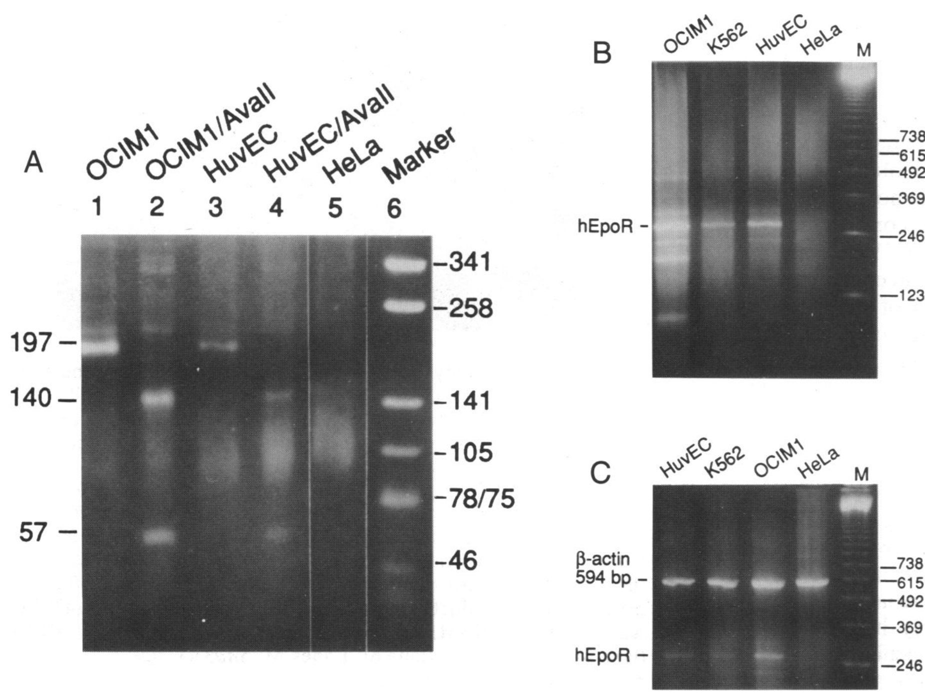

FIG. 1. Analysis of hEpoR mRNA in HUVECs by PCR. cDNA was obtained by reverse transcription of mRNA from HUVECs and erythroid (OCIM1) and nonerythroid (HeLa) cell lines. (*A*) Lanes 1 and 3 show PCR products obtained with hEpoR-specific primers for OCIM1 cells and HUVECs, respectively, after 30 cycles of amplification. Specificity of PCR products was demonstrated by generation of a 57-bp and a 140-bp fragment after *Ava* II digestion for OCIM1 cells and HUVECs in lanes 2 and 4, respectively. The PCR products obtained with HeLa cells are shown in lane 5. (*B* and *C*) For coamplification of hEpoR without (*B*) and with (*C*) β-actin as control, another set of specific primers, giving a PCR product of 265 bp, was used. Coamplification of β-actin and hEpoR cDNAs (*C*) provides an assessment of hEpoR mRNA levels by comparison of the β-actin-specific fragment of 594 bp with the hEpoR-specific fragment of 265 bp for each lane. Lane M, size markers.

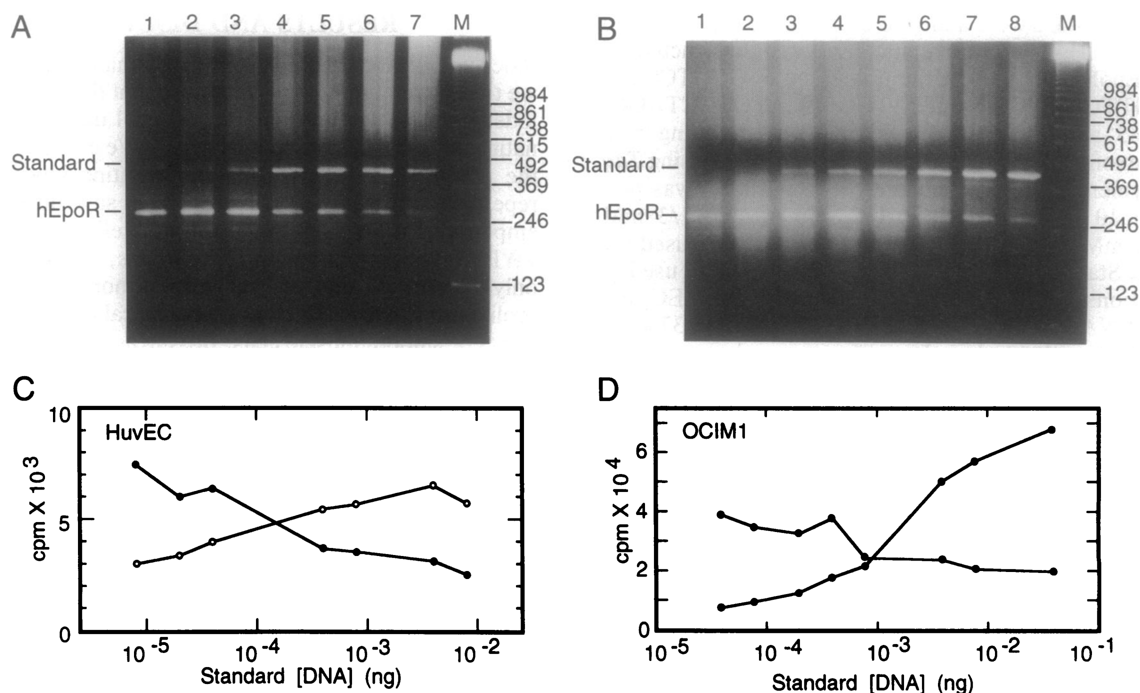

FIG. 2. Quantification of hEpoR mRNA by PCR in HUVECs and OCIM1 cells. cDNA was reverse transcribed from mRNA isolated from HUVECs (*A*) and OCIM1 cells (*B*). Multiple reactions (lanes 1–7 for HUVECs and lanes 1–8 for OCIM1 cells) for hEpoR-specific PCR amplification of cDNA were carried out with hEpoR primers. Prior to PCR amplification, increasing amounts of standard DNA were added to each reaction. At low amounts of standard the hEpoR-specific PCR product (hEpoR) was dominant. At high amounts of standard, the PCR product of 427 bp corresponding to the standard was dominant. Lane M, size markers. (*C* and *D*) To quantify the PCR products, [α-^{32}P]dCTP was added to each PCR mixture for HUVEC (*C*) and OCIM1 (*D*) cDNA. ○, Internal standard; ●, hEpoR-specific PCR product. The amounts of radioactivity recovered in the hEpoR and standard fragments are plotted against the amount of standard added to each reaction mixture. The amount of standard corresponding to the point at which the two PCR products are equal is an indication of the amount of hEpoR cDNA present.

with an internal standard constructed from an overlapping segment of the hEpoR target sequence (12). The amount of cDNA/μg of mRNA of duplicate quantitative PCR experiments was 3.0×10^{-4} ng for HUVECs, 4.8×10^{-3} ng for OCIM1, and 2.3×10^{-4} ng for K562 cells.

To further exclude the possibility that the hEpoR mRNA detected was produced by small numbers of hematopoietic progenitors that remained undetected in our clonal cultures, we added to our standard HUVEC cultures (6) hematopoietic growth factors at concentrations similar to those of our clonal cultures, changing the supplemented medium every 3 days. After 10 days, all cells were harvested and their mRNA was extracted and analyzed by PCR for the presence of α- and γ-globin mRNA, using reverse transcriptase (for conversion to cDNA) and primer pairs specific for each globin type (Fig. 3). PCR products corresponding to α-globin and γ-globin transcripts were found in OCIM1 cells but not in HUVECs or HeLa cells. These results provide further confirmation of the absence of erythroid precursors in our HUVEC cultures.

Immunostaining of umbilical cord and placental vessels with antibody to vWF showed strong reactivity of the endothelium of umbilical veins and of placental blood vessels having muscle walls (Fig. 4 *C* and *D*). Staining of septal and villous thin capillaries of placental tissue was weak and inconsistent. Similar results were obtained when umbilical cord and placenta tissue sections were reacted with mouse anti-hEpoR monoclonal antibody mh2er/16.5.1. This antibody binds to the soluble version of hEpoR on several receptor-positive cell lines and inhibits binding of Epo to its receptor (13). The endothelium of umbilical veins (Fig. 4*A*) and of placental septal blood vessels containing muscle wall (Fig. 4*B*) showed strong EpoR positivity. Placental capillaries and other cells were negative (Fig. 4*B*). Vascular endothelial cells are remarkably heterogeneous in terms of morphology, expression of molecular markers, and biological function (14) and many capillaries are vWF negative

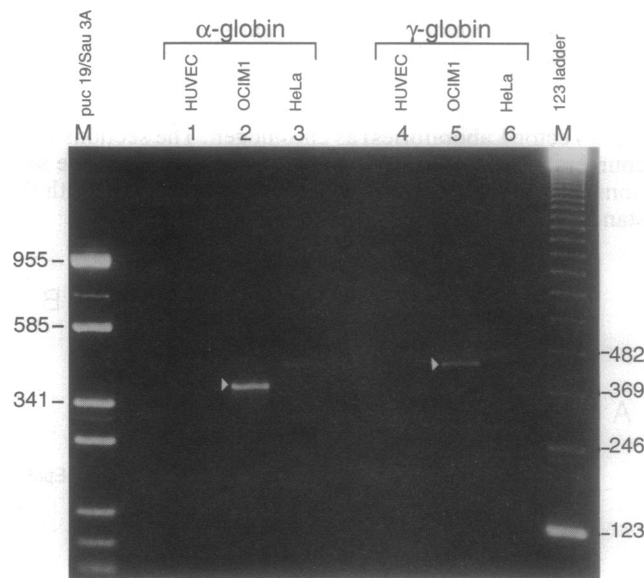

FIG. 3. PCR analysis of HUVEC mRNA for globin gene transcripts. mRNA was prepared from HUVECs, OCIM1 cells, and HeLa cells. PCR analysis for α-globin and γ-globin transcripts was carried out using reverse transcriptase and primers specific for α-globin cDNA (lanes 1–3) and γ-globin cDNA (lanes 4–6). The results for HUVECs are in lanes 1 and 4, for OCIM1 cells in lanes 2 and 5, and for HeLa cells in lanes 3 and 6. PCR product corresponding to α-globin transcripts (372 bp) was detected in OCIM1 cells (lane 2, arrowhead) but not in HUVECs (lane 1) or HeLa cells (lane 3). The background bands in lanes 1 and 3 are nonspecific PCR products. PCR product corresponding to γ-globin transcripts (433 bp) was detected in OCIM1 cells (lane 5, arrowhead) but not in HUVECs (lane 4) or HeLa cells (lane 6). Lanes M, *Sau*3A1-digested pUC19/plasmid (left) and a 123-bp ladder (BRL) (right).

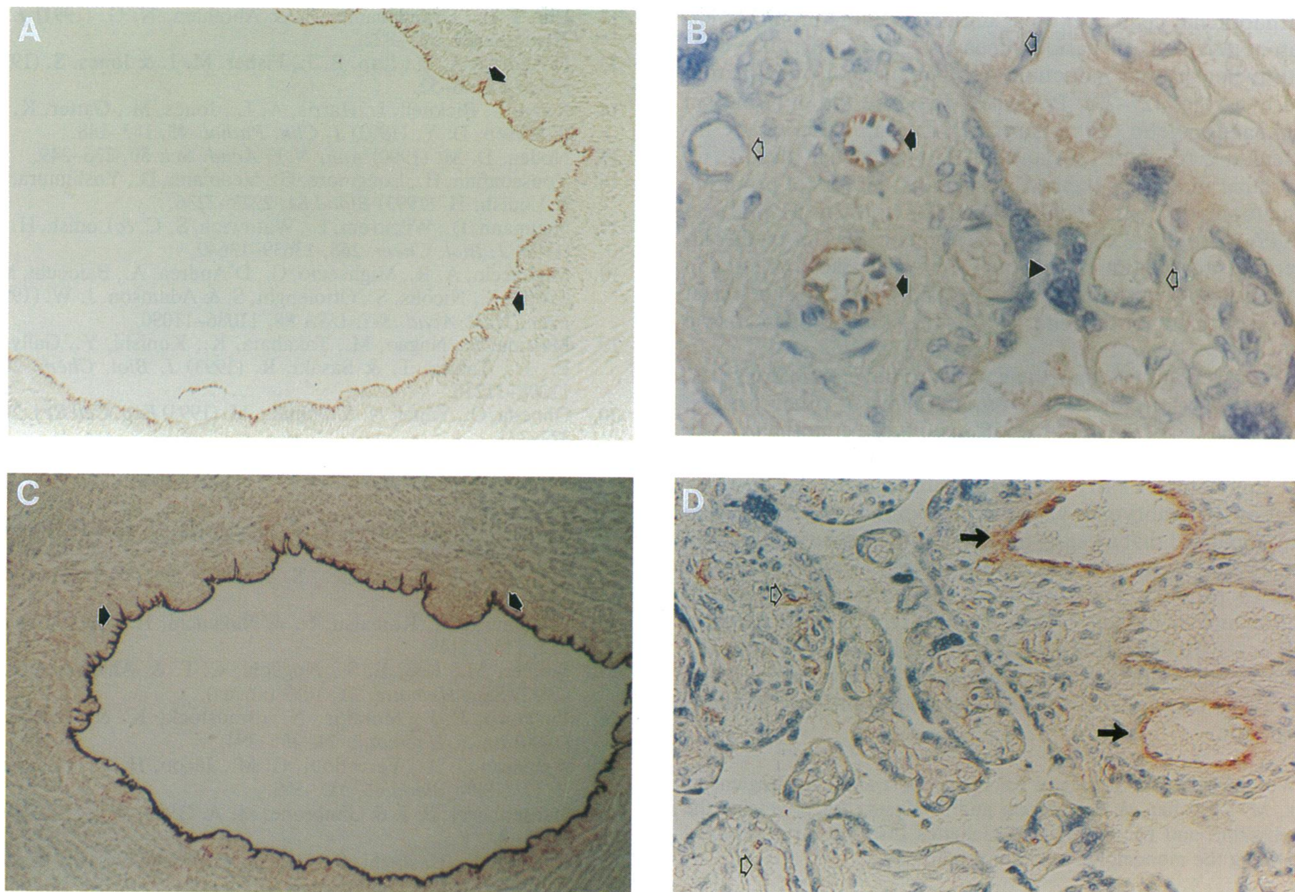

FIG. 4. Expression of vWF (*C* and *D*) and EpoR (*A* and *B*) in umbilical cord vein endothelial cells (*A* and *C*) and placenta (*B* and *D*). Acid ethanol-fixed, paraffin-embedded tissues were treated with anti-vWF or anti-EpoR antibody followed by a peroxidase-conjugated secondary antibody system. (*A*) Umbilical cord vein endothelial cells are strongly decorated with anti-hEpoR peroxidase (arrow). Smooth muscle of the wall is negative. (×35.) (*B*) Placental septal blood vessels with muscle wall show EpoR reactivity (filled arrow) while villous and septal capillaries are negative (open arrow). The interstitial tissue of septa and villi and the syncytiotrophoblastic cells are negative (arrowhead). (×220.) (*C*) Umbilical cord vein endothelial cells are decorated with anti-vWF peroxidase (arrow). (×35.) (*D*) Placental septal blood vessels with muscle wall are strongly positive for vWF (filled arrow) while the septal and villous thin capillaries show weak and inconsistent staining (open arrow). (×90.)

(15). Although the histochemical staining pattern of anti-hEpoR seems similar to that of anti-vWF, we do not yet have detailed comparative studies in other tissues to know whether this congruence extends to other vascular areas.

Our previous studies have shown that HUVECs have many more EpoRs than OCIM1 cells or other erythroid cells; however, the quantity of hEpoR mRNA in HUVECs as shown in this study is less than that in OCIM1 cells. We have no experimental evidence to explain this discrepancy, but several explanations can be suggested. In erythroid cells, the mature EpoR has an exceedingly short half-life (45–60 min), in sharp contrast to other receptors—e.g., transferrin, insulin, and asialoglycoprotein receptors, which have half-lives from 7 to 20 hr. Less than 5% of the EpoRs are found on the cell surface (16, 17). Migliaccio *et al.* (18) have proposed that it is the cellular environment in which the EpoR gene is expressed, and not simply its expression, that determines the erythroid-specific processing and function of EpoR.

The concept of strict erythroid or even hematopoietic specificity of EpoR is being challenged not only by our studies but also those of Masuda *et al.* (19), who have recently described the presence of EpoR in two rodent cell lines of neural origin. Ohneda *et al.* (20) also observed a dose-dependent mitogenic response to Epo of murine fetal liver stromal cells and detected EpoR mRNA in those cells. They hypothesized a possible involvement of Epo in the development of the fetal hematopoietic microenvironment.

Although functional high-affinity EpoR is expressed mostly at the erythroid-colony-forming unit and proerythroblast level—i.e., in erythroid cells of very late maturation stage (1)—Heberlein *et al.* (21) observed low levels of EpoR gene transcripts in embryonal or multipotential cell lines. These primitive cells had 10–100 times less EpoR mRNA than more mature stages of erythroid precursors. In fact, the EpoR gene seems to be expressed at low levels before either hematopoietic or erythroid commitment has occurred (21, 22), giving rise to speculation that EpoR in these early cells can transmit a proliferative signal either by itself or in synergy with other cytokine receptors (21).

Epo exerts primarily a mitogenic effect on early erythroid precursors and a mostly differentiating effect on later erythroid precursors (1). Erythroid cells or cell lines which respond to exogenous Epo by proliferation only (or are unresponsive) often have a single class of low-affinity receptors (23). Primitive erythroid progenitor cells ($CD34^+$, $CD71^-$) contain a truncated form of the receptor capable of transducing a mitogenic signal, whereas more mature erythroid cells ($CD34^-$, $CD71^+$) contain the full-length receptor (24). The mitogenic effect of Epo on HUVECs seems similar to that on primitive stem cells. In HUVECs, we found an Epo-induced increase in cell number and [^{3}H]thymidine incorporation (3) and a decrease in the phosphorylation of $p34^{cdc2}$ (25), all in association with a high number of low-affinity hEpoRs.

Endothelial cells coexpress antigens characteristic of hematopoietic cells of various lineages (26) and produce constitutively, or after induction, hematopoietic growth factors and cytokines (27-29). Presently, they are the only human nonhematopoietic cells expressing the p55 chain of the interleukin 2 receptor (30) and, as shown by us, hEpoR. Of particular interest is their expression of markers of primitive hematopoietic cells, such as the CD34 antigen (31, 32) and GATA-2 protein (33). GATA-1 is recognized as an erythroid-specific transcription factor, but GATA-2 is also expressed in more primitive progenitor cells, possibly signaling the onset of commitment of mesoderm to form hematopoietic tissue (34).

The vascular system is the earliest to develop in embryos, to best serve the metabolic requirements of other tissues. Although the presence of hEpoR in cultured endothelial cells cannot be *a priori* equated to *in vivo* situations, it does suggest that Epo controls early mitogenic pathways for the development of the embryonic vasculature. Schmitt *et al.* (22) have also suggested that an early action of Epo may be to stimulate the development and activation of embryonic endothelial cells to produce a varied array of hematopoietic growth factors and cytokines. In fact the first recognizable hematopoietic tissue, the blood islands of the yolk sac, are composed of only endothelial cells and blood cells (35, 36). At this time, however, the *in vivo* role of endothelial hEpoR remains enigmatic.

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