Expression of the Erythropoietin Receptor by Trophoblast Cells in the Human Placenta

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ABSTRACT

Nonclassical sites of erythropoietin (EPO) and erythropoietin receptor (EPO-R) expression have been described that suggest new physiological roles for this hormone unrelated to erythropoiesis. The recent finding of EPO expression by trophoblast cells in the human placenta prompted us to consider whether these cells also express EPO-R. With use of immunocytochemistry, EPO-R was identified in villous and extravillous cytotrophoblast cells, as well as in the syncytiotrophoblast at all gestational ages. EPO-R was also expressed by cells within the villous core, including endothelial cells of fetoplacental blood vessels. Placental tissues and isolated and immunopurified trophoblast cells, as well as trophoblast-derived choriocarcinoma Jar cells, expressed immunoreactive EPO-R on Western blot. EPO-R mRNA was also detected in the same placental tissues and trophoblast cells by nested-primer reverse transcription-polymerase chain reaction. Finally, EPO-R was functional insofar as the receptor was phosphorylated on tyrosine residues in response to exogenous EPO treatment of cultured trophoblast or Jar cells. Thus, the present findings support the hypothesis that trophoblast cells of the human placenta express EPO-R. In view of these results, taken together with previous work demonstrating EPO expression by the same cells, an autocrine role for this hormone in the survival, proliferation, or differentiation of placental trophoblast cells is proposed.

INTRODUCTION

Erythropoietin is a 30.4-kDa glycoprotein critical to the survival, proliferation, and differentiation of erythroid precursor cells [1–3]. The major sites of erythropoietin (EPO) production are the kidney and liver [1, 2, 4, 5]. However, other sites of EPO expression have been reported, including spleen and lung [6], bone marrow macrophages [7], early colony-forming cells [8], umbilical cord monocytes differentiated into a macrophage phenotype in vitro [9], and brain astrocytes [10]. Most recently, our laboratory has reported that trophoblast cells of the human placenta express EPO [11], and we have speculated that, analogous to the effects of other colony-stimulating factors produced by and acting upon trophoblast cells [12], locally derived EPO is involved in placental development. Of course, fundamental to this hypothesis would be the demonstration of EPO receptor expression by trophoblast cells.

The erythropoietin receptor (EPO-R), classically found in erythroid precursor cells [1–3], now has been described in other cell types [13–18] including endothelial cells [19–21]. These novel and nonclassical sites of EPO and EPO-R expression raise the possibility of physiological roles for this hormone not necessarily related to erythropoiesis. The recent finding that human placental trophoblast cells express EPO prompted us to investigate whether they also express EPO-R. If so, this hormonal system may have a potentially important autocrine role in the growth and differentiation of the placenta.

MATERIALS AND METHODS

Human Placentas

Human placentas were obtained from women with normal pregnancies at term immediately after cesarean section for breech presentation or repeat cesarean section. Placentas were also collected from nulliparous women with the diagnosis of preeclampsia immediately after cesarean section (in the absence of any labor). Preeclampsia was defined according to standard criteria: onset of hypertension during late pregnancy with systolic and diastolic blood pressure > 140/90 on at least two occasions and urinary protein > 2+ on dipstick or > 0.3 g/24 h [22]. First- and second-trimester placentas were obtained after elective terminations by vacuum evacuation and dilatation and curettage, respectively. A total of 20 placentas were investigated. Collection of placentas was approved by the Institutional Internal Review Board of the Magee-Womens Hospital.

Placental Processing

Pieces of placental villi from beneath the chorionic and basal plates, as well as basal plate, were quickly dissected (approximately 0.5 g each), rinsed in Dulbecco’s phosphate buffered saline (PBS), snap frozen in liquid nitrogen, and stored at −80°C for RNA and protein extraction. Small placental pieces were also fixed overnight at 4°C in 0.1 N phosphate buffer containing 4% paraformaldehyde, washed in ice-cold phosphate buffer, incubated in phosphate buffer containing 30% sucrose overnight at 4°C, embedded in OCT compound (Bayer, Elkhart, IN), snap frozen in liquid nitrogen, and stored at −80°C until cryosection. Frozen sections (12 μm) were cut and mounted on Fisher Superfrost/Plus (Pittsburgh, PA) glass slides.

Cell Isolation and Culture Procedures

TF-1, HeLa, and Jar cells were obtained from American Type Culture Collection (Rockville, MD). The erythroblastic, bone marrow-derived TF-1 cells served as a positive control for EPO-R expression [23]. These nonadherent cells were seeded in 75-cm² vented tissue culture flasks and grown in RPMI-1640 medium with 1–5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF: Pepro Tech, Rocky Hill, NJ), 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin. The Jar trophoblast-derived choriocarcinoma cells were seeded in 75-cm² vented culture flasks and grown to confluency in RPMI-1640...
medium, 10% FBS, L-glutamine, and penicillin/streptomycin; the HeLa cervical carcinoma cells were grown in Eagle’s Minimum Essential Medium with nonessential amino acids, Earle’s balanced salt solution, 10% FBS, L-glutamine, and penicillin/streptomycin. The HeLa cells have been reported not to express EPO-R [20] and thus served as a negative control. Another positive control cell type for EPO-R expression was fetal mouse liver cells obtained from Day 14 fetal mice and isolated immediately upon dissection by mechanical dispersion [24].

The procedure for trophoblast isolation was based on the method of Kliman et al. [25] with modifications. Briefly, the basal plate was removed, and approximately 50 g of villous tissue was harvested and teased into small fragments. Villous trophoblast cells were released by four sequential trypsin digestions (trypsin grade III; Sigma Chemical Co., St. Louis, MO). DNase I (grade II; Boehringer Mannheim, Indianapolis, IN) was used to prevent cellular aggregation. The first digestion was routinely discarded, as it contained few trophoblast cells and mainly red blood cells and leukocytes. The digests were layered over FBS and centrifuged to remove cellular debris and inactivate trypsin. The trophoblast cells were first separated from other cell types by Percoll (Pharmacia and Upjohn, Kalamazoo, MI) gradient centrifugation and then further purified using magnetic beads (PerSeptive Diagnostics, Cambridge, MA) coupled to anti-human leukocyte antigen-Class I (HLA-A,B,C) (Dako, Carpinteria, CA) for third-trimester placentas, and BioMag anti-CD45 antibody (PerSeptives Diagnostics) for first-trimester placentas. Purity was routinely > 97% as determined by immunocytochemical criteria including positive staining for cytokeratin, and viability was routinely > 95% by Trypan Blue staining. Freshly isolated cells were either snap frozen in liquid nitrogen and stored at −80°C until extraction of RNA or protein, or were immediately placed in 75-cm² tissue culture flasks (8–10 x 10⁶ cells per flask) for a 30-min preincubation at 37°C in RPMI-1640 medium containing 0.5% BSA for analysis of EPO-R phosphorylation.

Immunocytochemistry

Two anti-EPO-R antibodies were used—a monoclonal antibody designated mh2er 16.5.1 from Genetics Institute (Cambridge, MA) directed against secreted recombinant human (rhu) EPO-R [26], and a sheep polyclonal antibody from Upstate Biotechnology Incorporated (Lake Placid, NY) raised against the extracellular domain of the human EPO-R. After permeabilization of the placental tissue sections or cultured cells with 0.3% Triton X-100, quenching of endogenous peroxidase with 0.6% hydrogen peroxide in methanol, and blocking with normal horse serum, the specimens were incubated with either the monoclonal or the polyclonal antibody for 1 h at room temperature. The monoclonal and polyclonal antibodies were used at concentrations of 3–30 μg/ml and 10 μg/ml, respectively, which proved to be optimal based on preliminary experiments. Negative controls were generated by substituting the same concentration of mouse IgG1k isotype for the mouse anti-human EPO-R monoclonal antibody or sheep IgG for the sheep anti-human EPO-R polyclonal antibody. Using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine peroxidase substrate solution, immunoreactive EPO-R was detected. For the sheep primary antibody, a biotinylated rabbit anti-goat secondary antibody was used (Dako). Tissue sections were not counterstained so as to permit demonstration of immunoreactivity in a black-and-white photographic format. After dehydration in ethanol and xylene solutions, a coverslip was applied using Cytoseal XYL (Stephens Scientific, Riverdale, NJ).

Preabsorption experiments were conducted with the mh2er 16.5.1 antibody from Genetics Institute. For these studies, the antibody was used at a final concentration of 3 μg/ml after incubation overnight at 4°C with 3-, 10-, or 30-fold molar excess of soluble EPO-R (Genetics Institute) or of an irrelevant receptor, soluble tumor necrosis factor receptor 1 (TNF-R1) (R & D Systems, Minneapolis, MN). Because of the potential for nonspecific interaction of monoclonal antibodies with cytoskeletal proteins such as cytokeratin [27], which are abundant in the placenta, the EPO-R antibody was also incubated overnight at 4°C with a 30-fold molar excess of the most abundant cytokeratin types in the human placenta, cytokeratins 8 and 18 (Cortex Biochem, San Leandro, CA), or an equal volume of vehicle. In parallel experiments, the monoclonal antibody for cytokeratin 18 (clone CY-90; Sigma) was preabsorbed with the cytokeratin 8/18 to confirm the binding potential of the antigen.

In order to determine whether EPO-R staining was localized to the trophoblast cells, immunocytochemical procedures were performed on adjacent tissue sections using a mouse anti-human cytokeratin monoclonal antibody (1.75 µg/ml; Sigma) to identify the syncytiotrophoblast layer and underlying cytotrophoblast cells of floating villi, as well as extravillous cytotrophoblast cells in the basal plate.

Western Analysis

Tissue homogenates were prepared by homogenizing frozen tissues or cell pellets for 1 min on ice with a Tekmar (Cincinnati, OH) homogenizer in twice the volume of homogenizing buffer (100 mM EDTA diluted 1:2 in methanol that was combined with an equal volume of 100 mM KCl, 50 mM Tris, 50 mM NaF, 10 mM EDTA, 0.5 mM ZnCl₂, 1 mM dithiothreitol, pH 6.8, and a cocktail of protease inhibitors: PMSF, antipain, leupeptin, pepstatin A, chymostatin, and soybean trypsin inhibitor). Samples were centrifuged at 4°C for 15 min at 3000 × g, and supernatants were subsequently centrifuged at 10,000 × g for another 15 min. Protein concentration was determined by the Lowry method. For SDS-PAGE, samples were combined with an equal volume of double-strength sample buffer (0.5% SDS, 5% glycerol, 5% β-mercaptoethanol, 0.005% bromophenol blue in 0.5 M Tris, pH 6.8), boiled for 4 min, and microcentrifuged briefly; then 20 µl was loaded onto either 5–20% gradient gels or single-percentage gels as appropriate and electrophoresed for 1 h at 200 V. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) for 1 h at ~1 mA/cm² using a semi-dry electrophoresis transfer system.

Membranes were rehydrated for 20 min in Tris-buffered saline (TBS, 10 mM Tris, 150 mM NaCl, pH 7.4) containing 0.5% Tween 20 before blocking for 30 min in 3% nonfat dried milk diluted in TBS. Immunoblots were then incubated overnight at 4°C with monoclonal anti-human EPO-R antibody (mh2er 7.9.2; Genetics Institute) diluted 1:1000 in blocking buffer. Although this antibody was also generated against secreted rhuEPO-R, it is not neutralizing, and it recognizes a different epitope than the mh2er 16.5.1 [25]. For negative control blots, the mouse IgG₁ isotype
was substituted for the primary antibody. After a 5-min wash in distilled H₂O and two 10-min washes with TBS plus 0.05% Tween (TBST), blots were incubated for 1 h at room temperature with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Promega, Madison, WI) diluted 1:7500 in TBST. The blots were again washed in distilled H₂O and TBST; this was followed by a TBS wash and equilibration in alkaline phosphatase detection buffer (100 mM Tris, pH 9.5, 150 mM NaCl, 5 mM MgCl₂) for 10 min. The chemiluminescent substrate reagent CDP-Star (Boehringer Mannheim), diluted 1:200 in alkaline phosphatase detection buffer, was reacted with the blots for 5 min, and the membrane was then exposed to X-OMAT (Eastman Kodak, Rochester, NY) film for signal detection.

Additional studies were performed to confirm the specificity of mh2er 7.9.2 binding. The first was a preabsorption, for 2 h at 20°C, of the EPO-R monoclonal antibody with 100-fold molar excess of soluble EPO receptor (Genetics Institute) or nonspecific antigen (recombinant human EPO- gen; Amgen, Thousand Oaks, CA). Additionally, Western blots were probed for expression of cytokeratin to confirm that the EPO-R bands observed were not cross-reacting. The anti-cytokeratin monoclonal antibody reagent used (AE1/AE3; Dako) recognizes cytokeratins designated #1–6, 8, 10, 14–16, 19, and 20 and was incubated at 1:1000 for 2 h at room temperature with the immunoblots.

Protein molecular masses were originally estimated by visualization relative to a prestained standard (Kairoscope broad range; Bio-Rad, Richmond, CA). More precise calculation of molecular mass was then performed by also running unstained SDS-PAGE standards (Bio-Rad), staining the blots with Coomassie blue, and then measuring the relative mobility of each stained band relative to the dye front. The molecular masses of positive EPO-R bands were then interpolated from the generated standard curve.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Restriction Enzyme Digestion

Total RNA was extracted from frozen placental tissues, isolated and immunopurified first- and third-trimester trophoblast cells, JAR trophoblast-derived choriocarcinoma cells, and freshly isolated mouse fetal liver cells by the acid phenol/guanidinium isothiocyanate extraction method [28]. RNA aliquots were stored in 2-propanol at −80°C. Precipitated RNA was redissolved in nuclease-free water and quantitated by determination of absorbancy at 260 nm.

Two micrograms of RNA was annealed with 30 ng of oligo(dT)₁₅ in nuclease-free water (total volume 11 μl). Using the Perkin Elmer GeneAmp PCR System 9600 (Foster City, CA), the mixture was heated at 75°C for 10 min; the temperature was then slowly decreased to 42°C over a 20-min period, and the mixture was maintained at this temperature for 15 min. RT was performed in a total volume of 20 μl containing RT buffer (Promega), dithiothreitol (10 mM final concentration), dNTP (1.0 mM), and avian myeloblastosis virus reverse transcriptase (0.25 U/μl; Promega). Using the Perkin Elmer GeneAmp PCR System 9600, the reaction was heated at 42°C for 45 min followed by 99°C for 5 min to denature the enzyme.

Nested-primer PCR reaction was then performed. The external primers were 5′-CGG-AGT-GTG-TGC-TGA-GCA-3′ and 5′-GGT-CAG-CAG-CAC-CAG-GAT-GAC-3′ corresponding to base pairs (bp) 394–441 in exon 3 and bp 828–848 in exon 7, respectively, and producing a PCR product of 455 bp. The internal primers were 5′-GCA-

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CCG-AGT-GTG-TGC-TGA-GCA-3′ and 5′-GGT-CAG-CAG-CAC-CAG-GAT-GAC-3′ corresponding to base pairs (bp) 605–625 in exon 5 and bp 781–801 in exon 7, respectively, and producing a PCR product of 197 bp. Although the external primer set yielded product in all tissues and cells examined, the nested-primer protocol improved both sensitivity and specificity. The external and internal primers were chosen to span several introns and exons such that amplification of any contaminating genomic DNA would produce products of 3202 and 280 bp, respectively. For the first-primer PCR, 2 μl of the RT product was amplified with Taq Thermosable DNA Polymerase (0.01 U/μl final concentration; Epicentre Technologies, Madison, WI) in a total volume of 20 μl containing PCR buffer without magnesium (Epicentre), external primers (0.2 μM each), dNTP (0.2 mM), and magnesium chloride (1.5 mM). The first-primer PCR reaction was performed in the Perkin Elmer GeneAmp PCR System 9600 for 40 cycles of 94°C (45 sec), 54°C (30 sec), 72°C (30 sec) each. For the second amplification, 1 μl of the first PCR reaction was amplified using the same conditions as described above, except that an annealing temperature of 58°C and 30 cycles were used. For negative controls, the RT reaction was conducted without RNA or without reverse transcriptase.

The PCR products were mixed with 0.1 volume of gel-loading buffer and subjected to electrophoresis on a 2% NuSieve GTG low-melting point agarose (FMC Bioproducts, Rockland, ME) and 1% agarose (Promega) gel in single-strength Tris-borate-EDTA containing 3 μg/ml ethidium bromide.

Restriction enzyme digestion was performed using 10 μl of the nested-primer PCR reaction. One microliter of AvaII was added (1.0 μl/μl final concentration; Promega), and the mixture was incubated for 2 h at 37°C. Both the intact material and cut material were then subjected to agarose gel electrophoresis as described above. Fragments of 140 and 57 bp were expected, if the 197-bp PCR product ultimately derived from EPO-R mRNA [20].

Protein Tyrosine Phosphorylation

Freshly isolated trophoblast cells from the normal term placenta or Jar cells (10 × 10⁶ per treatment group), deprived of serum by incubation in RPMI-1640 containing only 0.5% BSA and antibiotics for 12–18 h, were used in this study. To initiate the experiments, the serum-free medium was replaced with RPMI-1640 with or without recombinant human EPO (40 U/ml; Amgen), and cells were incubated at 37°C for 2 or 5 min. Time zero controls were cells collected after preincubation in serum-free conditions. To quench experiments, flasks containing monolayer cultures of Jar cells were placed on ice, medium was aspirated, cells were rinsed with 2 ml cold PBS+0.1 mM Na₃VO₄, and the monolayer was then scraped into tubes and centrifuged for 1 min at 1800 × g. Trophoblast cells remained in a suspended form, and therefore the collected cells were pelleted by centrifugation for 5 min at 800 × g. Cell pellets were then resuspended in lysis buffer containing 20 mM Tris, pH 7.5, 1% Nonidet-P-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF; and 1 mM Na₃VO₄ with protease inhibitors. After incubation for 20 min at 4°C, lysates were centrifuged for 15 min at 10 000 × g, and the supernatant was stored at −80°C.

Immunoprecipitation was performed by incubating 150 μg protein from the cellular lysates with 4 μg mouse anti-EPO-R monoclonal antibody (clone mh2er 7.9.2) or sub-
Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitors) for 2 h at 4°C. 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.2 mM

binding) in a final volume of 0.5 ml containing immuno-

Labs., West Grove, PA) diluted in 1% BSA in TBST for 1

(TBS. Blots were then incubated with secondary antibody

ments: PY99 monoclonal antibody; Santa Cruz Biotech-

Laboratories, Lexington, KY, and trophoblast cell experi-

temperature with anti-phosphotyrosine antibody (Jar cell

BSA in TBS, the blots were incubated for 2 h at room

polyvinylidene difluoride membrane and blocking in 5%

FIG. 1. Localization of immunoreactive EPO-R in first-trimester human placenta using mouse monoclonal antibody clone mh2er 16.5.1 at 10 μg/ml. A, B) Demonstration of immunoreactivity associated with the villous cytotrophoblast (small arrowhead), syncytiotrophoblast (medium arrowhead), and the endothelium of a fetoplacental vessel (large arrowhead) within the villous core and other villous core cells. Substitution of mouse IgG1k isotype for the primary antibody was used for the negative control depicted in C. See Materials and Methods for details. Original magnification: ×400 (reproduced at 59%).

stituted IgG1k isotype (negative control for nonspecific binding) in a final volume of 0.5 ml containing immuno-

precipitation buffer (10 mM Tris, pH 7.5, 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.2 mM Na<sub>3</sub>VO<sub>4</sub>, and protease inhibitors) for 2 h at 4°C with gentle

rocking. Lysates were further incubated for 2 h after the

addition of 20 μl of Protein A/G PLUS-Agarose (Santa

Cruz Biotechnology, Santa Cruz, CA) per immunoprecipita-

tion reaction. The lysates were then centrifuged for 5 min

at 10 000 × g; pellets were washed four times in immu-

noprecipitation buffer before final resuspension in 20 μl of

double-strength sample buffer and then boiled for separa-

tion by SDS-PAGE as described above. After transfer onto

polyvinylidene difluoride membrane and blocking in 5%

BSA in TBS, the blots were incubated for 2 h at room

temperature with anti-phosphotyrosine antibody (Jar cell

experiments: PY20 monoclonal antibody; Transduction

Laboratories, Lexington, KY, and trophoblast cell experimen-
ts: PY99 monoclonal antibody; Santa Cruz Biotechnology)

followed by two 10-min washes in TBST and then

TBS. Blots were then incubated with secondary antibody

(rabbit anti-mouse Ig, peroxidase-conjugated; Jackson

Labs., West Grove, PA) diluted in 1% BSA in TBST for 1

h followed by another series of washes. Detection was per-

formed using Renaissance Chemiluminescence Reagent

(NEN Life Sciences, Boston, MA) and BioMax MR film

(Eastman Kodak).

RESULTS

Detection of EPO-R Immunoreactivity

Figures 1–3 portray the expression of immunoreactive EPO-R in the human placenta and are representative of three different placentas tested at each trimester of pregnancy. In the first-trimester placentas, immunoreactive EPO-R detected by the monoclonal antibody clone mh2er 16.5.1 was expressed by villous cytotrophoblast cells, syn-
cytiotrophoblast, and fetoplacental vascular endothelium, as well as other cells scattered throughout the villous core (Fig. 1, A and B). The staining was particularly intense at

the membrane interface between syncytiotrophoblast and

underlying cytotrophoblast. Similar results were obtained

with the sheep polyclonal antibody evaluated on the first-

trimester placentas, although the staining was generally less intense but clearly distinguishable from the negative control (data not shown). Using the same monoclonal antibody,

immunoreactive EPO-R was expressed by the syncytiotro-

phoblast, cytotrophoblast cell columns, and villous core

cells in the second-trimester placentas (Fig. 2, A and B).

Endovascular and intravascular cytotrophoblast cells were also positive for EPO-R immunoreactivity (data not shown). Immunoreactive EPO-R was also observed in the
trophoblast layer and fetoplacental vascular endothelium, as

well as in cells within the villous core and basal plate of

third-trimester placentas using monoclonal antibody clone

mh2er 16.5.1 (Fig. 3, A and B). All negative controls gen-
erated by substituting the mouse IgG1k isotype for the

primary antibody were virtually devoid of any staining (Figs.

1C, 2C, and 3C). In preabsorption studies, 3-, 10-, and 30-

fold molar excess of soluble TNF-R1 was without effect (Fig. 4, A–C). Finally, 30-fold molar excess of cytokeratin 8/18 did not affect

EPO-R immunoreactivity (Fig. 4D), whereas cytokeratin

staining after preabsorption was typically reduced by 50%

(data not shown).

Figure 5 shows the detection of a 66-kDa band by West-

ern analysis in the human placenta and is representative of

three different placentas tested at each trimester of normal

FIG. 2. Localization of immunoreactive EPO-R in second-trimester hu-

man placenta using mouse monoclonal antibody clone mh2er 16.5.1 at 30 μg/ml. A) Cytokeratin staining identifying the cytotrophoblast cell columns (large arrowhead) emanating from an anchoring villus into the basal plate. B) EPO-R immunoreactivity in the cytotrophoblast cell columns; C) the negative control generated by substituting mouse IgG1k isotype for the primary antibody. Original magnification: ×200 (reproduced at 62%).

FIG. 3. Localization of immunoreactive EPO-R in third-trimester human placenta using mouse monoclonal antibody clone mh2er 16.5.1 at 30 μg/ml. A) Cells expressing immunoreactivity within the basal plate (small arrowhead). B) EPO-R immunoreactivity associated with the endothelium of fetoplacental vessels (large arrowhead) and villous trophoblast (medi-

imum arrowhead). Substitution of mouse IgG1k isotype for the primary antibody was used for the negative control depicted in C. Original magnification: ×200 (reproduced at 61%).
pregnancy and at preeclamptic pregnancy. Moreover, immunoreactive EPO-R was evident in isolated, immunopurified first- and third-trimester cytotrophoblast cells. A band of lesser intensity was observed in placental homogenates, the molecular size of which was calculated to be $\sim 78$ kDa. Figure 6 portrays the detection of the immunoreactive 66-kDa EPO-R by Western analysis in the positive control TF-1 cells and in Jar trophoblast-derived choriocarcinoma cells, but not in the negative control HeLa cells. Negative control blots (Fig. 5B and Fig. 6, right panel) generated by substituting mouse IgG1 isotype for the primary antibody were virtually devoid of any bands. As a further control in Western blotting experiments, the 7.9.2 monoclonal antibody was also preabsorbed with soluble EPO-R (Fig. 7). The intensity of the 66-kDa band in TF-1 cells and purified trophoblast cells was considerably reduced compared to that obtained after incubation with 7.9.2 antibody alone, and the EPO-R band was eliminated altogether in placental homogenates. Band intensity was not affected when the EPO-R antibody was preabsorbed with a nonspecific antigen (rhEPO, data not shown). A larger molecular size band was also apparent ($\sim 90$ kDa), but it was not diminished by preabsorption of the 7.9.2 monoclonal antibody with soluble EPO-R. In contrast, a lower molecular size band ($\sim 45$ kDa, possibly the truncated EPO-R; see Discussion) was eliminated by the preabsorption protocol (data not shown). Finally, to ensure that the EPO-R monoclonal antibody was not cross-reacting with cytokeratins, simultaneous detection of EPO-R and placental cytokeratin with a pan-antibody was performed (Fig. 8, A and B). Immunoreactive EPO-R and cytokeratins bands were not overlapping. Although the pan-cytokeratin antibody does not detect cytokeratin 9, which is 64 kDa, this cytokeratin has been found exclusively in the suprabasal epidermis of the footpad [29].
Detection of EPO-R mRNA

By loading 20 μg of total RNA or poly(A)⁺ RNA from the villous placenta and from isolated first- and third-trimester trophoblast cells, we were able to detect a faint hybridization signal by Northern analysis at approximately 1.8 kilobases (data not shown). However, we used RT-PCR to verify the expression of placental EPO-R mRNA (Figs. 9 and 10). A 197-bp PCR product was amplified from reverse-transcribed RNA extracted from first- and third-trimester placentas, Jar trophoblast-derived choriocarcinoma cells, isolated and immunopurified first- and third-trimester trophoblast cells, and the positive control fetal mouse liver cells (Figs. 9A and 10A). The 197-bp product was predicted based on the internal set of primers [20]. Deletion of RNA or reverse transcriptase from the RT reaction yielded no demonstrable bands by ethidium bromide detection. As additional evidence that the amplified 197-bp PCR product was ultimately derived from EPO-R mRNA in these placental tissues and trophoblast cells, restriction enzyme digestion was performed using AvaII (Figs. 9B and 10B). The predicted and observed sizes of the two restriction enzyme fragments of 140 and 57 bp were identical. These were also observed for the positive control fetal mouse liver cells.

Protein Tyrosine Phosphorylation

Figure 11A is representative of three different experiments showing that the EPO-R of Jar trophoblast-derived choriocarcinoma cells undergoes phosphorylation on tyrosine residues in response to treatment with exogenous rhuEPO for 2 and 5 min (40 U/ml). After immunoprecipitation of cell lysates with monoclonal anti-EPO-R antibody clone mh2er 7.9.2, and probing of the immunoblot with anti-phosphotyrosine antibody, a prominent band of ≈ 80 kDa was observed. Low amounts of basal tyrosine phosphorylation of the receptor were also detectable in cells prior to EPO treatment. Analysis of tyrosine phosphorylation of the EPO-R in immunopurified third-trimester placental cells was also performed (Fig. 11B). As with Jar cells, purified trophoblast cells express a phosphoprotein that can be immunoprecipitated with an anti-EPO-R antibody, and the degree of intensity is increased with exogenous EPO treatment. In this representative blot of three experiments, an intense signal was observed in the freshly isolated trophoblast cells (time zero); however, in subsequent experiments, a band of lesser intensity was detected. In addition, a higher molecular weight band was observed, but this band was also apparent when IgG1 was substituted for primary antibody. Molecular weight standards × 10⁻³ are indicated on the left.

**DISCUSSION**

The recent report of EPO expression by trophoblast cells in the human placenta [11] prompted us to test whether the same cells also express the EPO-R. If so, then by analogy...
In the present investigation, we confirmed the localization of EPO-R to the endothelium of fetoplacental vessels by immunocytochemistry as originally described by Anagnostou et al. [20] using the same m2her 16.5.1 monoclonal antibody. Indeed, this confirmation was an important “positive control” for our work. In the same term placental tissue sections, however, we also observed EPO-R immunoreactivity associated with the trophoblast layer (Fig. 3), a finding not reported by Anagnostou and coworkers. The explanation for these partly discrepant results may relate to different procedures of tissue fixation and processing, as well as to the generally lower intensity of staining in the syncytiotrophoblast as compared to the fetoplacental endothelium. There was no evaluation of placentas of earlier gestational ages or of basal plate from the term placenta in the previous report, so the observation of EPO-R expression associated with extravillous cytotrophoblast, villous cytotrophoblast, and cytotrophoblast cell columns as reported herein is also new (Figs. 1–3). Further, the present results obtained with the m2her 16.5.1 antibody were observed with a sheep polyclonal antibody. Consistent with our observation of EPO-R expression by various populations of trophoblast cells in the human placenta is the finding of EPO-R immunoreactivity associated with the trophoblast giant cells in the mouse placenta [30].

A prominent band of 66 kDa was observed on Western blot for placental tissues and trophoblast cells, confirming the immunocytochemical results demonstrating EPO-R expression by these tissues (Figs. 5 and 6). Importantly, this detection of immunoreactive EPO-R by Western blot was achieved using another monoclonal antibody that recognized a different epitope from the one employed in the immunocytochemistry [26]. Although intact placental tissues might be expected to express EPO-R by Western blot because they contain fetoplacental vascular endothelium and fetal erythroid precursors, the expression of EPO-R by Western blot was also observed in isolated and immunopurified first-trimester and term trophoblast cells, as well as in the Jar trophoblast-derived choriocarcinoma cell line.

The 66-kDa form of the EPO-R reportedly represents the Golgi form of the EPO-R and is the predominant one found in other tissues [31], including the placenta (current study). In immunoblots of placental homogenates, a less intense band of ~76–78 kDa was also observed. This protein is presumably the plasma membrane form of the receptor, which has a reported size range of 70–78 kDa [31]. The TF-1 cells, an erythroleukemic cell line known to express high levels of EPO-R [23], also demonstrated a prominent band of corresponding size, thus serving as our positive control. A recent investigation has suggested that TF-1 cells also express an abnormal transcript due to a translocation breakpoint in exon VIII, resulting in a truncated form of EPO-R (~46 kDa) [32]. Human bone marrow cells express another truncated receptor that lacks most of the cytoplasmic domain coded in exon VIII and that derives from the inclusion of the 95-bp intron VII, resulting in a premature stop codon [33]. Further, the soluble form of EPO-R of ~34 kDa lacks both the transmembrane and cytoplasmic domains and derives from the insertion of the terminal 104-bp sequence of intron IV, which contains a stop codon [34, 35]. In this regard, lower molecular mass bands were observed for the TF-1 and Jar trophoblast-derived choriocarcinoma cells on Western blot analysis (Fig. 6). Because the expression of these different forms of EPO-R, as well as of the full-length EPO-R, may vary according to the stage of erythroid differentiation, and potentially regulate the cellular action of EPO [33, 36, 37], it may be important to consider the significance of these different EPO-R forms in the context of placental function and development.

FIG. 11. A) EPO-induced tyrosine phosphorylation of the EPO-R in trophoblast-derived choriocarcinoma Jar cells. The EPO-R was immunoprecipitated with anti-EPO-R antibody (mh2er 7.9.2) from Jar cell lysates at various times after treatment with recombinant human EPO (40 U/ml) and separated on 5–20% gradient SDS-PAGE; then immunoblots were probed with anti-phosphotyrosine antibody (PY20). See Materials and Methods for further details. Nonstimulated Jar cells after an overnight preincubation in serum-free conditions were considered to be Time 0 (Lane 1). Lanes 2 and 3 are Jar cells after 2 min without (lane 2) or with EPO treatment (lane 3), and after 5 min without (lane 4) and with EPO treatment (lane 5). Lane 6 represents Jar cells at Time 0 that were immunoprecipitated with a mouse IgG1 isotypic control. B) EPO-induced tyrosine phosphorylation of the EPO-R in freshly isolated, immunopurified trophoblast cells from the term placenta. EPO-R was immunoprecipitated with anti-EPO-R antibody (7.9.2) from cell lysates, and immunoblots were probed with anti-phosphotyrosine antibody (PY99). Lanes 1–6 are as follows: negative control immunoprecipitation with mouse IgG1, untreated trophoblast cells at time zero, untreated trophoblast cells after 5-min incubation at 37°C, trophoblast cells incubated for 5 min with rhEPO (40 U/ml), Jar cells incubated 5 min with no treatment, Jar cells incubated 5 min with rhEPO (40 U/ml), and Jar cells were incubated for 5 min with rhEPO (40 U/ml), and the EPO-R was immunoprecipitated from lysates with either monoclonal antibody mh2er 7.9.2 (lane 1) or 16.5.1 (lane 2). Immunoblots were probed with anti-phosphotyrosine antibody, PY99. Molecular weight standards x 10^(-2) are indicated on the left.

to erythroid precursors, important functions for this hormone related to survival, proliferation, and/or differentiation of placental trophoblast cells are possible. The findings of the present work provide evidence for the expression of EPO-R by trophoblast cells: 1) villous and extravillous cytotrophoblast cells, as well as syncytiotrophoblast at all gestational stages, expressed immunoreactive EPO-R identified by immunohistochemistry; 2) placental tissues and isolated, immunopurified trophoblast cells of various gestational ages, as well as Jar trophoblast-derived choriocarcinoma cells, also expressed immunoreactive EPO-R by Western blot; 3) EPO-R mRNA was detected in the same placental tissues and trophoblast cells by RT-PCR; and 4) the EPO-R was shown to be functional, insofar as tyrosine phosphorylation of the receptor increased in response to exogenously administered EPO.

In the present investigation, we confirmed the localization of EPO-R to the endothelium of fetoplacental vessels by immunocytochemistry as originally described by Anagnostou et al. [20] using the same m2her 16.5.1 monoclonal antibody. Indeed, this confirmation was an important “positive control” for our work. In the same term placental tissue sections, however, we also observed EPO-R immunoreactivity associated with the trophoblast layer (Fig. 3), a finding not reported by Anagnostou and coworkers. The explanation for these partly discrepant results may relate to different procedures of tissue fixation and processing, as well as to the generally lower intensity of staining in the syncytiotrophoblast as compared to the fetoplacental endothelium. There was no evaluation of placentas of earlier gestational ages or of basal plate from the term placenta in the previous report, so the observation of EPO-R expression associated with extravillous cytotrophoblast, villous cytotrophoblast, and cytotrophoblast cell columns as reported herein is also new (Figs. 1–3). Further, the present results obtained with the m2her 16.5.1 antibody were observed with a sheep polyclonal antibody. Consistent with our observation of EPO-R expression by various populations of trophoblast cells in the human placenta is the finding of EPO-R immunoreactivity associated with the trophoblast giant cells in the mouse placenta [30].

A prominent band of 66 kDa was observed on Western blot for placental tissues and trophoblast cells, confirming the immunocytochemical results demonstrating EPO-R expression by these tissues (Figs. 5 and 6). Importantly, this detection of immunoreactive EPO-R by Western blot was achieved using another monoclonal antibody that recognized a different epitope from the one employed in the immunocytochemistry [26]. Although intact placental tissues might be expected to express EPO-R by Western blot because they contain fetoplacental vascular endothelium and fetal erythroid precursors, the expression of EPO-R by Western blot was also observed in isolated and immunopurified first-trimester and term trophoblast cells, as well as in the Jar trophoblast-derived choriocarcinoma cell line.

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determine whether the truncated and soluble forms of EPO-R are expressed by trophoblast cells.

In addition to identification of EPO-R protein, we also detected EPO-R mRNA in placental tissues and trophoblast cells using RT-PCR. Levels of EPO-R message do not seem to be very abundant, as others have reported its detection by Northern analysis in positive control TF-1 cells when 10 μg of poly(A)+ RNA was used [38]. We noted only faint hybridization signals in placental villous tissues and isolated first- and third-trimester trophoblast cells. To improve both the specificity and sensitivity of the assay, we used nested-primer RT-PCR, which revealed an intense, solitary product of 197 bp by agarose gel electrophoresis and ethidium bromide staining (Figs. 9 and 10). Using restriction enzyme digestion, we verified that the 197-bp amplicon was derived from EPO-R mRNA in the placental tissues and trophoblast cells, since the predicted and observed sizes of the two fragments generated by incubation with AvaII, 140 and 57 bp, were identical. The nested-primer PCR approach that we employed did not permit us to determine whether alternative splice forms of EPO-R mRNA consistent with the soluble or truncated EPO-R are present in placental tissues and trophoblast cells because the internal primers did not span the insertion sites for these alternative species. Finally, we cannot completely exclude the possibility that the EPO-R mRNA faintly detected on Northern analysis and robustly by RT-PCR was derived solely from contaminating cells in the immunopurified trophoblast preparation (≤ 3%). However, the presence of mRNA in the Jar trophoblast-derived choriocarcinoma cell line, as well as protein expression by trophoblast cells in placental tissues on immunocytochemistry, makes this possibility an unlikely one.

The elucidation of potential binding sites and transport of EPO across the placental barrier has clinical significance in terms of assessing safety to the fetus with rhEPO administration to anemic pregnant women (refer to [39]). In addition, measurement of circulating EPO may serve as a marker of fetal distress since increased nucleated red blood cell counts can be measured in the neonate from complicated pregnancies [40]. Previous studies have resulted in contrasting results with no demonstrable binding of radio-labeled EPO by the human placenta [41] and no transport between the maternal and fetal compartments in a model of perfused cotyledons [42]. Interestingly, in the latter study the authors measured a 50% loss of EPO from the perfusate after 5 h, suggesting that the administered rhEPO was being bound without placental transfer. Our laboratory has also attempted to identify specific EPO binding sites on isolated trophoblast cells using 125I-labeled ligand without success (data not shown). However, since these cells express EPO, prior occupancy of the receptors may mask binding.

A proximal event in the signal transduction of EPO in erythroid precursor cells is the phosphorylation of the EPO-R on tyrosine residues (see [43], and citations therein). Like other members of the cytokine receptor superfamily, EPO-R itself does not contain a tyrosine kinase domain. Rather, interaction with EPO results in homodimerization and activation of the Janus tyrosine kinase, JAK 2, which is bound to the proximal cytoplasmic region of EPO-R resulting in tyrosine phosphorylation of several proteins including EPO-R itself [43]. In the present investigation, we provide evidence that the EPO-R on trophoblast cells and trophoblast-derived Jar cells is functional, insofar as it is phosphorylated on tyrosine residues in response to exogeneous EPO (Fig. 11). Interestingly, some basal tyrosine phosphorylation was also evident, perhaps reflecting the autocrine action of endogenously produced EPO, since we have previously reported that Jar trophoblast-derived choriocarcinoma cells secrete EPO [11].

The finding of EPO and EPO-R expression by the placenta adds to the growing list of hematopoietic growth factors and their receptors expressed by this organ. Various populations of placental trophoblast, villous core, and uterine decidual cells have been found to express colony-stimulating factor (CSF)-1 and its receptor, c-fms product [44–48], GM-CSF and GM-CSF receptor [49, 50], granulocyte-CSF and granulocyte-CSF receptor [51–53], and stem cell factor and its receptor, c-kit product [54], frequently in a gestational age-specific fashion. These hematopoietic growth factors are likely to exert paracrine and/or autocrine actions in the human placenta. For example, using cultured human trophoblast cells from first-trimester placenta, CSF-1 stimulated syncytiotrophoblast formation and concomitant production of hCG and human placental lactogen [55]. Similarly, spontaneous syncytial formation and production of these hormones by trophoblast cells in culture were prevented by addition of antibody directed against the CSF-1 receptor, c-fms product [55]. GM-CSF also possesses these differentiating effects on cultured term placental trophoblast cells [56]. It will be interesting in future studies to determine whether endogenously derived EPO has a similar role in promoting placental cell growth or differentiation. In this regard, the placentas associated with EPO−/− and EPO-R−/− knockout mice were reported to be of normal size at gestational Days 13–15 when the embryos died in utero apparently of severe anemia [57]. However, it was not reported whether the morphology and distribution of the various populations of trophoblast cells were normal. An autocrine role for EPO, whereby the hormone contributes to the survival, proliferation, and differentiation of trophoblast cells, may be analogous to its role in early colony-forming [8] and erythroleukemic cells [58], as well as human hepatocellular carcinoma cells [59].

Placental trophoblast and other cells expressed both EPO and EPO-R with the only apparent exception being the fetoplacental vascular endothelium, which expressed the receptor, but not the ligand, as assessed by immunocytochemistry ([11, 20]; present study). In addition to putative autocrine roles, another potential action of trophoblast-derived EPO is that it might interact with EPO-R on endothelium to stimulate fetoplacental and uterine vascular development, since EPO has a capacity to promote angiogenesis [19, 60]. Although the expression of EPO has been recently described in the endodermal cells of the mouse vascular yolk sac [18], possibly trophoblast-derived EPO contributes to primitive erythropoiesis and vasculogenesis in the human yolk sac or promotes the differentiation of hemangioblasts identified in the villous core [61]. Further, results of elegant studies by Dancis and colleagues [62] have suggested that perhaps the trophoblast cell has the capability to respond to erythroid differentiation signals, since reconstitution of irradiated isogenic mice with placental cells leads to the formation of hematopoietic colonies in the spleen. Clearly, this new and nonclassical site of EPO and EPO-R expression on cells composing the human placenta opens up the possibility of physiological roles for this hormone in addition to erythropoiesis.

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