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# Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells

**(hematopoietic growth factors)**

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ABSTRACT **Erythropoietin is known to be a hematopoietic growth factor with a singularly specific action on the proliferation and differentiation of erythroid progenitor cells. We have observed a dose-dependent proliferative action of human recombinant erythropoietin on human umbilical vein endothelial cells and bovine adrenal capillary endothelial cells. Binding studies with radioiodinated recombinant human erythropoietin revealed a large number (≈27,000) of an apparent single class of receptors with an affinity in the $10^{-9}$ M range. Linkage of the radiolabeled ligand to its receptor via a bifunctional crosslinking agent allowed us to identify an endothelial cell protein of 45 kDa as the principal receptor associated with this mitogenic effect of erythropoietin. Recombinant human erythropoietin also enhanced the migration of endothelial cells.**

Human umbilical vein endothelial cells (HUVECs) have been reported to produce a variety of colony-stimulating factors and lymphokines, although not erythropoietin (Epo) (1–5). Epo has been considered unique among the other hematopoietic stimulators because its only generally accepted action has been the proliferation and differentiation of cells of erythroid lineage (6). Colony-stimulating factors, on the other hand, are known to affect a variety of hematopoietic cells, and even cells of nonhematopoietic origin (6–8). Recombinant human Epo (rHuEpo) has been used therapeutically in thousands of uremic, anemic patients, with the only side effects arising from the vascular system (hypertension and thrombosis) (9).

In experiments designed originally to study additional mechanisms of the vascular manifestations (thrombosis and hypertension) of Epo treatment (aside from the ones ascribed to increased blood viscosity), we serendipitously noticed significant cell proliferation when rHuEpo from Integrated Genetics (Framingham, MA) was added to HUVEC cultures. We pursued this observation with a series of new experiments, verifying that rHuEpo from various manufacturers has indeed a mitogenic effect on both HUVECs and bovine adrenal capillary endothelial cells (BACECs). We further analyzed the number and molecular weight of the putative receptor through which this action may be exerted. Since many agents with a mitogenic effect on endothelial cells have also an effect on endothelial cell migration, we investigated this aspect, finding again that the rHuEpo preparations enhanced the chemotaxis of both HUVECs and BACECs.

## MATERIALS AND METHODS

**Cell Culture.** Cesarian section-derived HUVECs were cultured at 37°C and in 5% $CO_2$/95% air by standard methodologies (10) in T 25-$cm^2$, 50 ml capacity, tissue culture flasks (Falcon, Becton Dickinson Labware) in the presence of heparin and endothelial cell growth supplement (Sigma). They were characterized by the homogeneous and typical cobblestone morphology, factor VIII antigen positivity, and the presence of Weibel–Palade bodies on electron microscopy. In general, HUVECs were used for our experiments after two to four passages. rHuEpo, initially from Integrated Genetics (Framingham, MA) and later from additional bioengineering companies, was added in various concentrations to cultures containing $3 \times 10^5$ cells per flask. The cell number of trypsinized HUVECs was determined daily both manually by hemocytometer and by Coulter Counter and additionally was verified in some experiments measuring the [$^3$H]thymidine incorporation into serum-starved HUVEC cultures. BACECs, a homogeneous population at passages 11–15 (donated by J. Folkman and K. Butterfield, Harvard University, Boston) were grown at 37°C under 10% $CO_2$/90% air in Dulbecco's modified Eagle's medium containing penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 10% (vol/vol) defined calf serum (Hyclone), and endothelial mitogen (25–50 μg/ml) prepared from bovine hypothalamus (Biomedical Technologies, Stoughton, MA) (11).

**Epo–Receptor Binding Studies.** For the Epo–receptor binding studies, human recombinant (3-[$^{125}$I]iodotyrosyl)erythropoietin ($^{125}$I-rHuEpo; Amersham) was used. When tested by us in a mouse marrow colony-forming unit-erythroid (CFU-E) assay, the $^{125}$I-rHuEpo was fully bioactive compared with Amgen rHuEpo (Amgen Biologicals) and sheep Epo Step III (Connaught Laboratories). The HUVECs were seeded in six-well plates in medium 199 (Sigma) with 10% (vol/vol) fetal bovine serum. The plates were incubated for 3–5 days, at which time cell numbers were found to be $2.5–3.5 \times 10^5$ per well (preconfluent). The cells were then washed with binding medium (0.05 M phosphate buffer containing 0.14 M NaCl, 1 mg of human serum albumin per ml, 68 mM $CaCl_2$, and 50 mM $MgSO_4$, pH 7.4). Each well received 1.0 ml of binding medium with 60,000 cpm of $^{125}$I-rHuEpo [specific activity, 0.36–0.56 Ci/mmol (1 Ci = 37 GBq); $M_r$, 34,000; radiochemical purity, <5% free $^{125}I^-$; potency, 70,000 units/mg)]. Binding was allowed for various periods of time in open air at room temperature. Experiments were terminated by six washes with ice-cold binding medium containing 1% fetal bovine serum rather than albumin. The cells then were lysed with 1.0 ml of 20 mM Hepes (pH 7.4) containing 1% Triton X-100, 10% (vol/vol) glycerol, and 0.1 mg of bovine serum albumin per ml. The Triton X-100 lysate was sampled after 20 min of incubation at room temperature.

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Abbreviations: HUVEC, human umbilical vein endothelial cell; BACEC, bovine adrenal capillary endothelial cell; Epo, erythropoietin; rHuEpo, recombinant human Epo; EGS, ethylene glycol bis(succinimidyl propionate).

The radioactivity of the lysates was determined in a $\gamma$ scintillation counter. Concentration dependence of the rHuEpo was shown by adding various amounts of nonradioactive rHuEpo (Integrated Genetics, Framingham, MA, or Amgen Biologicals) premixed with $^{125}$I-rHuEpo in binding medium to the culture wells and incubating them for 3 hr at room temperature. Termination of incubation and preparation of the cells for assay of radioactivity were as described above. After correcting the cpm for nonspecific absorption, they were plotted according to Scatchard (12). Nonspecific binding (<10%) was determined in the presence of a 200- to 300-fold excess of nonradioactive rHuEpo and was subtracted from all binding data.

**Crosslinking and Immunoblotting Studies.** HUVECs were grown to near-confluence in T 25-cm$^2$ flasks. The cells of four flasks were harvested by scraping. A total of $3.2 \times 10^6$ cells were resuspended in 0.05 M phosphate buffer containing 0.14 M NaCl, 0.3% bovine serum albumin, and 100 mg of D-glucose per dl (pH 7.4) (PBS) at a final concentration of $\approx 10 \times 10^6$ cells per ml. The cell suspensions were incubated at 4°C with 0.4 mCi of $^{125}$I-rHuEpo (specific activity, 0.56 Ci/mmol) or with the same amount of radioactive ligand plus 100 units of nonradioactive rHuEpo. All rHuEpo preparations were supplied in bovine serum albumin-containing buffer. After 60 min, ethylene glycol bis(succinimidyl propionate) (EGS), was added to a final concentration of 0.5 mM. Incubation was continued for 30 min at 4°C. The reaction was then quenched by addition of 50 mM lysine/50 mM *N*-ethylmaleimide. After 5 min at 30°C, the cells were washed twice with PBS and then were solubilized in 0.0625 M Tris containing 1 mM $Na_2EDTA$ and 1% sodium dodecyl sulfate (SDS). The solubilized proteins were reduced with 60 mM dithiothreitol and resolved by SDS/polyacrylamide gradient gel electrophoresis (SDS/PAGE). After immunoblotting (Western blotting) onto poly(vinylidine difluoride) (Immobilon-P, Milipore, Bedford, MA), autoradiographs were prepared (13). Protein was visualized by a silver-staining method. For the immunologic identification of the electrophoretically separated proteins, Immobilon-P transfer membrane was treated with a monoclonal antibody (IgG) to rHuEpo (raised in mice against a 26-amino acid synthetic analogue; Genzyme). Gold-conjugated anti-mouse IgG was used as a second antibody. A silver enhancement stain was used to intensify the gold-conjugated IgG.

**Cell Migration Studies.** Chemotaxis assays were carried out essentially as described by Connolly *et al.* (14) with a 48-microwell chamber (Neuroprobe, Cabin John, MD). Polycarbonate filters (5-$\mu$m pore size, polyvinylpyrrolidone-free) were washed with PBS, incubated overnight with 0.01% gelatin, and air-dried. Various doses of rHuEpo in RPMI 1640 medium containing 10% fetal bovine serum or control medium (without Epo) were loaded into each bottom well. Endothelial cell suspensions ($\approx 4.5 \times 10^4$ cells per well) were loaded into each top chamber of the microwell plate, and the temperature-equilibrated apparatus was incubated at 37°C under 5% $CO_2$/95% air for 5 hr. Then the chamber was disassembled, the filters were rinsed and stained, and cell migration was determined at a magnification of ×430. After coding samples, three fields were counted for each well, and five replicate wells were counted for each of three experimental protocols. Fibrinogen at a concentration of 1 mg/ml was used as the standard chemoattractant for endothelial cells (15). It exerted a 70% increase in migration above that induced by culture medium alone (control). The mean number ± SEM of control endothelial cells was 230 ± 9.4. Differences between control and experimental samples were evaluated by the Student *t* test.

## RESULTS

**The Effects of Epo on Endothelial Cell Proliferation.** When we added different concentrations of rHuEpo from Integrated Genetics (Framingham, MA) to HUVECs ($3 \times 10^5$ cells per 25-cm$^2$ flask) cultured by standard methods, a dose-dependent increase in cell proliferation was readily observed (Fig. 1). This unexpected effect was also observed with rHuEpo from other manufacturers as well as capillary endothelial cell cultures derived from bovine adrenals (BACECs). With the addition of rHuEpo at 5 units/ml to the HUVECs, the doubling time of the Epo-treated cells averaged 36 hr versus 72 hr for the control group. The stimulation of cell proliferation by rHuEpo was optimal at 5 units/ml, with concentrations of up to 20 units/ml tested. With rHuEpo at 5 units/ml, there was a 52.6 ± 12% (mean ± SEM) increase in cell number above the control value (without Epo) at 2 days, and a 256 ± 10% increase ($n = 6$, $P < 0.001$) at 7 days, whereas with 2.5 units/ml there was a smaller increase in cell number, ranging from 14 ± 3% on day 2 to 141 ± 12% ($n = 4$, $P < 0.001$) on day 7. The $K_m$ calculated from these data was 3000 units/liter. rHuEpo obtained from two other bioengineering companies (Amgen Biologicals and Genetics Institute, Cambridge, MA) also exhibited a consistent proliferative action on HUVECs, although with a smaller maximal biological effect [mean value ± SEM of increase in cell number above the control value was 28 ± 3.5% with a range of 14–60% ($n = 4$, $P < 0.005$), which was observed at 5 units/ml for the Genetics Institute (Cambridge, MA) rHuEpo, and at 0.5 units/ml for the Amgen Biologicals product]. Addition of 0.5 unit of Amgen Biologicals rHuEpo per ml to serum-starved HUVEC cultures produced a 2-fold increase in [$^3$H]thymidine incorporation over control (synchronized) cultures refed with 20% fetal bovine serum (data not shown). When BACECs were the target cells, there was at 7 days a mean ± SEM increase of 35 ± 7% in cell number above the control value ($n = 4$, $P < 0.005$; range 7–60%) with rHuEpo from Amgen at 0.5 unit/ml or rHuEpo from Genetics Institute (Cambridge, MA) at 5 units/ml (data not shown).

**Binding of Epo to HUVECs.** The time dependence of the binding of $^{125}$I-rHuEpo to the HUVECs is shown in Fig. 2 and demonstrates specific binding that increases with time and reaches equilibrium in about 4 hr. The concentration dependence of the rHuEpo binding was measured over the range from 0.15 to 15 nM. Specific binding was found to be saturable and reversible and involved at equilibrium an

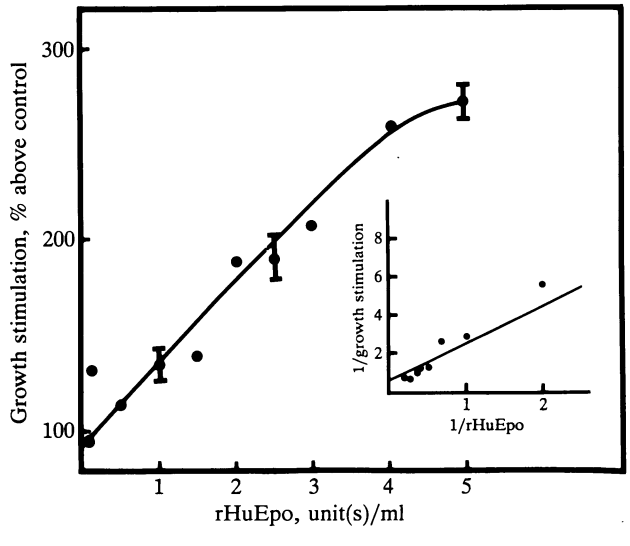


FIG. 1. HUVEC proliferation (expressed as percent of control) after addition of increasing concentrations of rHuEpo (Integrated Genetics). Cell numbers represent mean ± SEM increase over optimally grown control cultures 7 days after addition of rHuEpo. Results represent the mean of six experiments. The insert is a double-reciprocal Lineweaver–Burk plot of the growth stimulation data.

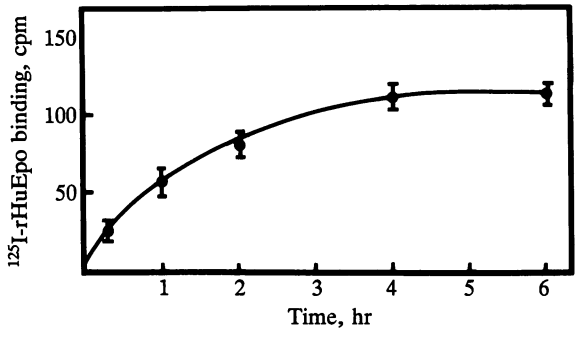


FIG. 2. Time dependence of $^{125}$I-rHuEpo binding to HUVECs. Results represent the mean ± SEM of four experiments.

apparent single class of receptors. The mean number of receptors per cell from our studies was calculated by Scatchard plot analysis (Fig. 3) to be 45,000, with a $K_d$ of 5.7 × $10^{-9}$ M. In the presence of 0.02% sodium azide, to prevent possible receptor–Epo complex internalization, the mean receptor number per cell decreased to 27,000 with a $K_d$ of 3.8 × $10^{-9}$ M. Binding studies with confluent HUVECs showed an average of 12,500 receptor sites per cell with a $K_d$ of 2.4 × $10^{-9}$ M. Our calculation of the $K_d$ is based on a potency of the Amersham rHuEpo of 70,000 units/mg. Attempts to conduct the binding experiments at 4°C revealed that, after 3 hr at this temperature, 80% of the HUVECs became nonviable, as determined by the trypan blue dye-exclusion test. The Scatchard analysis also showed a scattering of data points at very low ligand concentration, raising the possibility of positive cooperativity. Artifactual sources for this upward convex plot (16) were thought unlikely because of our aforementioned demonstration that the radioactive ligand had equal biological activity to nonradioiodinated rHuEpo, and because prolonged exposure at 37°C was needed to measure that effect, we feel confident that the radioactive rHuEpo had

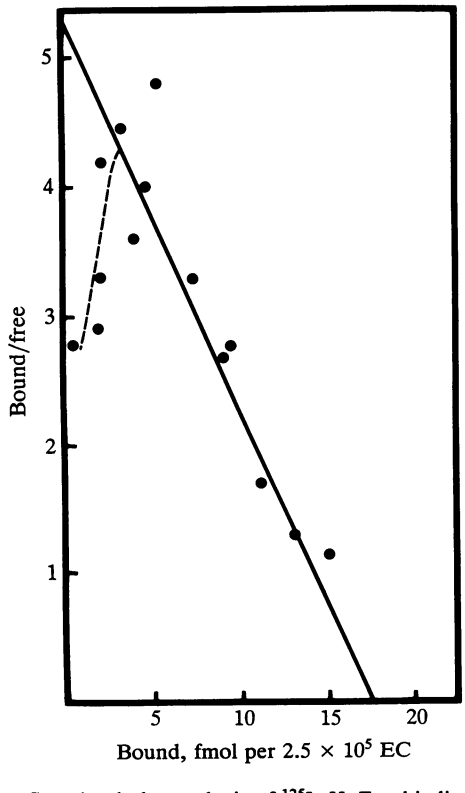


FIG. 3. Scatchard plot analysis of $^{125}$I-rHuEpo binding to endothelial cells (EC). Values of the ordinate axis (bound/free) are × $10^2$.

binding characteristics and chemical stability equal to that of the nonradioactive substance. Most significant, however, was the fact that binding studies performed solely with radioiodinated rHuEpo gave an identical upward curving plot. Others are also utilizing the same product without loss of biologic activity or other problems (17–21).

**Identification of Epo Receptors on HUVECs.** To identify the Epo receptor(s) of HUVECs, $^{125}$I-rHuEpo was linked to its receptor(s) with a homobifunctional crosslinking agent, EGS (Fig. 4 *Left*, lane A). One prominent band ≈79 kDa was clearly recognizable. Two very faint bands of 97 kDa and 165 kDa could be seen only by direct inspection of the autoradiograph. Subtraction of the molecular mass of rHuEpo (≈34 kDa) yields a molecular mass for the putative receptor of 45 kDa. Specificity of the binding process in our study could be shown by the almost complete suppression of the receptor–$^{125}$I-rHuEpo complex formation by the addition of a 200-fold excess of nonradioactive rHuEpo (Fig. 4 *Left*, lane B). The two radioactive bands > 200 kDa represent crosslinked complexes that have failed to enter or are at the top of the running gel.

A direct comparison of the three different rHuEpo preparations used in this study is shown in Fig. 4 *Center* and *Right*. Apparent differences in the specific activities of these preparations can be recognized easily as each lane of the gel was loaded with the same number of units of rHuEpo. Lane a, loaded with rHuEpo of Amgen, shows the faintest band upon silver staining, whereas that of the Integrated Genetics rHuEpo shows the strongest. The equivalence of the erythropoietin lots with respect to their reactivity with monoclonal antibody is shown in Fig. 4 *Right*. Significant differences in the migration rate of the three preparations of erythropoietin are evident, which may be due to different glycosylation of the three preparations (22, 23).

**Effect of Epo on Endothelial Cell Migration.** When rHuEpo preparations from Amgen Biologicals and Genetics Institute were tested on endothelial cells for chemotactic effect (14), they both increased cell migration (Fig. 5) of HUVECs and BACECs [mean ± SEM increase of HUVEC migration over control was 69 ± 16% for Genetics Institute rHuEpo ($n$ = 3, $P < 0.001$) and 49 ± 11% for Amgen Biologicals rHuEpo ($n$ = 3, $P < 0.005$); for BACECs the values were 36 ± 9% for Genetics Institute rHuEpo ($n$ = 3, $P < 0.005$) and 41 ± 6% for Amgen rHuEpo ($n$ = 3, $P < 0.005$)].

## DISCUSSION

Endothelial cell proliferation is presently an area of major scientific interest because of the intriguing role of angiogenesis in neoplastic processes (24). Many angiogenic substances have been identified that exert a proliferative and chemoattractive effect on endothelial cells (25), and one may speculate whether hematopoietic growth factors also play a role in the normal development of the vascular system or its alteration in disease states. Some of the hematopoietic growth factors produced by endothelial cells have already been reported to enhance endothelial cell proliferation and migration (26). Nonetheless, a great number of studies spanning more than three decades have not shaken the belief that the only well-documented action of Epo is on the proliferation and differentiation of erythroid cells, notwithstanding observations that suggest an effect on thrombopoiesis (27) and possibly on early hematopoietic stem cells (28). Thus, the mitogenic effect of rHuEpo on the nonhematopoietic endothelial cells was most surprising to us.

Studies of receptors for Epo on erythroid progenitor cells or erythroid cell lines have shown only a small number of surface receptors (usually <1000 per cell) either of a single class (21, 29, 30) or of both low- and high-affinity types (31, 32). A low number of receptors per cell is common in studies

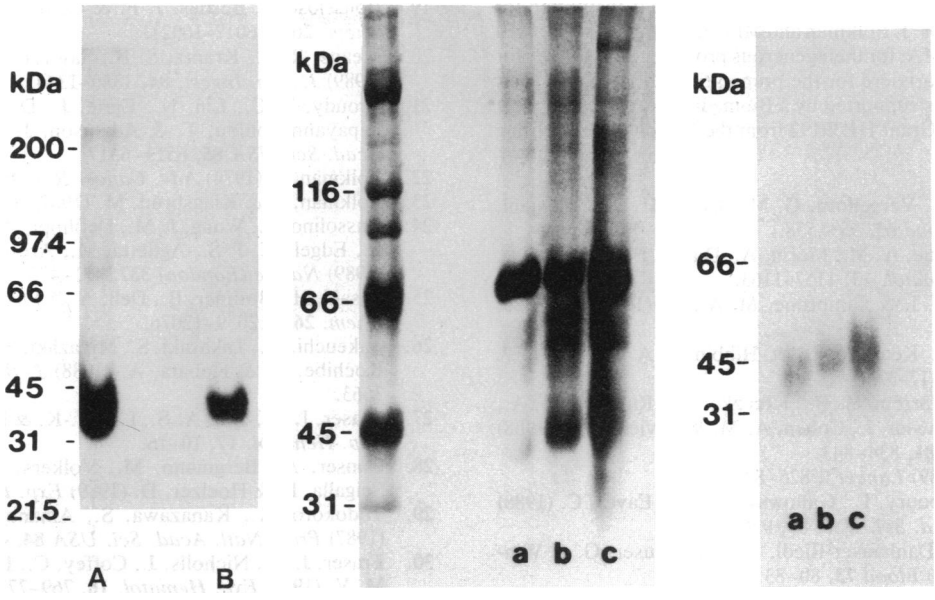


FIG. 4. Crosslinking $^{125}$I-rHuEpo to HUVECs with EGS (*Left*), characterization of the various rHuEpo preparations used in our experiments by SDS/PAGE (*Center*), and immunological analysis by reaction with the monoclonal antibody to rHuEpo (*Right*). Lanes in *Left*: A, not suppressed; B, suppressed with excess rHuEpo. In *Center* and *Right*, amounts equivalent to 20 units of Epo of each preparation were applied. Lanes: a, rHuEpo from Amgen; b, rHuEpo from Genetics Institute; c, rHuEpo from Integrated Genetics. Molecular mass standards, shown in the left-most lane of each panel, were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21.5 kDa).

of factors whose main action is differentiation of hematopoietic cells (17). It has been speculated that high-affinity receptors are necessary for the differentiating effect of Epo on hematopoietic cells and cell lines, whereas low-affinity receptors may be involved in its proliferative action (33–35). Our finding of a high number of relatively low-affinity binding sites is thus mindful of studies with platelet-derived growth factor, the mitogenic action of which on fibroblasts or smooth muscle cells is associated with a single class of receptors with a $K_d$ in the $10^{-9}$ M range and $3 \times 10^5$ binding sites per cell (36). The decrease in the number of receptor sites with increasing confluence of cells as seen in our studies agrees with studies with transforming growth factor β, platelet-derived growth factor, and epidermal growth factor, in which the binding of these peptides to their receptors on various cell lines decreases as cell density increases (37). This has been shown to be due to reduction in the number of receptors and not to a decrease in receptor affinity or cell cycle-dependent regulation. This density-related down-regulation of growth-factor receptor sites has been thought (37) to possibly serve as a mechanism keeping cell proliferation under control.

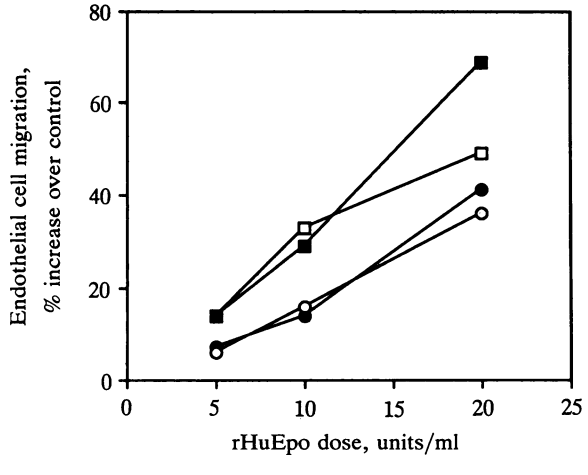


FIG. 5. Effect of rHuEpo from two different manufacturers (Amgen and Genetics Institute) on HUVEC and BACEC migration. Results are the mean values from three replicate experiments for each rHuEpo and each cell type. The numerical values are detailed in *Results*. □, Amgen rHuEpo with HUVECs; ■, Genetics Institute rHuEpo with HUVECs; ○, Genetics Institute rHuEpo with BACECs; ●, Amgen rHuEpo with BACECs.

Our studies suggest a kDa of 45 for the putative receptor of Epo on endothelial cells. Previous studies on the structure of the Epo receptor on Epo-responsive cells or cell lines report two proteins of 100 and 85 kDa (34, 38). Other investigators, in studies on both Epo-responsive and -unresponsive cells, have variously reported three subunits of 119, 94, and 63 kDa (Epo-unresponsive cells) (29); 110 and 95 kDa (39); and 85 and 41 kDa (40). Thus, the molecular mass of the receptor demonstrated in our study differs from the molecular mass of receptors found on cells on which Epo exerts a differentiation/maturation effect. Furthermore, we believe that our data raise the possibility that the receptor mediating the mitogenic action interacts with a site on the Epo molecule that differs from that involved in the differentiation of cells. This may explain our observation that Epo preparations of equal cell-differentiating activity display unequal mitogenic ability. A comparison of the apparent molecular masses of the three Epo preparations we used showed considerable differences, which we assume are related to the different degrees of glycosylation of the Epo molecule.

The physiological concentration of Epo in most healthy animals and humans is in the range of 4–30 milliunits/ml of plasma, but 100-fold increases can be seen in severe anemias. Thousands of patients have already received treatment with rHuEpo with beneficial results on their hematocrit and their general well-being. The side effects have been few and easily controllable, mostly from the vascular system (9). Our *in vitro* studies raise the question whether some of the observed vascular complications could be related to an effect of Epo on endothelial cells.

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rHuEpo preparations. We thank Dr. M. Miller in our Institution for her overall support; Dr. J. Folkman and Mrs. K. Butterfield, Harvard University, Boston, MA, for their generous provision of BACECs for our studies; and J. Lariviere for the preparation of the manuscript. This investigation was supported by a Biomedical Research Support Grant and in part by Grant HL39192 from the National Heart, Lung and Blood Institute.

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