

Recombinant Human Erythropoietin α Targets Intratumoral Blood Vessels, Improving Chemotherapy in Human Xenograft Models

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Abstract

Recombinant human erythropoietin (rHuEPO) is widely used for correction of hemoglobin level in cancer patients. However, apart from hematopoiesis, rHuEPO reportedly has an effect on endothelial cells. We describe here how rHuEPO α can modulate tumor vasculature in human squamous cell (A431) and colorectal carcinoma (HT25) xenograft models. *In vivo* rHuEPO treatment of xenografts at human-equivalent dose significantly increased the proliferation index of the tumor-associated endothelial cells and the size of CD31-positive intratumoral blood vessels, whereas the pericyte coverage became fragmented. Moreover, rHuEPO administration resulted in decreased expression of vascular endothelial growth factor both by cancer cells and tumor stroma, measured by quantitative PCR. Due to the morphologic alterations in tumoral microvessels, DNA-binding agents (Hoechst and Doxorubicin) labeled significantly larger areas in the tumor mass. Furthermore, rHuEPO treatment led to a significantly improved efficacy of 5-fluorouracil (5-FU) chemotherapy in the case of both tumor xenografts. Meanwhile, rHuEPO had no effect on the *in vitro* proliferation of erythropoietin receptor-positive tumor cells, and did not interfere with the effects of 5-FU either. These data reveal a new effect of rHuEPO administration: remodeling tumoral microvessels, suppressing vascular endothelial growth factor expression, thereby augmenting antitumor effects of a cancer drug, 5-FU, even in erythropoietin receptor-positive human cancer xenografts. (Cancer Res 2005; 65(16): 7186-93)

Introduction

Anemia, a common complication in cancer patients, induces fatigue, weakness, impaired concentration, resulting in diminished physical capacity and poor quality of life (1). Numerous clinical data suggest that hypoxia in cancers enhances their aggressiveness and promotes malignant progression (2). Hypoxia is an independent prognostic factor in some malignant cancers, such as cervical carcinoma and head and neck tumors (3). However, the correction of anemia and the increased oxygen level inside the tumor not only result in an improvement in the quality of life but enhance the success of cancer therapy as well, leading to improved survival of patients (4). Recombinant human erythropoietin (rHuEPO) is

widely used for correction of hemoglobin level by increasing the number of RBC (5). Recent studies show that, beside hematopoietic progenitor cells, numerous other cell types (endothelial and cancer cells) express erythropoietin receptor, and rHuEPO may affect their functions (6, 7). However, clinical and experimental data are contradictory on the way by which exogenous rHuEPO treatment can influence cancer growth and therapy (8–10).

Here, we have studied the effects of exogenous rHuEPO on *in vitro* and *in vivo* growth of human epidermoid (A431) and colorectal carcinoma (HT25) xenografts and on the efficacy of 5-fluorouracil (5-FU) treatment. Previous data indicated that the oxygen level in the tumor tissue influences the success of anticancer therapy (4). On the other hand, data also suggested that rHuEPO can increase the oxygen level in the tumor mass independently of hemoglobin level (11). These data together suggested that rHuEPO may influence tumor perfusion through the tumor vasculature. Therefore, we examined the tumoral blood vessels after rHuEPO treatment of xenotransplanted human cancers in severe combined immunodeficient (SCID) mice.

Materials and Methods

Cell lines. A431 human epidermoid carcinoma and HT25 human colon carcinoma were cultured in DMEM or RPMI, respectively (Sigma Chemical Co., St. Louis, MO), supplemented with 5% fetal bovine serum (FBS, Sigma) at 37°C humidified atmosphere with 5% CO₂. Human brain endothelial cells (HBE 70-4) were cultured in RPMI and F12 (Sigma) 1:1 mixture containing 20% FBS.

***In vitro* proliferation test.** Two thousand A431 and HT25 tumor cells per well were plated into 96-well tissue culture plate (Greiner, Frickenhausen, Germany) in medium containing 5% serum, and 24 hours later were incubated with human recombinant erythropoietin α (rHuEPO, epoetinum α ; Janssen-Cilag, Shaffhausen, Switzerland) at different concentrations (0.1–10 IU/mL) for 72 hours in 200 μ L serum-containing or serum-free medium, respectively. At the end of incubation, 20 μ L of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) was added to the cell medium for 4 hours at 37°C, and the medium was removed and tetrazolium crystals were dissolved in 100 μ L DMSO (Sigma). Absorbance was measured at 570 nm using ELISA Microplate Reader (Bio-Rad, Hercules, CA).

For the detection of the effect of 5-FU (TEVA, Pharmachemie BV, Haarlem, the Netherlands) and rHuEPO combinations on the cell proliferation, A431 tumor cells were treated at different doses with 5-FU (0.2–20 μ g/mL) and epoetinum α (0.1–10 IU/mL) simultaneously.

Mice and tumors. SCID mice were bred and maintained in our specific pathogen-free mouse colony and housed 10 in each cage. Xenografts were produced by s.c. inoculation of 10⁶ tumor cells in single-cell suspension in 0.1 mL serum-free DMEM into the back of female mice. All animal experiments were conducted following standards and procedures approved by the Animal Care and Use Committee of National Institute of Oncology, Budapest.

Real-time PCR of human erythropoietin receptor and vascular endothelial growth factors. For erythropoietin receptor real-time PCR, total RNA was isolated from *in vitro* growing cell lines using TRI Reagent

Note: J. Tóvári is a recipient of the Bolyai Research Fellowship Grant of the Hungarian Academy of Sciences.

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doi:10.1158/0008-5472.CAN-04-2498

(Sigma) according to the manufacturer's instructions. For eliminating the possible DNA contamination, we used TURBO DNA-free kit (Ambion, Austin, TX). Vascular endothelial growth factor (VEGF) expression was determined from the RNA isolated from the experimental tumor samples of treated and control animals using NucleoSpin RNAII kit (Macherey-Nagel, Düren, Germany).

For reverse transcription, we added 1 μ L of 10 mmol/L deoxynucleotide triphosphate mix (Finnzyme, Espoo, Finland) and 1 μ L of random primer-oligo(dT) combination for a final concentration of 2.5 μ mol/L and used 2 μ g of the purified total RNA in both cases. After incubating at 70°C for 10 minutes, we added 2 μ L of 10 \times M-MLV Reverse Transcriptase Buffer (Finnzyme), 1 μ L of M-MLV Reverse Transcriptase (200 units/ μ L, Finnzyme), 0.5 μ L RNase Inhibitor (40 units/ μ L, Promega, Madison, WI), and 6.5 μ L diethyl pyrocarbonate-treated water for a final volume of 20 μ L, then incubated at 37°C for 50 minutes and 85°C for 10 minutes. The occurrence of reverse transcription was checked by carrying out PCR with β -actin primers as a housekeeping gene. RNA of the same sample was used as negative control for detection of DNA contamination, and diethyl pyrocarbonate-treated water as nontemplate control.

For quantitative PCR measurements, each 25 μ L reaction mixture contained 12.5 μ L 2 \times iQ SYBR Green Supermix (Bio-Rad), 0.5 to 0.5 μ L of each primer for a final concentration of 200 nmol/L, and 11.5 μ L of the diluted cDNA. The cycling conditions comprise of 3 minutes of iTaq DNA polymerase activation at 95°C, 40 cycles at 95°C for 30 seconds, 60°C (erythropoietin) or 64°C (VEGF) for 30 seconds, 72°C for 1 minute. Starting quantities were defined on the basis of standard 5-fold dilution series (1 \times -625 \times) done with control cDNA. Controls were A431 for human erythropoietin receptor and mouse B16a for the mouse-specific primers and the human Kaposi sarcoma (KS Imm) cell lines for human-specific primers in the case of VEGF examination. Relative human erythropoietin receptor (primers: TCT GGC ATC TCA ACT GAC TAC A and CCA TCC CTG TTC CAT AAG TCT TG) and VEGF (human-specific primers: CTT GCC TTG CTG CTC TAC CT and TGA TGT TGG ACT CCT CAG TGG, mouse-specific primers: CTA CTG CCG TCC GAT TGA GA and CAT CTG CTG TGC TGT AGG AAG) expression was determined by normalizing the starting quantities to the housekeeping gene β -actin from the same cDNA sample (β -actin primers for human erythropoietin receptor determinations: GTG GGG CGC CCC AGG CAC CA and CTC CTT AAT GTC ACG CAC GAT TTC, and for VEGF determinations: AGA CAA CAT TGG CAT GGC TT and AAT GAA GTA TTA AGG CGG AAG ATT). Species specificity of VEGF primers was proved by carrying out reactions of human sample-mouse primer and mouse sample-human primer combinations. Real-time PCRs were measured by Bio-Rad MyiQ Single color real-time PCR detection system.

Flow cytometry of tumor cells. Tumor cells were detached with EDTA (Sigma), washed with serum-free RPMI, and fixed with methanol for 20 minutes. After fixation, cells were blocked with 3% bovine serum albumin/PBS (Sigma) for 30 minutes and then incubated with anti-human erythropoietin receptor monoclonal antibody (1:20 in PBS, R&D Systems, Minneapolis, MN) or control mouse IgG2b antibody (Sigma). Bound primary antibody was detected with goat anti-mouse immunoglobulins conjugated with R-phycoerythrin (1:10 in PBS, DakoCytomation, Glostrup, Denmark). Fluorescence of labeled cells was measured in CyFlow flow cytometer (Partec, Münster, Germany) and the percentage of positive cells was determined.

Recombinant human erythropoietin and 5-fluorouracil treatment *in vivo*. Tumor-bearing animals were treated by rHuEPO at human-equivalent dose (150 IU/kg in physiologic salt solution, final volume 0.1 mL) thrice per week i.p. from day 1 till the end of 5-FU treatment (day 16). Mice were treated with 5-FU i.p. from day 12 after tumor cell inoculation at a dose of 750 mg/m² every day for 5 days (average tumor volumes were 50 to 70 mm³ at the start of chemotherapy). Control animals were treated with physiologic salt. Tumor volumes were measured in two diameters by caliper thrice a week during 5-FU treatment, and were calculated by using the following formula: length \times width² \times π /6.

Measurement of RBC number and hematocrit. Mice were anesthetized by i.p. administration of Nembutal, and blood was taken from the heart every 3 to 4 days. Fresh blood was kept in glass tubes in 3.8% sodium

citrate solution (citrate/blood ratio = 1:10 v/v). The samples were centrifuged for 15 minutes at 4,000 rpm and the RBC count and hematocrit level were determined in SYSMEX SF 3000 hematologic machine.

Morphometry of tumor-associated blood vessels. S.c. growing tumors were collected freshly (on days 7, 10, 14, 17, 21, and 24 after tumor inoculation), and frozen sections of 5 to 7 μ m thickness were used for immunohistochemistry. Slides were incubated with monoclonal rat anti-mouse CD31 antibody (diluted 1:20, Becton Dickinson Bioscience, San Jose, CA) followed by biotinylated anti-rat IgG and streptavidin-FITC (Vector Laboratories, Burlingame, CA). The nuclei were identified with propidium iodide (Sigma). Five 300 \times 400 μ m-sized living tumor areas per slide were photographed and analyzed under a confocal microscope (MRC 1024, Bio-Rad) and computer-aided morphometry (CUE2 software, Olympus Vanox, Tokyo, Japan). The average Feret's diameter (defined as the greatest distance possible between two points along the boundary of a region of interest, and was calculated by the software by averaging the measurement for each vessel at projection angles of 0°, 45°, 90°, and 135°), perimeter, and density were determined in five tumors per experimental group. The number of CD31⁺/propidium iodide⁺ endothelial cell nuclei per 100 μ m vessel perimeter unit was determined in five different areas of each tumor.

The pericytes of the vessel wall were identified using immunohistochemical labeling with anti-smooth muscle actin antibody (DakoCytomation), and visualized by anti-mouse TRITC (Sigma).

Functional imaging of tumor tissue using Hoechst 33342 dye and Doxorubicin. One milligram per milliliter of Hoechst 33342 dye (Molecular Probes, Eugene, OR; blue) or 2 mg/mL Doxorubicin (Adriablastina PFS, Pharmacia Italia, S.p.A., Italy) were inoculated in a volume of 0.2 mL into rHuEPO-treated and control tumor-bearing animals i.v. on day 21 after tumor inoculation. After 15-minute incubation, the tumors were removed and 5- to 7- μ m-thick frozen sections were analyzed immediately or following nuclear labeling with Hoechst 33342 under fluorescent microscope (Eclipse E6000, Nikon Optoteam, Vienna, Austria). Ten stained areas were analyzed with morphometric software (ImageTool, University of Texas Health Science Center, San Antonio, TX) to determine the perfused area in the living tumor mass.

Measurement of proliferation index. Tumor-bearing animals on day 18 were injected i.p. with 5-bromo-2'-deoxyuridine (BrdU, 200 mg/kg; Sigma). After 6 hours of incubation, 5- to 7- μ m-thick frozen sections of fresh tumors were used to detect BrdU-positive cells using anti-BrdUrd monoclonal antibody (Becton Dickinson) according to the manufacturer's instruction. Positive cells were visualized with TRITC-conjugated anti-mouse IgG (1:100, Sigma). To discriminate tumor cells from endothelial ones, the vessels were stained with rat anti-mouse CD31 antibody as described above, and nuclei were stained with Hoechst 33342 dye (Molecular Probes). The labeling index of A431 tumor cells and mouse endothelial cells in the tumor-associated vessels was determined in 10 independent areas of five different tumors.

Statistical analysis. Data from *in vitro* experiments and from morphometric analyses were compared using Student's *t* test; *P* < 0.05 was considered significant. The *in vivo* data were compared using Mann-Whitney *U* test. All statistical analyses were done using CSTAT statistical software program.

Results

Expression of erythropoietin receptor on human tumor cell lines and *in vitro* effect of recombinant human erythropoietin treatment. Expression of erythropoietin receptor was shown at mRNA and protein level in A431 human epidermoid carcinoma and HT25 colorectal carcinoma cells *in vitro*, using real-time PCR technique and flow cytometry (Fig. 1A). The human *erythropoietin receptor*-specific primer pair amplified a 224-bp product from both cell lines, and there was no amplification in the case of mouse skin and muscle samples (data not shown), indicating the human specificity of the primers. Real-time PCR study revealed that HT25 cells expressed 3.1-fold more *erythropoietin receptor* mRNA than

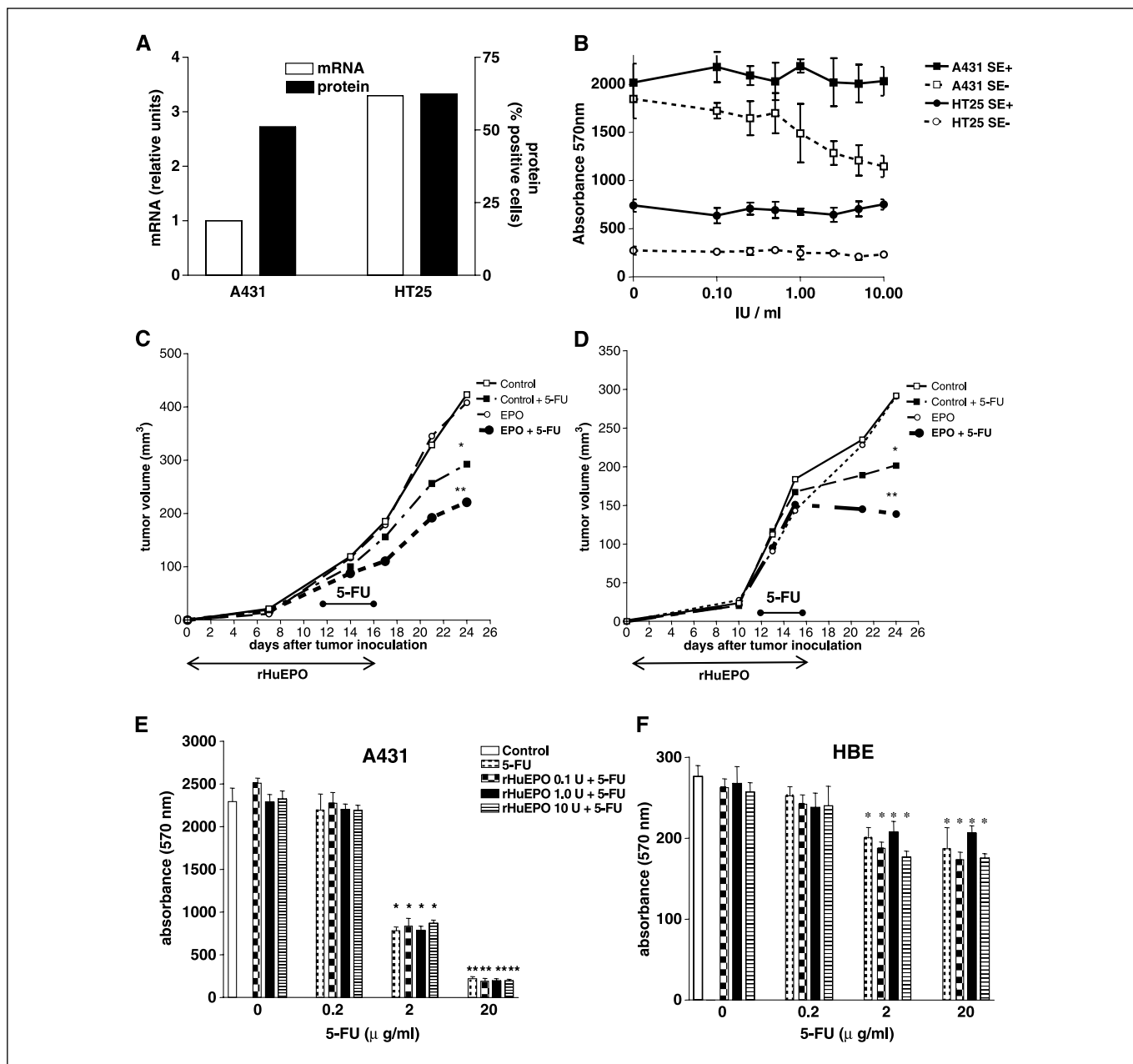


Figure 1. Effect of rHuEPO on proliferation and growth of erythropoietin receptor-expressing A431 and HT25 cells and tumor xenografts. *A*, expression of human erythropoietin receptor at mRNA and protein level. Erythropoietin receptor was measured by real-time PCR and expressed in arbitrary units. Cellular erythropoietin receptor protein was measured by flow cytometry and expressed in percentage of positive cells. *B*, effect of 72-hour treatment with rHuEPO on the *in vitro* proliferation of A431 and HT25 cells (0.1-10.0 IU/mL; SE+/SE- = 5% serum or serum-free conditions). Points, mean; bars, SD ($n = 6$). *C* and *D*, *in vivo* effects of rHuEPO (150 IU/kg, thrice per week) and 5-FU treatments (750 mg/m², five times per week) on A431 (*C*) and HT25 (*D*) xenografts ($*P < 0.05$, 5-FU versus control; $**P < 0.05$, 5-FU + rHuEPO versus 5-FU, Mann-Whitney *U* test, $n = 10$). *E* to *F*, rHuEPO (0.1-10 IU/mL) did not modulate the *in vitro* effect of 5-FU on A431 (*E*) or HBE 70-4 (*F*) cell in a 72-hour proliferation test. Columns, mean; bars, SD ($n = 6$, $*P < 0.05$; $**P < 0.01$, 5-FU versus control, Student's *t* test).

A431 cells. Immunocytochemistry indicated that >50% of A431 and HT25 cells expressed the erythropoietin receptor protein analyzed by flow cytometry (Fig. 1A). rHuEPO treatment at a dose range of 0.1 to 10 IU/mL had no effect on the *in vitro* proliferation of A431 and HT25 cells, except for a moderate inhibition of A431 cells in serum-free conditions at higher concentrations (Fig. 1B).

Effect of recombinant human erythropoietin on RBC number and hematocrit of tumor-bearing severe combined immunodeficient mice. We have administered rHuEPO i.p. into SCID mice at human-equivalent dose (150 IU/kg, thrice per week)

from day 1 of the s.c. inoculation of A431 and HT25 cells till the end of the experiments (day 22). During the treatment period, we have monitored RBC count and hematocrit level in the tumor-bearing mice, and found that the growing A431 xenograft induced a decrease in both parameters, which was corrected by rHuEPO treatment ($P < 0.001$; Table 1).

Effect of recombinant human erythropoietin on the anti-tumor activity of 5-fluorouracil *in vivo* and *in vitro*. We found that rHuEPO administration *in vivo* at human-equivalent dose had no significant effect on the growth of A431 and HT25 xenografts

Table 1. Effect of rHuEPO treatment on RBC number and hematocrit during A431 tumor xenograft growth

	Days after tumor inoculation						
	0	5	8	12	15	19	22
RBC (M/μL)							
Tumor free	8.1 \pm 0.8	7.7 \pm 0.1	7.7 \pm 0.6	8.3 \pm 0.2	7.7 \pm 0.3	8.5 \pm 0.9	7.6 \pm 0.3
Tumor control		7.3 \pm 0.6	7.6 \pm 0.4	7.9 \pm 0.2	7.5 \pm 0.4	7.4 \pm 0.4	7.2 \pm 0.3
Tumor + rHuEPO		8.1 \pm 0.8	8.1 \pm 0.5	8.7 \pm 0.4*	8.8 \pm 0.4*	8.5 \pm 0.3*	8.4 \pm 0.1*
Hematocrit (%)							
Tumor free	39.4 \pm 4.1	37.4 \pm 0.2	37.3 \pm 3.6	39.8 \pm 0.9	37.4 \pm 1.7	41.5 \pm 4.3	36.6 \pm 1.5
Tumor control		35.6 \pm 3.1	36.6 \pm 1.9	38.1 \pm 0.7	36.2 \pm 2.7	35.1 \pm 1.9	33.2 \pm 1.7
Tumor + rHuEPO		34.7 \pm 3.3	40.0 \pm 3.3	43.6 \pm 2.4*	43.7 \pm 2.4*	41.3 \pm 1.7*	41.2 \pm 0.4*

NOTE: Data are mean \pm SD ($n = 3$).* $P < 0.001$.

(Fig. 1C and D). 5-FU administration at human dose (750 mg/m²/d for 5 days) was started on day 12 following tumor cell inoculation (when the average tumor size reached 50-70 mm³). Whereas 5-FU administration alone induced moderate but significant ($P < 0.05$) inhibition in the growth of both tumor xenografts, rHuEPO treatment significantly increased this growth inhibitory effect ($P < 0.05$, Fig. 1C and D). By comparison, 5-FU (0.2-20 μ g/mL, 72 hours) had a dose-dependent antiproliferative effect on A431 cells *in vitro*, and rHuEPO (0.1-10 IU/mL) did not modify this effect (Fig. 1E). We also determined the possible modulating effect of rHuEPO on the blocking of endothelial cell proliferation by 5-FU. Compared to A431 tumor cells, 5-FU had a weaker inhibitory effect on the well-differentiated, slowly growing human brain endothelial (HBE 70-4) cell line, which was not influenced by rHuEPO (Fig. 1F).

Effect of recombinant human erythropoietin treatment on tumoral blood vessels. Histologic analysis of rHuEPO-treated A431 and HT25 xenografts between days 1 and 24 following tumor inoculation revealed that the intratumoral microvessels enlarged, the endothelial cells became more rounded, and their nuclei bulged into the vessel lumen (not shown). Therefore, microvessels have been labeled by CD31 marker, and morphometry was done in frozen samples from A431 and HT25 tumors. Treatment of mice with rHuEPO at human-equivalent dose resulted in significant blood vessel enlargement (the average Feret's diameter in A431 tumor increased with 45%, $P < 0.05$) inside the living, nonnecrotic A431 and HT25 tumors at day 17 (Fig. 2A-D and F). More than 95% of intratumoral vessels were dilated in rHuEPO-treated tumors compared with controls according to morphometric analysis by calculating average Feret's diameters. However, intratumoral vessel density remained similar to that in controls (Fig. 2E). The development of this effect on vessels took \sim 2 weeks, because on day 10 or 14 (Fig. 2F and data not shown) after tumor inoculation there was no difference in the size of the intratumoral vessels of rHuEPO-treated animals compared with control.

Effect of recombinant human erythropoietin treatment on cancer and endothelial cell proliferation *in vivo*. Cryosections of 18-day-old A431 tumors were used to determine the proliferation of tumor and endothelial cells. We have injected BrdU (200 mg/kg) i.p. into tumor-bearing mice 6 hours before the termination of the experiment. To identify the proliferating endothelial cells, we have

stained blood vessels with the endothelial cell marker CD31. Data indicated that rHuEPO treatment did not influence significantly the proliferation of A431 tumor cells (Fig. 3A-C). However, rHuEPO significantly increased the BrdU labeling index of endothelial cells by up to \sim 100% compared with untreated tumors (Fig. 3A-C). Parallel to these changes, rHuEPO administration resulted in a 2-fold increase in the number of endothelial cells per vessel perimeter, measured by computer-aided morphometry ($P < 0.001$; Fig. 3D).

Because the major endothelial mitogen is VEGF, we have analyzed the effect of *in vivo* rHuEPO treatment on its expression level in A431 tumor xenografts. In the xenograft, stromal components of the tumor tissue derives from the murine host; therefore, we can determine VEGF expression by the stroma and the cancer cells separately using species-specific primers (Fig. 3E and F, insets). Quantitative PCR analysis of VEGF mRNA deriving from the host and the xenograft indicated that rHuEPO treatment significantly decreased the expression of VEGF *in vivo* (Fig. 3E and F).

Effect of recombinant human erythropoietin treatment on pericytes. Pericytes were detected in the tumoral vessels using smooth muscle actin immunohistochemistry. In control A431 tumors, pericytes continuously covered the endothelial cells of the intratumoral blood vessels, but following rHuEPO treatment this pericyte coverage became discontinuous (data not shown). By morphometry, we found that the average pericyte coverage was significantly reduced in rHuEPO-treated tumors compared with controls (59.5 \pm 12.0% and 85.1 \pm 10.1%, respectively, $P = 0.004$).

Effect of recombinant human erythropoietin treatment on *in vivo* labeling of cancer cells by DNA-binding agents. The observed increased efficacy of chemotherapy and enlargement of blood vessels after rHuEPO treatment raised the possibility that rHuEPO may affect the bioavailability of DNA-binding agents in the tumor tissue. Therefore, we have injected a supravital DNA adduct (Hoechst 33342) into the tail vein of the A431 tumor-bearing SCID mice on the 21st day after tumor implantation, and 15 minutes later examined the labeled nuclei in cryosectioned tumor tissues by fluorescent microscopy. Results indicate that the total Hoechst-labeled tumor area inside the A431 xenograft increased >2 -fold in rHuEPO-treated animals compared with controls ($P < 0.01$; Fig. 4A, B, and E). Next, we used a molecular imaging technique to prove

that rHuEPO modulates the labeling of cancer cells by a chemotherapeutic agent. For this purpose, we used Doxorubicin, a chemotherapeutic agent with red autofluorescence when excited by green light. We applied Doxorubicin (2 mg/mL in 0.2 mL) in A431 tumor-bearing animals 15 minutes before termination of the experiment, and cryosections of A431 tumors were analyzed again by fluorescent microscope. The result of this experiment was highly similar to that of the Hoechst dye labeling: the Doxorubicin-labeled area of A431 xenograft (identified by nuclear red fluorescence) was >2-fold larger in rHuEPO-treated than in control animals ($P < 0.01$; Fig. 4C-E). To compensate the effect of parallel vessel enlargement, we have also calculated the ratio of average labeled tumor area/vessel perimeter unit: In the rHuEPO-treated tumors, this parameter was also found to be increased significantly ($P < 0.05$) compared with controls (Fig. 4F).

Discussion

Several *in vitro* and *in vivo* experiments showed that rHuEPO treatment modulates tumor growth and the effectiveness of cancer therapy. rHuEPO enhanced the antitumor efficacy of photodynamic therapy in mice (12), and restored anemia-induced reduction of cyclophosphamide cytotoxicity in rat tumors (13). Moreover, rHuEPO alone induced tumor regression and antitumor immune response in murine myeloma models (8). Furthermore, various

chemotherapeutic agents were more effective in the presence of oxygen (14), and their antitumor effects correlated with the tumor perfusion (15). However, novel studies questioned the benefit of rHuEPO therapy. Certain cancer types may express erythropoietin and erythropoietin receptor, and this cytokine signaling system may promote cancer growth (7, 16, 17). Furthermore, the inhibition of erythropoietin signaling destroyed ovarian and uterine cancer xenografts (18). Recently, in a controversial clinical trial, researchers discovered that patients with metastatic breast cancer, receiving chemotherapy and rHuEPO to prevent anemia, had a higher mortality rate than the placebo-treated group (9).

In our human epidermoid and colorectal cancer xenograft models, rHuEPO prevented the decrease in RBC and hematocrit values, had no effect on the growth of tumors, but significantly increased the antitumor efficacy of 5-FU. Although we detected erythropoietin receptor in both A431 and HT25 human tumor cells, in contrast to the suggestion of others (7, 16, 17), rHuEPO treatment did not stimulate cancer cell proliferation *in vitro* and *in vivo* at human-equivalent doses. Because rHuEPO did not influence the *in vitro* antiproliferative effect of 5-FU either, we suggested that the *in vivo* effects of rHuEPO treatment were not mediated by direct effects on tumor cells.

The major side effect of erythropoietin, beside influencing hematopoiesis, is its effect on endothelial cell proliferation. It is well documented that endothelial cells express erythropoietin

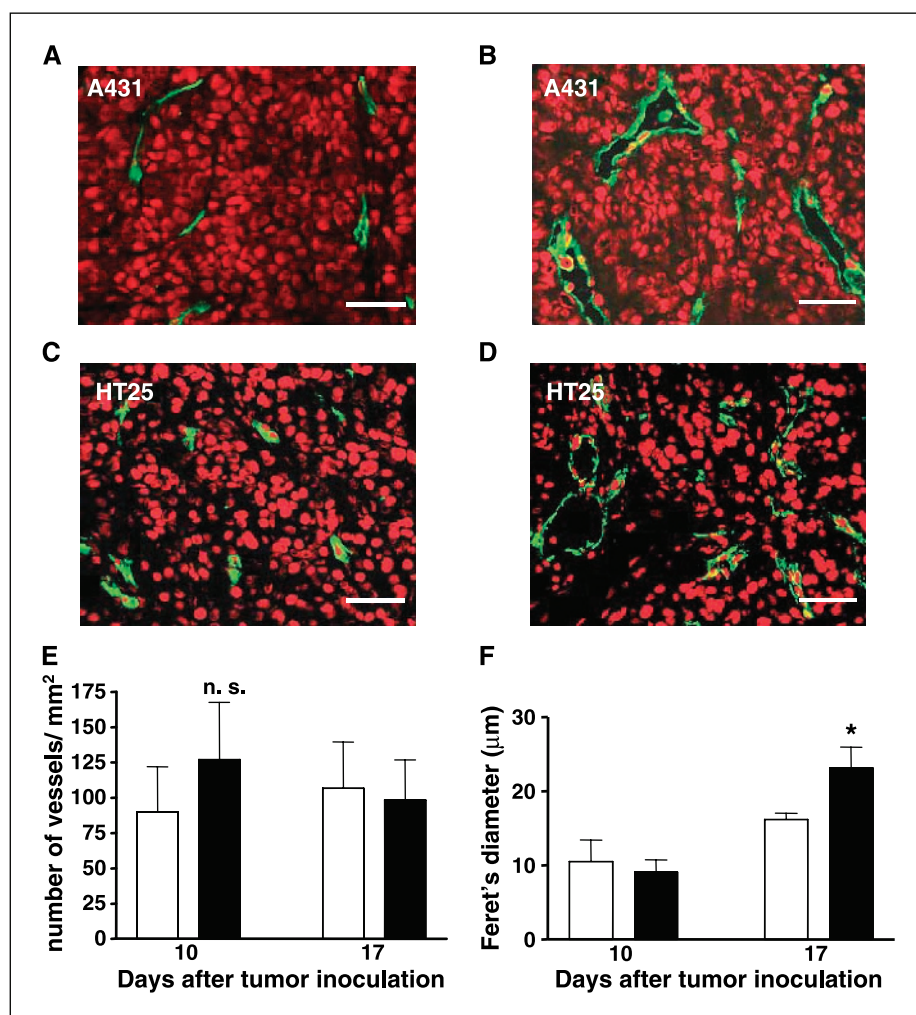


Figure 2. Effect of rHuEPO treatment on microvessels in A431 and HT25 human tumor xenografts (for rHuEPO treatment protocol, see Fig. 1C and D). A to D, immunofluorescence of microvessels labeled by CD31. On day 17 after tumor inoculation, rHuEPO (B and D) at human-equivalent dose (150 IU/kg, thrice per week) increased the size of microvessels compared with control (A and C) in growing tumor xenografts. Microvessels were identified by anti-CD31-FITC (green) and nuclei by propidium iodide (red). Bar, 50 μ m. E, quantification of CD31⁺ microvessels in A431 tumors. rHuEPO treatment had no significant effect on the intratumoral vessel density on day 10 or 17. Columns, mean; bars, SD ($n = 10$; open columns, control; filled columns, rHuEPO-treated tumors). F, comparison of Feret's diameter of microvessels at days 10 and 17 in A431 tumors. Larger vessels were developed by the 17th day after tumor inoculation in rHuEPO-treated tumors. Columns, mean; bars, SD ($n = 10$; open columns, control; filled columns, rHuEPO-treated sample; * $P < 0.05$).

receptor, and several data indicated that erythropoietin inhibited apoptosis, and induced proliferation and migration of these cells (19, 20). Erythropoietin is a potent angiogenic factor comparable in its effect to VEGF (21). rHuEPO stimulated the endothelial tube formation *in vitro* and angiogenesis in chicken chorioallantois membrane assay *in vivo* (22). rHuEPO treatment did not affect the microvessel density of human cancer xenografts in our study but induced a significant vessel enlargement. To explore the background of the rHuEPO-induced increase in vessel size, we measured the proliferation index in the tumor mass using BrdU. Notwithstanding that A431 tumor cells express the erythropoietin receptor, their proliferation index did not change upon rHuEPO treatment, corresponding to the results of our *in vitro* proliferation tests. On the other hand, rHuEPO significantly increased the endothelial cell proliferation inside the living tumor mass without affecting pericytes, resulting in incompletely covered endothelial channels in the tumor tissue. Our result that both the host- and tumor-derived VEGF expression had decreased significantly in

rHuEPO-treated xenografts compared with controls suggest that the proliferation promoting effect of rHuEPO on tumoral endothelial cells is independent of VEGF production. Furthermore, this finding also suggests that the rHuEPO-corrected RBC and hematocrit levels (as well as hypoxia; ref. 12) may control tumoral VEGF expression.

Because the antitumor efficacy of 5-FU was significantly improved by rHuEPO, it is important to define the possible mechanisms and direct drug delivery to tumor cells could be one option. rHuEPO treatment increased 2-fold the supravital DNA-adduct labelings (Hoechst or Doxorubicin) of cancer cells in the xenografts. Our data that rHuEPO treatment not only increased the labeled area in the xenografts, but also increased significantly the labeled tumor area relative to vessel perimeter suggest that this treatment may affect drug perfusion independently of the alteration of vessel size. Previous studies showed that erythropoietin treatment increases O₂ perfusion of xenografts independently of hemoglobin level (11). These observations, together with the

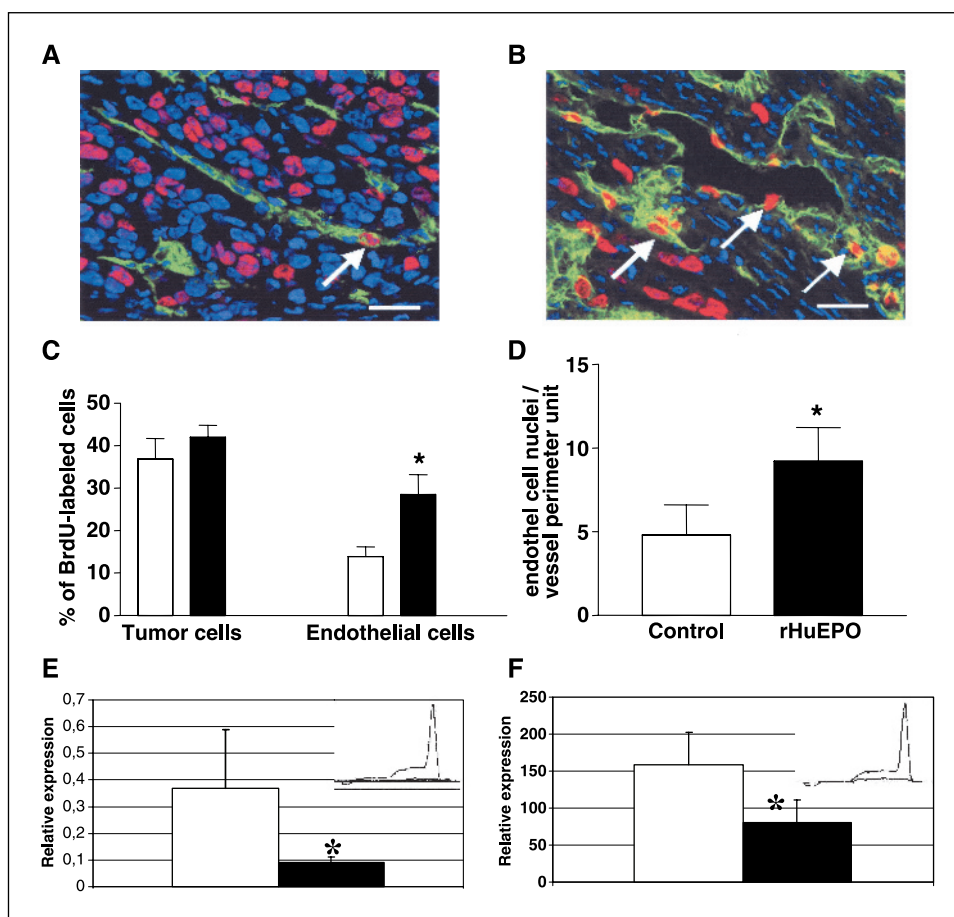


Figure 3. Effect of rHuEPO on BrdU labeling index of A431 human tumor cells and mouse endothelial cells (for rHuEPO treatment protocol, see Fig. 1C). *A* and *B*, immunofluorescence microscopy: BrdU (red), CD31 (green), and Hoechst 33342 (nuclear staining, blue) triple labeling of control (*A*) and rHuEPO-treated (*B*) tumors on day 18 following tumor inoculation. Arrows, CD31⁺/BrdUrd⁺ endothelial cells; bar, 25 μ m. *C*, quantification of BrdUrd labeling index of tumor and endothelial cells *in vivo* in 18-day-old A431 tumors. rHuEPO treatment significantly increased microvascular endothelial cell labeling in the tumor, but it had no effect on the perivascular tumor cell population. Columns, mean; bars, SD, $n = 10$ (open columns, control; filled columns, rHuEPO-treated sample; * $P < 0.001$). *D*, quantification of CD31⁺/Hoechst⁺ endothelial cell nuclei per unit (100 μ m) of vessel perimeter. Results are representative of different areas per tumor from five animals (number of microvessels >100 per sample). Columns, mean; bars, SD (* $P < 0.001$). *E* and *F*, the relative expression of VEGF showed significant (* $P < 0.05$) decrease in the case of rHuEPO-treated animals in the tumor (*E*) and in the host tissue (*F*) as well (open columns, control; filled columns, rHuEPO-treated tumors). Columns, mean of results derived from three mice calculated as the average of three amplification reactions done on a single cDNA sample from each animal; bars, SD. Melting curve analysis showed that VEGF primer pairs amplified a single and species-specific product: on human sample (Kaposi sarcoma, *E*, inset)—the human primers gave a single, well-defined peak, whereas there was no amplification using mouse primers; mouse sample (B16 murine melanoma, *F*, inset)—single peak was only detectable with mouse VEGF primers and not with human ones.

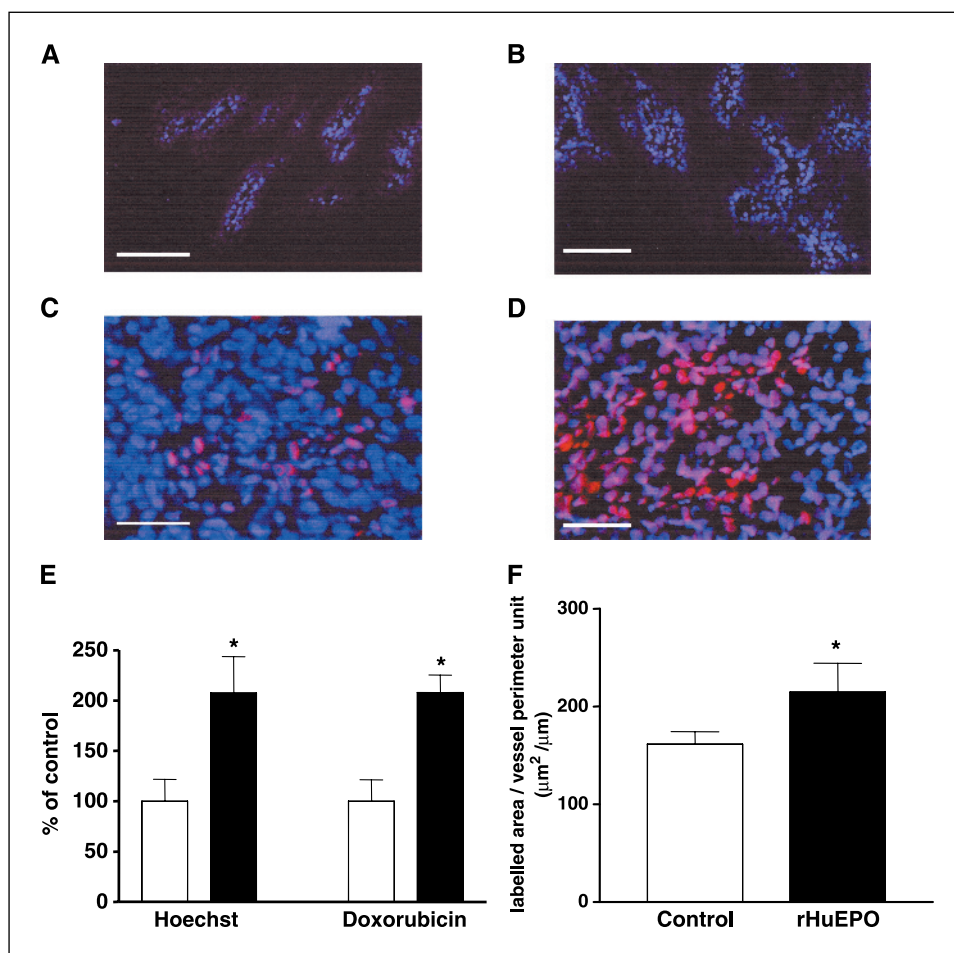


Figure 4. Functional imaging of DNA-adduct labeling of A431 tumor xenograft with fluorescent microscopy (for rHuEPO treatment protocol; see Fig. 1C). A and B, Hoechst 33342 nuclear labeling (blue) 15 minutes before termination of the experiment in control (A) and rHuEPO-treated (B) tumor tissues on day 21 after tumor inoculation (bar, 50 µm). C and D, Doxorubicin labeling (red) 15 minutes before termination of the experiment in control (C) and rHuEPO-treated (D) tumor tissues. Nuclei were labeled on crysections by Hoechst 33342 dye (blue, bar, 50 µm). E, morphometry of Hoechst- and Doxorubicin-labeled areas in A431 tumors. Columns, mean percentage of control; bars, SD (n = 10; open columns, control; filled columns, rHuEPO-treated tumors; *P < 0.05). F, changes in average labeled area per vessel perimeter unit during rHuEPO treatment. Columns, mean; bars, SD (n = 10; *P < 0.05).

results of our DNA-adduct studies, suggest that rHuEPO may modulate blood perfusion in tumors, increasing the bioavailability of anticancer agents.

Taken together, these data show that rHuEPO had profound effect on tumoral blood vessels in human epidermoid and colorectal cancer xenograft models. rHuEPO did not influence the density of microvessels, but increased their size due to the selectively enhanced proliferation of endothelial cells. Our observation in the A431 xenograft model correlates well with our previous data where we have shown that in murine cutaneous melanoma model the newly formed endothelial cells within the tumor, due to their increased proliferation index, participate in vessel dilation only instead of providing a source for new vessel production (23). In the present study, the increased vessel surface resulted in improved drug delivery to tumor cells and augmented its antitumor effectiveness. These effects of rHuEPO were restricted to tumoral vessels because there were no alterations observed in

microvessels of mouse muscle or hepatic tissues (data not shown). A possible explanation for the differential effect of rHuEPO on tumor microvessels compared with normal ones is the severe hypoxic conditions in the tumor tissue, which might increase the expression of erythropoietin receptor on intratumoral endothelial cells (24). Based on these results, studies are urgently needed to test the general nature of these observations in rHuEPO-treated cancers.

Acknowledgments

Received 7/15/2004; revised 4/27/2005; accepted 5/5/2005.

Grant support: Ministry of Education, Hungary, grant NKFP-1/48/2001 and National Research Foundation grants OTKA D048519 and F046501.

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We thank Katalin Derecskei and Ibolya Sinkáné for excellent technical assistance and Andrea Ladányi for critical review of the manuscript.

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