ERYTHROPOIETIN AND G-CSF RECEPTORS IN HUMAN TUMOR CELLS: EXPRESSION AND ASPECTS REGARDING FUNCTIONALITY

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Aims and background: Recombinant human erythropoietin (Epo) and granulocyte-colony-stimulating factor (G-CSF) are used to stimulate hematopoiesis in patients with malignant diseases. These cytokines transduce their biological signal via the Epo receptor (EpoR) and G-CSF receptor (G-CSF-R) into the cell. We therefore investigated in human tumor cell lines the expression of these receptors in tumor cells as well as their response to Epo and G-CSF.
Methods and study design: The expression of EpoR and G-CSF-R mRNA was analyzed with reverse transcription-polymerase chain reaction (RT-PCR). EpoR protein expression was further monitored with Western blot and immunocytochemistry analysis. The cellular response to various concentrations of Epo was evaluated using ^{3}[H]-thymidine uptake, Northern blot of c-fos expression and tyrosine kinase activity assay. The proliferation after G-CSF incubation was analyzed with the MTS assay.
Results: In this study EpoR mRNA and protein were detected in various human tumor cell lines. Treatment with Epo did not influence the proliferation rate of examined EpoR-positive tumor cell lines.Epo did not stimulate the tyrosine kinase activity nor did it affect the c-fos mRNA in these cell lines.G-CSF-R mRNA was only detected in two myeloid cell lines. Treatment with G-CSF did not increase the proliferation of these cells.
Conclusions: These results demonstrate that Epo and G-CSF did not modulate the growth rate of examined receptor-positive tumor cell lines; the presence of the Epo receptor seems not essential for cell growth of these tumor cells in cell culture.

Key words: Epo, G-CSF, human Epo receptor (EpoR), human G-CSF receptor, human tumor cell lines.

Introduction

The cytokine erythropoietin (Epo) is the principal regulator of erythropoiesis; it stimulates in an endocrine way the proliferation and differentiation of the erythroid precursor cells[1]. The glycoprotein is produced in the adult kidney and in the fetal liver. Recombinant erythropoietin (Epo) is indicated for the correction of anemic conditions in patients with malignant diseases. Use of Epo may substitute or reduce blood transfusions in these patients. The efficiency of such an approach in cancer patients is currently under investigation[2,3]. Epo exerts its effect by association with a specific cell surface receptor, the Epo receptor (EpoR), a member of the cytokine receptor superfamily[4]. Following ligand binding EpoR forms a homodimer and activates cytoplasmic protein tyrosine kinases (PTKs) to induce downstream signaling pathways: phosphorylated Janus kinases (JAKs) rapidly induce signal transduction and activation of transcription (STAT) proteins that translocate to the nucleus and induce gene expression[4]. Recent studies have provided evidence that in the central nervous system and in the uterus a paracrine Epo/EpoR system exists which is independent of the endocrine erythropoietic system. In the brain Epo has a neuroprotective effect under hypoxic conditions[5-7]. Furthermore, Epo is present in human milk and EpoR is detectable in enterocytes postnatally, indicating another function of Epo: influence on the neonate's intestinal function[8]. EpoR expression is known to be present in many non-erythroid tissues *in vivo*: megakaryocytes[9], endothelial cells[10], Leydig cells[11], embryonal stem cells[12], stromal cells of fetal liver[13], and placenta[14]. Expression of EpoR has been shown in human erythroid cell lines including OCIM1, JK-1, KU-812 and RM10, but also in non-erythroid tumor cell lines (eg the myeloma cell line MM-S1)[15]. In UT-7 cells EpoR is overexpressed (about 10,000 receptors/cell) and may play a role in leukemogenesis[16]. Expression of EpoR has also been reported in renal cell lines[17], in melanoma cells[18], in neuronal cell lines[19], and in astrocytes of the brain[20]. However, the role of EpoR in these cells is not yet fully understood[21], and it is therefore important to investigate the function of EpoR in tumor cells.

The cytokine granulocyte colony-stimulating factor (G-CSF) is the main factor for the development of the granulocytic lineage and stimulates – like Epo – the proliferation and differentiation of late precursor cells

in bone marrow. Moreover, G-CSF leads to mobilization of early precursor cells[22]. These effects indicate a broad application range of G-CSF, often in combination with Epo, to accelerate the reconstitution of hematopoietic cells after bone marrow transplantation and chemotherapy. The expression of the G-CSF receptor has also been examined in malignant cells, for example U-937[23] and ovarian cancer cells[24].

In the present study we have investigated the effects of Epo on human tumor cells and suggest a safety profile for the treatment of cancer patients. The expression of the EpoR at mRNA and protein level in human tumor cell lines was analyzed with reverse transcription-polymerase chain reaction (RT-PCR), Western blot and immunocytochemistry. The cellular response to various concentrations of Epo using ^{3}[H]-thymidine uptake, Northern blot of c-fos and tyrosine kinase activity assay was evaluated. The expression of G-CSF-R mRNA in tumor cells was examined with RT-PCR, and the MTS assay was chosen to analyze proliferation after stimulation of the cells with G-CSF.

Material and methods

Cell cultures

Tumor cell lines were provided by the tumor bank of the German Cancer Research Center (DKFZ), Heidelberg, Germany (for a description see Table 1). The UT-

Table 1 - Expression of hEpo-Receptor mRNA and protein in different benign and malignant cell lines.The mRNA analyses were performed with qualitative RT-PCR, the protein analyses were carried out with Western Blot [ ] and immunfluorescence staining

Cell line	Cell type	hEpoR mRNA	hEpoR protein
BA/F3-hEpoR	Mouse pro-B-cell line	nd	[+]
CX-1	Colon adenocarcinoma	(+)	(+)
Dan-G	Pancreatic carcinoma	+	+
DU145	Prostate carcinoma	+	+
HBTPL-1	Urinary bladder carcinoma	+	+
HeLa	Cervix carcinoma	+	nd [+]
HepG2	Hepatoma	+	+ [+]
HL-60	Promyelocytic leukemia	+	+
HT-29	Colon adenocarcinoma	(+)	+ [+]
K562	Erythroleukemia	+	+ [+]
KG-1a	Acute myelogenous leukemia	(+)	(+) [+]
KTCTL-26/-103	Kidney carcinoma (both)	+	nd
KTCTL-30	Kidney carcinoma	+	+ [+]
MCF-7	Breast carcinoma	+	+ [+]
NMB	Neuroblastoma	+	+ [+]
PLC	Hepatoma	+	+
Raji	Burkitt's lymphoma	–	nd
RD	Rhabdomyosarcoma	+	nd
RT112	Urinary bladder carcinoma	–	+ [+]
S117	Thyroid sarcoma	+	+ [+]
Sk-Mel-5	Melanoma	+	+ [+]
Sk-NMC	Neuroblastoma	nd	+ [+]
THP-1	Acute monocytic leukemia	+	nd
UT-7/Epo	Megakaryoblastic cells	+	+ [+]
HaCat	Immortalized keratinocytes	–	+ [+]
Keratinocytes	Primary cell culture	–	(+) [–]
Bone marrow	Normal tissue	+	nd

(+), + is the intensity of the ethidium bromide signal of the DNAband in the gel or the fluorescent signal of the immuncytochemical staining; – indicates no staining of the cells; nd, no data available.

7/Epo cell line was provided by HF Bunn and M Showers (Brigham and Women's Hospital, Boston, MA, USA), BA/F3-hEpoR by the University of Bonn (Germany), and NMB by Y Wollman. Cell lines were maintained in RPMI 1640 medium (Gibco and Sigma, Germany), and UT-7/Epo was cultivated in IMDM (Gibco). The medium was supplemented with 10% FCS (Sigma), 1% glutamine and (except BA/F3-hEpoR) 1% penicillin-streptomycin (Gibco). The cell lines BA/F3-hEpoR and UT-7/Epo depend on 1 IU Epo/mL medium and the mouse myeloid NFS-60 cell line on 10 ng/mL G-CSF. The cell lines used for thymidine uptake, Northern blot analysis and tyrosine kinase activity assay were grown during experiments in DMEM/Ham's F12 mix medium with 10, 1 and 0.1% FCS to exclude cross-reaction of FCS and Epo or G-CSF. The cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C. Recombinant human Epo and G-CSF were purchased from Roche (Boehringer Mannheim). Bone marrow was isolated from donation samples.

RNA preparation and reverse transcription PCR (RT-PCR)

Total cellular RNA was isolated by a single-step guanidinium thiocyanate-phenol-chloroform extraction using RNAzol^{TM} B (AGS, Heidelberg) according to the manufacturer's protocol[25]. The RNA was quantified spectroscopically and its purity controlled by the ratio 260/230 nm and 260/280 nm. Primers were designed according to EpoR mRNA sequence (accession number: M34986) with the computer program HUSAR (German Cancer Research Center, Heidelberg). The sequence of the EpoR forward primer is 5'–AGT TCG AGA GCA AAG CGG CCT–3' and of the EpoR reverse primer 5'–ACG CGC AAC TCTAGG GGC AC–3'; the amplicon has a length of 271 bp. In the same way primers for detection of G-CSF-R message were chosen from G-CSF-R mRNA sequence (accession number: X55721): forward primer 5'–CCA TAT TCT GGT ACA ACA GCA GG–3' and reverse primer 5'–ATC ATC AAG CAG AAC TGC AGC CA–3'. Primers span different exons to exclude false-positive amplification signals caused by DNA contamination.

For cDNA synthesis 1 µg of total RNAand 100 ng of the reverse primer or 250 pmol of poly(dT)$_{15}$ (Roche) in a total volume of 6 µL were heated to 72 °C for 4 mins, cooled to 37 °C prior to addition of 9 µL reaction mixture (1.5 µL 10 x cDNA buffer (500 mM Tris-HCl, 60 mM $MgCl_2$, 400 mM KCl, pH 8.3), 2 µL dNTPs (10 mM), 10 units RNAsin (Roche), 5 units AMV Reverse Transcriptase (Roche) and sterile water to adjust to 9 µL). The reaction was sustained for 60 mins at 37 °C and stopped by heating to 94 °C for 10 mins.

One microliter of cDNA was used in 25 µL PCR amplification reaction mixture containing 2.5 µL 10 x PCR buffer (100 mM Tris HCl, 20 mM $MgCl_2$, 500 mM KCl, 1 mg/mL gelatin, pH 8.3), 4 µL dNTPs (10 mM), 100 ng of each primer, 2.5 units Taq-Polymerase (Roche) and sterile water for volume adjustment to 25

μL. The reaction mix was overlaid with 50 μL mineral oil. Thirty-five cycles of amplification were performed in a Perkin Elmer Cetus thermal cycler under the following conditions: 1 min denaturation at 94 °C, 30 s annealing at 61 °C (using EpoR primers) or at 63 °C (using G-CSF-R primers), 30 s elongation at 72 °C and a final extension at 72 °C for 10 mins. Nested primers were used to identify the amplification product. The amplified cDNA product was electophoresed on a 3% Metaphor agarose gel in TBE buffer or on 8% polyacrylamide gel in TBE buffer.

Western blot analysis

Cell lysates were prepared by lysis of 2.5×10^4 cells in buffer containing 1% Triton X-100, 10 mM Tris-HCl pH 6.8, 2 mM PMSF, 10 μM leupeptin, 2 mM o-phenanthrolin, 2 mM EDTA, and 10^3 U/mL aprotinin for 40 mins; insoluble fragments were removed by centrifugation at 15,000 x g for 10 mins. Lysates were boiled in SDS sample buffer under reducing conditions (2.5% 2-mercaptoethanol) for 5 mins. After SDS-PAGE (10%) samples were electroblotted onto Hybond C membrane (Amersham, Braunschweig), blocked with 3% low-fat milk in PBS (Glücksklee, Nestle, Germany) for 20 min and incubated with 3 μg/mL MoAb anti-hEpoR (mh2er/16.5.1, supplied by Genetics Institute Cambridge, MA, USA) at 4 °C overnight. After washing the blots were probed with a 1 : 10,000 dilution of a goat anti-mouse horseradish-peroxidase (HRP) conjugated antibody (DAKO, Hamburg) for 2 hrs. Blots were then washed and incubated with Enhanced-Chemoluminescence (ECL) substrate (Amersham) to visualize the immunoreactive bands. Experiments were performed three times to confirm the results.

Immunocytochemistry

Adherent growing cells were seeded on silanized slides and grown at 37 °C in a humidified atmosphere with 5% CO_2 for 24 hrs. Suspension cells were adhered by cytocentrifugation (250 rpm for 3 mins; Shandon Cytospin 2). A more careful method was the use of Cell-Tak adhesive (Becton Dickinson, Heidelberg, Germany), which coats a substrate to immobilize cells or tissues. Fixation of the cells was carried out in freshly prepared 4% paraformaldehyde/PBS solution at room temperature for 10 mins, followed by washing the slides twice in PBS. The cells were incubated with 10% fetal calf serum in PBS. After blocking the localization of EpoR was assessed using mouse monoclonal antibody (MoAb) anti-hEpoR (mh2er/16.5.1, supplied by Genetics Institute Cambridge, MA, USA): 2 μg antibody for each reaction was diluted in 1% BSA/PBS mixture and incubated at 37 °C for 1 hr. After subsequent washing in PBS, the cells were incubated at 37 °C for 1 hr with an anti-mouse secondary antibody labeled with Alexa-TM488 (Molecular Probes, Göttingen, Germany). If the secondary antibody was only biotinylated and not conjugated with a fluorochrome, an incubation with the streptavidin-conjugated fluorochrome fluorescein-isoth-

iocyanate (FITC) in a dilution of 1:40 (rat anti-mouse IgG, Dianova) in 1% BSA/PBS at room temperature for 1 hour followed. Finally, after washing steps with PBS, the cell nuclei were counterstained with propidium iodide (1 : 1000, 1 μg/mL) for 10 mins. After washing in PBS the cells were mounted with Vectashield (Linaris, Germany) and visualized using a Zeiss Axiophot microscope equipped with an epifluorescence unit. Photographs were taken on a Kodak E100S color slide film. Further primary anti-EpoR antibodies used were mh2er/7.9.2 (supplied by Genetics Institute Cambridge, MA) and anti-EpoR, Cat# MAB307 (R&D Systems, Wiesbaden, Germany). Each staining was compared with staining yielded by IgG isotype controls (DAKO, Hamburg, Germany) that were used in the same protein concentration as the primary antibody. The results were reproduced three times.

Direct immunocytochemistry was performed to exclude staining artifacts caused by the use of a secondary antibody and background effects. The primary anti-EpoR antibody (mh2er/16.5.1) was labeled with the fluorescent dye Alexa™568. The labeling reaction was done according to the manufacturer's recommendation (Alexa™568 Protein Labeling Kit (A-10238), Molecular Probes). 2 μg labeled antibody was used for each reaction in 1% BSA/PBS mixture with incubation at 37 °C for 1 hr. After subsequent washing in PBS the cells were mounted with Vectashield.

Confocal laser scanning microscopy (CLSM)

Confocal laser scanning images were taken with a Leica laser scanning microscope (TCS 40, Leica, Germany), objective 40 x /1.0, oil immersion. Double staining of EpoR was evaluated with a Zeiss SLM 410, objective 63 x /1.41 PlanApo, oil immersion. Both were equipped with an Argon Krypton laser. Cells were grown on 170 ± 10 μm thick, 15 mm x 15 mm coverslips (Assistent, Germany). Each section was averaged eight times and each image consisted of 512 x 512 x Z pixels (Z: axial number of planes). The images were filtered with a 3 x 3 x 3 median filter which assigns to each pixel the mean intensity value of the immediately adjacent pixels. Background reduction was achieved by subtracting from each pixel the mean intensity of the whole image.

Tyrosine kinase assay

Tyrosine kinase activity was quantitatively detected with the Tyroscan Tyrosine Kinase EIA-Kit (Eurodiagnostics of the Netherlands). Cell samples were solubilized after incubation with Epo (5 IU/mL) for 0, 1, 5, 10, 15 and 30 mins. After removal of cell debris and nuclei at 4 °C with 800 x g for 10 mins the supernatant was separated into cytosolic and membrane fractions by ultracentrifugation at 4 °C with 48,000 x g for 10 mins and further processed according to the manufacturer's protocol. The microplates were coated with PGT (poly (Glu, Tyr) 4:1). Protein tyrosine kinases (PTK) of the samples react enzymatically with PGT: phosphate is

transferred from ATP to tyrosine and an anti-phosphoty-rosine antibody can bind specifically to the phosphorylated tyrosine molecules. A secondary antibody conjugated with HRP can bind to this complex and HRP catalyzes a color reaction.

Northern blot analysis

Cells were grown to 70-80% confluence, then trypsinized and plated out to a concentration of 7.5 x 10^5 up to 2 x 10^6 cells per flask. The cells were subsequently cultured for 48 hours in media containing different levels of FCS (10%, 1% or 0.1%). Subsequently, the cells were incubated with 100 IU Epo per mL for different intervals (0.5, 1, 2 or 6 hours). After such stimulation the total RNA was immediately isolated by the RNAzol™ B method. The total RNA (10 µg) was separated by electrophoresis in 1% agarose/formaldehyde denaturing gels, and blotted to nitrocellulose filters. Finally the blots were hybridized with probes labeled with ^{32}P (in vitro transcription). The v-fos probe was an 800 bp sequence of v-fos originating from mouse. As control for equal mRNA amounts a ß-actin probe was analyzed simultaneously.

[3H]-Thymidine uptake

The proliferation assay was performed by plating 2 x 10^4 cells/well (200µL) in a 96-well plate and subsequent cultivation for 48 hours in media containing different levels of FCS (10, 1 and 0.1%). 1000, 100 or 10 IU/mL of Epo was added and incubated for 16 hours, followed by pulse labeling with [3H]-thymidine (1 µCi/mL) for 4 hrs. Finally, the cells were transferred to a matrix and the incorporation of [3H]-thymidine was measured in a ß-counter. Since it was not possilbe to use this laboratory equipment for the experiments with G-CSF, we used the MTS assay.

Proliferation assay (MTS assay)

The CellTiter 96™ A_{queous} Non-radioactive Cell Proliferation Test (Promega, Germany) was used to test proliferation after incubation with G-CSF (the [3H]-thymidine technique was no longer available in the laboratory). Cells were seeded in a 96-well microtiter plate and incubated with G-CSF in a dilution range of 0, 3.85, 7.7, 15.4, 30.8, 61.6, 123, 246, 493, 986 and 1972 ng/mL. After 72 hours the MTS (dimethylthiazol-carboxymethoxyphenyl-sulfophenyl-2H tetrazolium) reaction was started with 20 µL of the MTS/PMS (phenanzine methosulfate) solution (2 mL MTS substance (333 µg/mL) + 1000 µL PMS solution (25 µM)). After incubation at 37 °C with 5% CO_2 for 75 mins, the plate was measured with a BioRad plate reader at a wavelength of 490 nm.

Epo-ELISA

To assess whether HepG2 produces Epo under the growth conditions that occur during experiments, the culture medium was collected at several points in time, and the concentration of Epo was analyzed using a commercial enzyme-linked immunosorbent assay (ELISA) to quantify Epo in medium (Roche, Germany). Growth medium of HepG2, KTCTL-30 and NMB cells was collected at 12, 36, 72 and 96 hours after seeding. The lower detection limit for this Epo-ELISAis 20 mIU/mL.

Results

Expression of EpoR and G-CSF-R in human tumor cell lines

With the reverse transcription polymerase chain reaction (RT-PCR) we demonstrated the expression of human Epo receptor mRNA in various tumor cell lines of different origin. Expression was not restricted to cell lines derived from the hematopoietic system. In many of the examined tumor cell lines the 271 bp-amplicon representing the mRNA of hEpoR was detected with RT-PCR. The ethidium bromide-stained gel showed the signal obtained from the mRNA of the cell lines HBT-PL-1, Sk-Mel-5, Dan-G, Du-145, and NMB (Figure 1). This signal was not found in an acute myelogenic leukemia cell line (KG-1a), in a cell line originating from a urinary bladder carcinoma (RT112), from Burkitt's lymphoma (Raji), from immortalized keratinocytes (HaCat), and in primary human keratinocytes. Also other cell lines (listed in Table 1) expressed the mRNA of the EpoR. Bone marrow, BA/F3-hEpoR cells and UT-7/Epo cells served as positive controls (Figure 1, Table 1). These cell lines were examined to obtain information about a wide spectrum of human malignant cell lines of different origin.

A corresponding experiment was carried out to detect mRNAof G-CSF-R in several tumor cell lines. RT-PCR showed expression of human G-CSF-R mRNA only in the myeloid cell lines KG-1a and THP-1. In all other cell lines of different origin (Table 1) no G-CSF-R mRNA was detectable with the established RT-PCR technique: CX-1, HepG2, HL-60, K562, KTCTL-30, PLC, RD, RT112, and S117 (Figure 2).

Detection of EpoR mRNA expression raised the question of whether the corresponding protein exists,
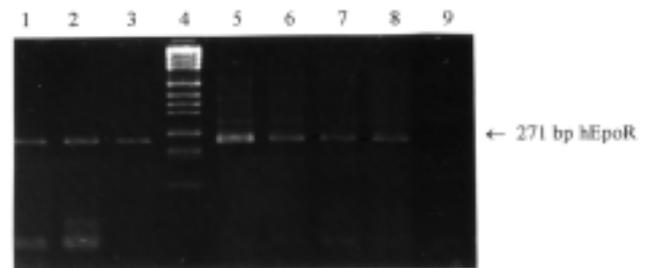

Figure 1 - RT-PCR analysis of human EpoR. PCR was performed with 35 cycles and samples were electrophoresed on a 3% Metaphor agarose gel (FMC). DNA was visualized by ethidium bromide staining. Each lane was loaded with 7.5 µLof PCR product; the 271 bp DNAband represents human EpoR. Lane 4 contains molecular size marker, the other lanes show the results obtained from isolated total RNA from the cells: (1) HBTPL-1, (2) Sk-Mel-5, (3) Dan-G, (5) UT-7/Epo, (6) DU145, (7) Dan-G, (8) NMB, (9) Raji. The reverse transcription was done with primer poly-dT$_{15}$ (lanes 1-3) and with reverse primer (lanes 5-9).

which is the relevant molecule for the cell. Western blot analyses and immunocytochemistry confirm the EpoR expression at the protein level in the human tumor cells tested (Table 1). A Western blot signal of 66 kD representing the hEpoR was visible in the cell lines RT112, HeLa, NMB, KG-1a, KTCTL-30, HepG2 and K562 (Figure 3). Bone marrow, BA/F3-hEpoR and UT-7/Epo cells served as positive controls, keratinocytes as negative control. We subsequently examined EpoR expression with immunocytochemistry to obtain knowledge about the localization and distribution pattern of the EpoR protein in the cell. The staining of HepG2 cells as shown in Figure 4 is representative of all tested cell lines. The fluorescent signal was characterized by the staining of granular structures that were spread throughout the cell. The same result was obtained with two other antibodies against human EpoR; the staining characteristics were identical, only the signal intensity between these antibodies differed (Table 1).

A confocal laser scanning microscope (CLSM) was used to locate the exact position of the fluorescent signal within the cells (Figures 5 and 6). An optical section of the center of a cell from an image series is shown in Figure 5. This image of KTCTL-30 cells is representa-
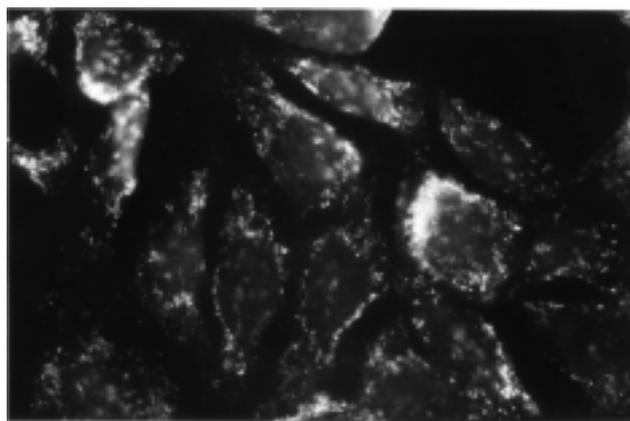

Figure 4 - Immunofluorescence analysis of EpoR in HepG2 cells. Indirect immunocytochemical staining was performed with MoAb anti-hEpoR (mh2er/16.5.1) and secondary antibody conjugated with Alexa™488. Magnification x 1000, image side length 1.3 mm.

Figure 2 - RT-PCR analysis of human G-CSF receptor. RT was done with poly-dT$_{15}$ and qualitative PCR was carried out with 35 cycles; amplification products were electrophoresed on an 8% polyacrylamide gel (TBE buffer). Each lane was loaded with 9 μLof PCR product and the resulting 460 bp band represents the G-CSF-R. Lanes 5 and 24 contain molecular size marker VI (Roche). The other lanes show the results obtained from isolated total RNAfrom the cell lines: (1, 2) KTCTL-30, (3, 4) PLC, (6, 7) RD, (8, 9) RT112, (10, 11) S117, (12, 13) THP-1, (14, 15) CX-1, (16, 17) HepG2, (18, 19) HL-60, (20, 21) K562, (22, 23) KG-1a, (25) human healthy bone marrow, (26) PCR-negative control.
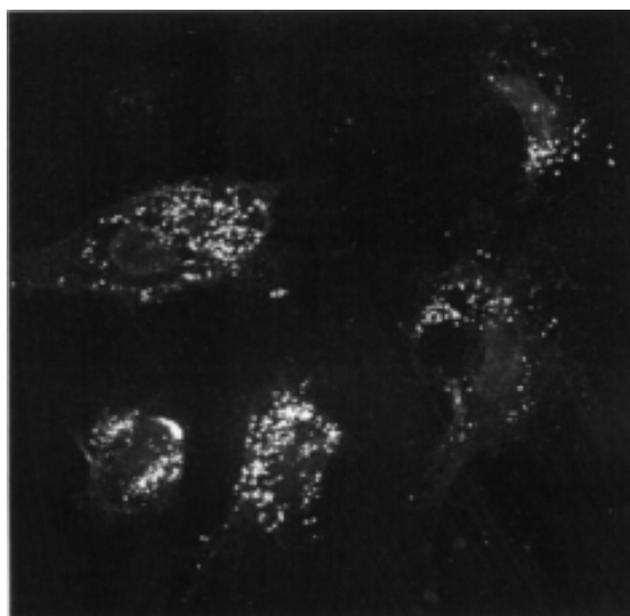

Figure 5 - Immuncytochemical detection of human EpoR. Confocal laser scanning image of KTCTL-30 cells. An optical section through the center of the cells; image side length 195 μm.
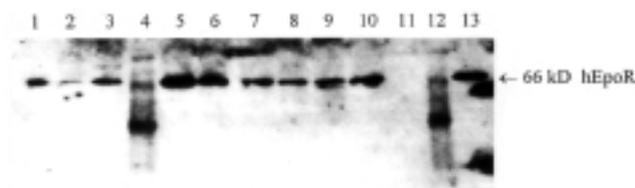

Figure 3 - Western blot analysis. Cell lysates were electrophoresed on 10% SDS-PAGE and analyzed by Western blotting with MoAb anti-hEpoR (mh2er/16.5.1). Lanes 4 and 12 contain SDS-PAGE standard solution (Sigma No. P-1677). Other lanes: (1) RT112, (2) BA/F3-hEpoR, (3) UT-7/Epo, (5, 6) HeLa, (7) NMB, (8) KG-1a, (9) KTCTL-30, (10) HepG2, (11) keratinocytes, (13) K562.

tive of all other cells investigated by immunocytochemistry. The double staining of EpoR with simultaneous staining of both direct and indirect immunocytochemistry is shown in Figure 6: analysis with CLSM gave identical staining patterns for the corresponding fluorescent signals Alexa™488 and Alexa™568. The staining characteristics in the Epo-dependent cell line UT-7/Epo growing in suspension were also similar to those of the adherent cell lines HepG2 or KTCTL-30 seen in Figures 4 and 5.
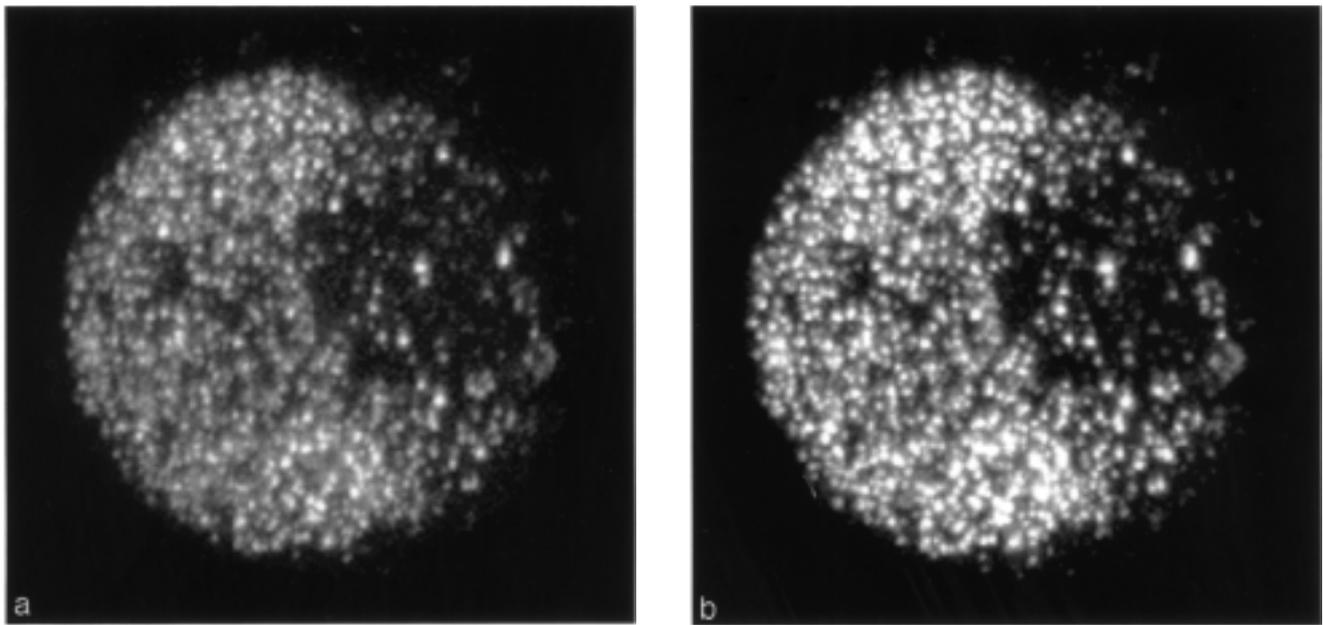
Figure 6 - Immunocytochemical detection of human EpoR in UT-7/Epo cells with MoAb against EpoR (mh2er/16.5.1). The primary antibody mh2er/16.5.1 is conjugated with Alexa[TM]568 (a) and the secondary antibody is conjugated with Alexa[TM]488 (b). Image side length 42 μm.

Influence of Epo on human tumor cell lines

The existence of receptors is a prerequisite for specific signal transduction. Therefore, in a second set of experiments we studied the influence of Epo on the cellular response of human tumor cell lines. To evaluate whether Epo had any effects on the EpoR-positive cells in particular and to clarify whether the cells express a functional EpoR we investigated ^{3}H-thymidine uptake, c-fos expression with Northern blot analysis and tyrosine kinase activity assay. The experiments were performed with the EpoR-positive cell lines HepG2, K562, KTCTL-30, NMB, and S117 and the EpoR-negative cell line RT112. Despite expression of EpoR none of these cells showed any response to Epo. The UT-7/Epo cell line served as positive control in these assays. The tyrosine assay was sensitive to all tyrosine kinases activated in the cell. Only the Epo-dependent cell lines UT-7/Epo (derived from megakaryoblasts) and BA/F3-hEpoR (a mouse B-cell line stably transfected with the human Epo receptor) showed an up to fivefold increase in tyrosine kinase activity after incubation with Epo and served as positive controls (Figure 7). Compared to the positive control only the protein tyrosine activity of UT-7/Epo showed significant activation. None of the other cell lines showed such induction of protein tyrosine activity (Table 2).

In addition, the expression of the immediate early gene c-fos was monitored with Northern blot analysis. As in the other experiments regarding the functionality of EpoR, an increase in c-fos mRNA was only observed in the positive control cells UT-7/Epo and BA/F3-hEpoR (Table 2).
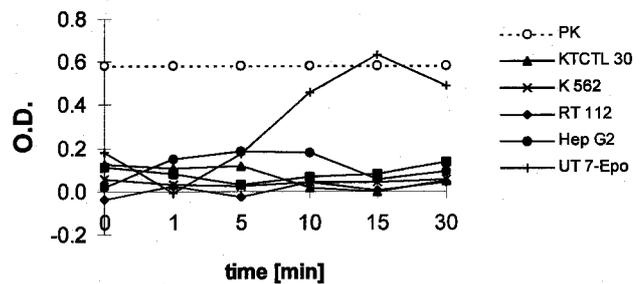

Figure 7 - The effect of Epo (5 IU/mL) on the phosphorylation of cytoplasmic tyrosine kinases. Cells were lysed and fractions were separated by differential centrifugation. PK is the positive control of the ELISAkit.

Table 2 - Cellular response after rhEpo treatment.The effect of rhEpo (5 IU/mL) on the phosphorylation of cytoplasmic tyrosine kinase activity. Northern Blot analysis using radiolabeled cRNA probes directed against c-fos mRNA. Proliferation assay was carried out by [^{3}H]-thymidine proliferation assay. Cells grown in culture medium with different FCS contents (10%, 1% and 0.1%) were incubated with 1000, 100 and 10 IU/mL rhEpo in these experiments (Northern blot and proliferation assay)

Cell line	c-fos (mRNA)	Proliferation assay	Tyrosine kinase activity
UT-7/Epo	+	+	+
HepG2	−	−	−
K562	−	−	−
KTCTL-30	−	−	−
NMB	−	−	nd
RT112	−	−	−
S117	−	−	nd

Different FCS concentrations (10%, 1%, 0.1%) in the culture medium had no influence on the results of signal transduction experiments. It was interesting to use also high Epo concentrations in the experiments because Epo occurred locally at high levels after application. No effect of Epo had been observed in any of the human tumor cells examined so far. Table 2 summarizes the results of three typical experiments.

To examine the functionality of the detected G-CSF receptor on the transduction of a proliferation signal, the growth of the G-CSF-R-positive cell lines KG-1a and THP-1 after stimulation with G-CSF was analyzed with the MTS assay. No difference in proliferation was observed. The growth rate of the G-CSF-dependent cell line NFS-60 (positive control) was increased after stimulation with G-CSF[26], but not that of the receptor-positive cell lines THP-1 and KG-1a (Figure 8).

The cell line HepG2 was used for many experiments and is known to produce Epo *in vitro* under hypoxic conditions[27]. To exclude that Epo synthesized by the cells itself did not interfere with in the experiments added Epo, we measured Epo in culture medium. However, under the growth conditions employed no Epo could be detected in the culture medium even after three days of growth. The sensitivity of the ELISA was 20 mIU/mL. The growth medium of NMB and KTCTL-30 cells was analyzed in the same way, but no Epo production could be observed either.

Discussion

The expression of EpoR in human tumor cell lines

In patients suffering from anemia of cancer, erythrocytes have a shortened survival in the circulation. The bone marrow fails to compensate this by increased red blood cell production, due to a low concentration of Epo or an inadequate Epo response[28]. This low level of Epo can be corrected by application of the recombinant cytokine Epo (reviewed in Erslev[29]). The interaction of Epo with tumor cells, especially its influence on quiet tumor tissue in patients, is a matter of concern and the growth modulation in nonhematopoietic tumor cells after Epo application should be characterized. It is therefore necessary to investigate whether Epo transduces signals into tumor cells. Berdel *et al.*[30] and Rosti *et al.*[31] studied the effect of Epo on growth regulation in several hematopoietic and nonhematopoietic tumor cell lines. They found no effect with techniques like clonogenic growth. However, for a specific interaction of cells with Epo the presence of the Epo receptor on the cell surface is essential. In the first part of our experiments we describe the expression of the human erythropoietin receptor (hEpoR). EpoR mRNA expression was found in a variety of human tumor cell lines. The design of primers was chosen not to distinguish the various forms of the Epo receptor (full length, truncated and soluble EpoR), but to amplify every Epo receptor form (Figure 1).

As shown, the human EpoR protein was detected in examined human tumor cell lines with immunocytochemistry and with Western blot (Table 1, Figures 3-6). Results with ligand immunoblot analysis support the results obtained with Western blot analysis.

When immunocytochemistry was performed only a weak fluorescent signal was seen on the cell surface, indicating a small number of receptor molecules on tumor cells, comparable to the number of receptors on erythroid precursor cells (300-1000 receptors per cell). Our results are similar to those obtained by Neumann *et al.*[32] regarding degradation of the Epo receptor. These authors found similar staining characteristics in NIH-3T3 cells stably transfected with EpoR as we did. They demonstrated that only 60% of the newly synthesized EpoR is processed to the glycosylated receptor protein in the Golgi apparatus; from then it has a half-time of 45-60 mins. It would therefore seem possible that the staining performed here showed not only the mature receptor but also the premature protein in vesicles of endoplasmic reticulum or Golgi apparatus. The fluorescently stained vesicles could also be lysosomes, where the receptor became degradated, while its intermediates could be recognized by the antibody against EpoR. The anti-EpoR antibody used in the presented experiments binds only the extracellular domain of human EpoR[33]. Moreover, a rapid degradation of receptor mRNA could be an explanation for the mRNA-negative but protein-positive results in the cell lines RT112, Raji and KG-1a. Expression of EpoR in the erythroleukemic cell lines K562 and HEL has been known since 1988[34,35]; binding studies showed 4-6 receptors per K562 cell and 30-35 receptors per HEL cell. Recent results have also demonstrated the synthesis of Epo by K562[36].

In previous experiments we found that EpoR is present in all lymphocytes isolated from leukemia patients (AML, ALL, CML, CLL), in lymphocytes of healthy persons, and in many samples from human skin tumors, but not in healthy skin tissue[37]. This finding, together with the overall expression of EpoR in human tumor
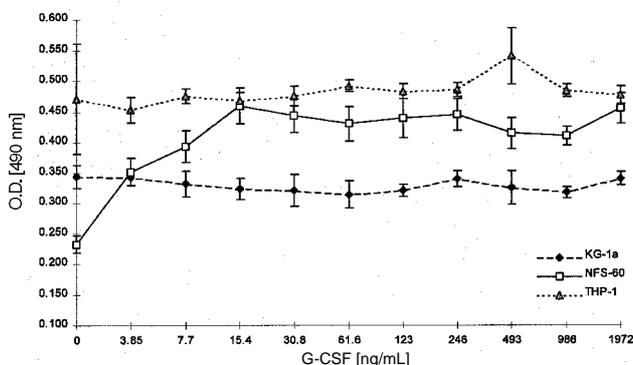

Figure 8 - Proliferation of G-CSF-positive cell lines in response to G-CSF. MTS test of the cell lines THP-1, KG-1a and NFS-60 after incubation with G-CSF. Cells were seeded in a 96-well plate and incubated with different G-CSF concentrations – arithmetic dilution. Two days after G-CSF exposure the MTS assay was performed. Each value represents the mean obtained from eight single readings.

cells, may suggest a relationship between EpoR expression and increased malignancy. Selzer *et al.*[18] also hypothesized that EpoR might be a progression marker for human melanoma cells.

Influence of Epo on tumor cell lines

The second part of the experiments concerns the effects of Epo on tumor cell lines: although hematopoietic and nonhematopoietic tumor cell lines express hEpoR, it was important to specify whether the receptor was functional in order to exclude the possibility that the antibodies against EpoR detected a truncated or mutated EpoR, which is unable to transduce the Epo signal. Epo could not stimulate proliferation of the tumor cell lines K562, KTCTL-30, HepG2, NMB, S117 and RT112 as shown by ^{3}[H]-thymidine uptake (Table 2). Only the Epo-dependent cell lines UT-7/Epo and BaF3/hEpoR, which served as positive controls, showed an increased proliferation rate. Also Kitamura *et al.*[38] were unable to detect an increase in the proliferation rate of TF-1 cells. By contrast, Lang *et al.*[39] showed increased proliferation in the human prostate cell lines PC-3 and DU145 after addition of Epo (0.1–1 mIU/mL) but not after incubation with G-CSF or IL-3. This result could be validated with the demonstrated detection of EpoR in DU145 cells (Table 1). Other groups did not observe any modulation of cell growth after Epo incubation. Increased proliferation was only found in some cytokine-dependent hematopoietic cell lines[30,31,40]. There has been recent evidence of stimulated proliferation in human renal carcinoma cell lines[17].

In further experiments presented here the signal transduction after cell stimulation with Epo was examined using the general activation for intracellular tyrosine kinases as an indicator of a successfully transducer Epo signal. Only in UT-7/Epo cells, which served as positive control, was an increase in tyrosine kinase activity observed. No induction of the intracellular tyrosine kinases was observed in the cell lines HepG2, K562, KTCTL-30 and RT112. The function of the receptor in these tumor cell lines therefore remains unknown. A possible cause of signal transduction inhibition could be a mutation of the receptor. Bittdorf *et al.*[41] described a truncated receptor form in the erythroleukemic mouse cell line F4N. However, such a mutation would not explain the presence of the receptor in this case because Western blot analysis showed the protein with its complete size of 66 kD (Figure 3), but other mutations of EpoR have been described in the literature and could be a reason for a negative response to treatment with Epo. All known mutations of EpoR cause a truncated receptor lacking the negative regulatory domain leading to erythrocytosis[42]. Only one exception was shown in a recent study on a child with primary familial and congenital polycythemia: no erythrocytosis developed despite a truncation of the intracellular domain of EpoR and hypersensitivity of erythroid progenitor cells to Epo[43].

Induction of the early response gene c-fos in the tumor cell lines was chosen as an indicator for signal transduction through EpoR triggered by Epo[44]. In contrast to the erythropoietin-dependent cell lines BaF3/hEpoR and UT-7/Epo as positive controls, c-fos could not be induced with Epo on tumor cell lines HepG2, K562, KTCTL-30, NMB, RT112 and S117, indicating that there is no signal transduction after Epo induction.

The cell line HepG2 was used for many experiments and is described as a model for the production of Epo *in vitro* under hypoxic conditions[27]. No Epo production could be observed in the growth medium of HepG2, NMB and KTCTL-30 cells under optimal growth conditions. Therefore additive effects of cell-produced and added Epo could be excluded in the described experiments. This is an important fact for the evaluation of the shown examination.

Experiments concerning effects of G-CSF on tumor cells (in vitro)

Our experiments show expression of human G-CSF-R mRNA only in the myeloid cell lines KG-1a and THP-1. Avalos *et al.*[45] described a functional G-CSF-R in two cell lines derived from a small cell lung carcinoma. Also the prostate cell lines DU145 and PC-3 expressed the G-CSF-R, but G-CSF could influence the growth and motility of PC-3 cells[39]. The same conditions pertain to some urinary bladder carcinoma-derived cell lines and in six of 26 tumor biopsies of the urinary bladder the G-CSF-R could be shown[46]. Similar to the presented Epo experiments it was not possible to modulate the proliferation rate of receptor-positive cells after incubation with G-CSF. However, it was shown that G-CSF as well as Epo can stimulate the growth rate in the leukemic cell lines HU-3 and M-07e[40].

Clinical significance of the results

Fifty to sixty per cent of patients with anemia of cancer can be treated successfully with Epo[47,48]. If Epo is used as a drug against anemia and for the treatment of cancer patients, it must not have any growth supporting effects on tumor cells. The obtained results support the safety profile of Epo because it seems to have no growth modulating effects on tumor cells at the actual level of knowledge. An exception was the acute myeloid leukemias (AML) due to the synergistic growth stimulating effect together with GM-CSF, G-CSF and IL-3[49]. It seems not useful to treat pediatric cancer patients with Epo because the anemia depends on a disturbed proliferation of the erythroid precursor cells[50]. The presented data do not suggest that G-CSF can modulate the growth of solid tumor cells *in vitro*. Nevertheless, recent studies have shown that G-CSF is secreted by some non hematopoietic malignant tumors including urinary bladder carcinoma, hepatoma and melanoma. Autocrine growth stimulation of the tumors cannot be excluded, and supervision of G-CSF therapy for cancer is therefore imperative[46].

More efficient than the separate application of Epo or G-CSF is a combination of these cytokines in some cases

for the reconstitution of blood cells after chemotherapy. Especially treatment of anemia in myelodysplastic syndromes (MDS) with Epo alone is poorly effective in most cases, and application of both factors results in a better response of the production of erythrocytes *in vivo* and *in vitro*[50,51]. However, another study with a combination of Epo and G-CSF did not show this synergistic effect[52].

Our *in vitro* data support the current findings that there is no dose-effect relationship between Epo concentration and tumor cell proliferation. The lack of expression of the protooncogene c-fos and the lack of tyrosine kinase activity in spite of EpoR expression indicate that the Epo signal is probably not transduced into the cell; both effects can be shown after successful signal transduction[4,44]. Differentiation processes cannot be excluded but have not been investigated in this study. Together with an Israeli group we found evidence that Epo might induce differentiation pathways in a neuroblastoma (NMB) cell line, as assessed by induction of the enzyme markers neutral endopeptidase, creatine kinase and dopamine uptake[54].

In summary, our experiments demonstrate on the one hand that EpoR mRNA and protein are expressed in most of the human tumor cell lines examined so far, and on the other hand that incubation with Epo does not lead to stimulation of proliferation in the EpoR-positive cell lines. The mRNA of the G-CSF receptor is only detectable in two cell lines, and G-CSF could not modulate the proliferation rate of G-CSF receptor-positive cells. Further insight into the different existing Epo/EpoR-systems in humans is required. On the basis of the currently available knowledge we conclude that our experimental data support the safety profile of Epo and G-CSF in the treatment of cancer patients, and that expression of the Epo receptor in tumor cells does not seem to be essential for the growth of these cells.

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