Abstract

Purpose: Erythropoietin (EPO) and EPO receptor (EPO-R) expression have been reported in solid tumors and are claimed to regulate tumor growth; however, no data have been published on this issue in B-cell malignancies or normal lymphoid cells. This report describes genomic/protein EPO-R expression and in vitro effects of recombinant human EPO (epoetin) in B-cell chronic lymphocytic leukemia (B-CLL), mantle-cell lymphoma (MCL), and multiple myeloma (MM).

Experimental Design: Blood samples were obtained from patients with B-CLL, MCL, and healthy volunteers, and bone marrow was obtained from MM patients. EPO-R mRNA was detected by reverse transcription-PCR. EPO-R surface expression was investigated by flow cytometry using digoxigenin-labeled epoetin and polyclonal rabbit anti–EPO-R antibody for intracellular receptor. Tumor cell stimulation was determined in vitro using [3H]thymidine incorporation and CD69 expression after exposure to epoetin α or β or darbepoetin α.

Results: EPO-R mRNA was detected in mononuclear cells from 32 of 41 (78%) B-CLL and 5 of 7 (71%) MCL patients, and 21 of 21 (100%) MM samples. Expression was also detected in highly purified T cells from six of eight B-CLL patients, four of four MM patients, and normal donor B and T cells. Surface EPO-R protein was not detected. Intracellular EPO-R staining with anti–EPO-R antibodies was unspecific. No tumor-stimulatory effect was observed with high epoetin concentrations.

Conclusions: EPO-R gene is frequently expressed in lymphoid malignancies and normal B and T cells. However, there was no surface protein expression and no epoetin-induced in vitro stimulation of tumor B cells, indicating that epoetin therapy in vivo is likely to be safe in patients with lymphoid malignancies.

Erythropoietin (EPO), the principal regulator of erythropoiesis, is a glycoprotein hormone produced by the kidney and fetal liver (1). EPO prevents apoptosis, stimulates growth, and promotes the differentiation of RBC progenitors by interacting with a specific transmembrane receptor (EPO-R) expressed on the cell surface (2). EPO-R is a member of a cytokine receptor family that lacks the tyrosine kinase domain (2). Upon activation, EPO-R homodimers undergo conformational changes and initiate a Janus kinase signal transducer, which, in turn, activates the transcription factor STAT5 that regulates cell proliferation and differentiation (2–4). EPO and EPO-R expression have recently been shown in several nonhematopoietic tissue types, including embryonic, nervous system, uterine, ovarian, and endothelial cells, suggesting a broader biological role for EPO signaling (5–8). The EPO–EPO-R pathway might be involved in a variety of cellular functions, including cellular growth, survival, and proliferation as well as in the promotion of angiogenesis and the prevention of ischemic and toxic-stress tissue injuries (5–14).

Great concern and debate have recently emerged about the potential role of EPO and recombinant human EPO in promoting tumorigenesis not only in vitro (2–4) but also in vivo (5–17). EPO and EPO-R were reported to be expressed in a variety of malignant human cell lines and solid tumors, including breast, prostate, ovarian, uterine, and renal cancers (3, 4, 18, 19). Moreover, in some reports, exogenously added epoetin was described as having a tumor cell growth–promoting effect (4, 18, 20, 21). Other studies have failed to detect such an effect in vitro (22–24). Further uncertainty was evident from two prospective randomized studies on epoetin therapy in head and neck cancer patients (25, 26). Without clearly establishing the potential for epoetin to promote tumor growth, it is important to further explore this issue.
Erythropoietin Receptor in B-Cell Malignancies

Materials and Methods

Patients. After informed consent according to the protocol approved by the institutional ethical committee and in keeping with the Helsinki Declaration on research on human subjects, peripheral blood samples from patients with B-CLL and MCL, and bone marrow aspirates from patients with MM, were obtained. The clinical characteristics of the patients are shown in Table 1 (31, 32). Peripheral blood was also obtained from six healthy men (mean age 32.5 ± 1.4 years) as controls.

Isolation of peripheral blood mononuclear cells and purification of CD19+ cells. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Amersham Pharmacia Biotech) density gradient centrifugation (33). Cells were washed thrice with PBS (pH 7.2; Gibco), applied on a nylon wool column (Biotest; ref. 33), and incubated at 37°C for 1 h. Unabsorbed cells were removed by extensive washing with warm (37°C) culture medium (RPMI 1640; Gibco). Cells were eluted from the column (33). The purity of CD19+ cells, determined by flow cytometry, was >98%. Purified B cells were then used for RNA extraction and subsequent reverse transcription-PCR (RT-PCR)–based EPO-R expression analysis, as well as for functional studies.

Isolation of bone marrow mononuclear cells and purification of CD19+ cells. Bone marrow aspirates (4-8 mL) from MM patients were diluted in equal volumes of RPMI, applied to a filter (pore size 40 or 70 μm) to remove debris and fat particles, and subjected to Ficoll separation (33) to isolate bone marrow mononuclear cells (BMMC). Cells were washed twice with PBS or Iscove’s modified Dulbecco’s medium (Sigma Aldrich) containing 2% FCS and 2 mmol/L EDTA, and the CD138+ cell portion was purified using MidiMACS columns (Miltenyi Biotec) according to the manufacturer’s instructions. Purity of the CD138+ fraction, determined by flow cytometry analysis or May-Grünwald-Giemsa staining and morphology, was >95%. The cells were resuspended in complete medium (RPMI 1640 supplemented with 10% heat-inactivated pooled human AB+ serum, 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin) and used for the proliferation assay and RNA extraction.

RT-PCR analysis. Total RNA was extracted from 2 × 10^6 PBMC or purified tumor cells with RNA-Be (BioSite) using the guanidine thiocyanate phenol-chloroform extraction or TRIzol reagent (Invitrogen) method, according to the manufacturer’s recommendation. RNA was denatured at 65°C for 5 min and immediately chilled on ice. First-strand cDNA synthesis was done in a 20 μL reaction mixture containing 0.5 μg RNA in a 10 μL volume, 4 μL 5× buffer (Invitrogen), 1.5 μL DTT (100 mmol/L), 2 μL deoxynucleotide triphosphates (5 mmol/L each; Amersham Biosciences), 1.0 μL random hexamer primers (100 pmol/μL, Amersham Biosciences), 0.5 μL distilled water, and 1.0 μL M-MLV reverse transcriptase (200 units/μL, Gibco). The reaction mixture was incubated at 42°C for 45 min followed by 5 min at 70°C to inactivate reverse transcriptase. Quality of cDNA was confirmed for all samples by RT-PCR for β-actin. cDNA samples were then stored at -20°C. RT-PCR was done in a 25 μL reaction mixture using 2.5 μL of 10× buffer, 1 μL magnesium chloride (25 mmol/L), 1.5 μL deoxynucleotide triphosphates (10 mmol/L), 5 pmol of each primer, and one unit of AmpliTaq Gold DNA polymerase (Perkin-Elmer/Applied Biosystems). Target (patient) cDNA (2 μL) was included in each reaction volume except for the negative controls. PCR was done in 35 concurrent cycles, using the temperature sequence 1 min at 94°C, 1 min at 61°C, and 1.5 min at 72°C.

For detection of EPO-R–specific cDNA, the following primer pairs were used: sense acgggacctaccaaatc and antisense gctttccttcgctgtcctt, resulting in an amplicon size of 485 bp (34). The PCR product was cloned, sequenced, and compared with EPO mRNA sequences available from GenBank.

Surface EPO-R flow cytometry. Digoxigenin-labeled epoetin binding in viable tumor cells was used to analyze EPO-R expression. The digoxigenin label was covalently bound to epoetin sialic acids via hydrazide linker. This modification did not affect its capacity to stimulate proliferation (data not shown). The UT-7 cell line served as a positive control for EPO-R expression (35). The specificity of epoetin-digoxigenin binding was shown by competitive inhibition in the presence of a 100× excess of unlabeled epoetin β (NeoRecormon, Roche Diagnostics GmbH) added 30 min before EPO-digoxigenin labeling (see Results). All staining steps were done on ice in 100 μL PBS/10% human AB plasma for 30 min, except the initial epoetin competitive inhibition test that was done in RPMI 1640/10% FCS. The washing steps were done with 3 mL PBS/10% human AB plasma at speed 400 × g for 10 min. Three concentrations of EPO-digoxigenin (1, 3, or 10 nmol/L) were used for staining.

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Table 1. Clinical characteristics of the patients

<table>
<thead>
<tr>
<th>B-CLL (n = 41)</th>
<th>MCL (n = 7)*</th>
<th>MM (n = 21)</th>
</tr>
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<tr>
<td>Median age, y (range)</td>
<td>67 (53-78)</td>
<td>66 (52-83)</td>
</tr>
<tr>
<td>Sex, n (M/F)</td>
<td>27/14</td>
<td>4/3</td>
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<tr>
<td>Clinical stage, n</td>
<td>0</td>
<td>1</td>
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<tr>
<td>I</td>
<td>11</td>
<td>0</td>
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<tr>
<td>II</td>
<td>10</td>
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<td>IV</td>
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<tr>
<td>Disease phase, n</td>
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<tr>
<td>Progressive</td>
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<td>3</td>
</tr>
<tr>
<td>Nonprogressive</td>
<td>29</td>
<td>4</td>
</tr>
<tr>
<td>Previous chemotherapy, n</td>
<td>Yes</td>
<td>12</td>
</tr>
<tr>
<td>No</td>
<td>29</td>
<td>6</td>
</tr>
</tbody>
</table>

*The leukemic MCL cell count ranged from 7.5 × 10^9/L to 130.0 × 10^9/L.
† Determined using the Durie and Salmon (31) and Rai/Ann Arbor (32) staging systems.
‡ Nonprogressive disease was defined as ≥6 mo period of clinically stable disease, either previously untreated or in unmaintained response/plateau phase after previous antitumor therapy.
Before staining, the cells were washed once and resuspended at a concentration of $5 \times 10^5$ cells/100 μL. The labeling steps were as follows: EPO-digoxigenin labeling for 5 h, wash; incubation with 10 μg biotin-labeled antidigoxigenin antibody (Jackson ImmunoResearch), wash; staining with 5 μg avidin-FITC (BD PharMingen), wash; incubation with 5 μg biotin-labeled murine IgG (DakoCytomation), wash; and staining with 7 μg FITC-labeled antimurine antibody (Caltag Laboratories). After the final washing step, the cells were resuspended in 300 μL PBS/10% human AB plasma, and 1 μg/mL (final concentration) of propidium iodide was added for dead cell staining. All stainings were done at 4°C. The EPO-R/EPO-digoxigenin complex is quite stable when located outside the cells (UT-7 cell line), and the detection level of this method is ~300 receptors per cell (data not shown).

The cells were analyzed on a fluorescence-activated cell sorter scan flow cytometer (BD Bioscience). The viable cell population was identified in the forward scatter/propidium iodide dot plot as propidium iodide–negative cells and the mean fluorescence of the FITC label was recorded.

**Intracellular EPO-R flow cytometry.** For indirect staining of intracellular EPO-R, the cells were fixed with 2% paraformaldehyde on ice for 10 min in the dark, permeabilized with 0.1% saponin in PBS, and incubated with the primary polyclonal rabbit anti–EPO-R antibody (Santa Cruz Biotechnology, SDS Biosciences) at different concentrations (from 8.0 to 0.02 μg) for 30 min at room temperature in the dark. Goat anti-rabbit FITC-conjugated secondary antibody was then added to washed cells and incubated for 15 min at 4°C in the dark. After a final wash, the cells were resuspended in PBS and analyzed using a FACScalibur flow cytometer (BD Biosciences) and CellQuest software. A minimum of 10,000 lymphocyte-gated events were acquired and cells were analyzed by forward and side scatter. Criteria for positive staining were set at fluorescence intensities displayed by <1% of cells stained with the isotype controls.

**Proliferation assay (DNA synthesis).** PBMC or purified tumor cells ($2 \times 10^5$) in complete medium were placed in a 96-well U-bottomed plate in triplicate and incubated at 37°C for 5 days. Cells were cultured either alone or with 100 IU/mL of epoetin α (Eprex, Janssen-Cilag AB), epoetin β, or the equivalent concentration of darbepoetin α (500 ng/mL; Amgen AB) in triplicate. This concentration is at least 5-fold the physiological concentration of EPO in human peripheral blood (36). $[^3]$H]thymidine (Amer shamPharmacia Biotech) was added to each well at a concentration of 1 μCi during the last 18 h of the culture period. The plates were harvested and the incorporated

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Patients tested, n</th>
<th>Positive for EPO-R mRNA expression, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-CLL (PBMC)</td>
<td>41</td>
<td>32 (78)</td>
</tr>
<tr>
<td>Mutated VH phenotype</td>
<td>9</td>
<td>7 (78)</td>
</tr>
<tr>
<td>Unmutated VH phenotype</td>
<td>7</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Purified B-CLL cell fraction</td>
<td>10</td>
<td>7 (70)</td>
</tr>
<tr>
<td>Purified T-cell fraction</td>
<td>8</td>
<td>6 (75)</td>
</tr>
<tr>
<td>MM (BMCC)</td>
<td>21</td>
<td>21 (100)</td>
</tr>
<tr>
<td>Purified myeloma plasma cell fraction (CD138+)</td>
<td>8</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Plasma cell depleted fraction</td>
<td>4</td>
<td>4 (100)</td>
</tr>
<tr>
<td>MCL PBMC</td>
<td>7</td>
<td>5 (71)</td>
</tr>
<tr>
<td>Normal PBMC</td>
<td>7</td>
<td>7 (100)</td>
</tr>
<tr>
<td>Purified B-cell fraction</td>
<td>4</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Purified T-cell fraction</td>
<td>6</td>
<td>6 (100)</td>
</tr>
</tbody>
</table>
**CD69 expression assay.** To assess if epoetin can induce expression of the activation marker CD69, 2 × 10⁶ PBMC were placed in each 24-well plate in a 1 mL volume with epoetin α, epoetin β, or darbepoetin α. PBMC or purified tumor cells were cultured alone as controls. In parallel on the same plate, 2 × 10⁵ irradiated CD40L-transfected fibroblasts were seeded in each 24-well plate 6 h before the experiment and PBMC were added alone or in combination with epoetin α, epoetin β, or darbepoetin α. The cells were incubated for 48 h, and CD69 expression was assessed by flow cytometry.

**Fig. 2.** Flow cytometry analysis of EPO-R protein expression on the surface of tumor cells from patients with B-CLL. Patient numbers (Pt #) one to nine; five patients were EPO-R mRNA-positive and four were negative. Three concentrations (1, 3, or 10 nmol/L) of EPO-digoxigenin (DIG; +antidigoxigenin FITC) were used without (unfilled columns) and with (filled columns) epoetin competition. UT-7, EPO-R-positive control cell line; auto, autofluorescence of cells; (DIG) ctrl, staining of cells with all reagents but without prior incubation with EPO-digoxigenin.

**Fig. 3.** Flow cytometry analysis of EPO-R protein expression on the surface of EPO-R mRNA-positive myeloma plasma cells from four patients with MM. Three concentrations (1, 3, and 10 nmol/L) of EPO-digoxigenin (+antidigoxigenin FITC) were used without (unfilled columns) and with (filled columns) epoetin competition.
**Results**

**Genomic expression of EPO-R in malignant cells**

The genomic expression of EPO-R in B-CLL, MCL, and MM tumor cells is summarized in Table 2. Figure 1A shows examples of EPO-R mRNA expression or nonexpression in PBMC from B-CLL patients, as detected by RT-PCR. Figure 1B shows a case of EPO-R mRNA expression in the enriched T cell, but not in the tumor B-cell fraction in PBMC from a patient with B-CLL. Figure 1C shows EPO-R mRNA expression in both T-cell and tumor B-cell fractions from a B-CLL patient, and EPO-R gene expression in the CD138+ fraction from a MM patient. Expression in PBMC from a patient with MCL as well as in T- and B-cell fractions from a healthy control are also shown.

In total, EPO-R mRNA was detected in PBMC of 32 of 41 (78%) B-CLL patients (Table 2). Positive results were confirmed in highly purified tumor B cells from 7 of 10 (70%) B-CLL patients. EPO-R expression was also detected in cells with mutated (7 of 9) and unmutated (7 of 7) immunoglobulin VH phenotypes. Repeated analyses from two individual patients revealed that EPO-R mRNA expression seemed not to be influenced by chemotherapy (data not shown). EPO-R mRNA was detected in the purified T-cell fraction of 6 of 8 (75%) B-CLL patients and, in samples from two B-CLL patients, EPO-R mRNA was detected in the T-cell fraction but not in the enriched tumor B-cell fraction (Fig. 1B). BMMC from 21 of 21 (100%) and highly purified plasma cells (CD138+) from 8 of 8 (100%) MM patients also expressed EPO-R mRNA, as well as PBMC from 5 of 7 (71%) MCL patients (Table 2). The tumor-depleted fractions of BMMC, containing mainly T cells, from four MM patients were positive for EPO-R mRNA expression. Purified normal B cells and purified T cells from four and six healthy donors, respectively, were all found to express EPO-R mRNA (Table 2).

**EPO-R protein expression**

**Surface staining.** The surface EPO-R detection technique using digoxigenin-labeled epoetin was done using the EPO-R–positive cell line UT-7 as a positive control. Figures 2 and 3 show the results of the flow cytometry analysis of EPO-R protein surface expression on UT-7 cells as well as on B-CLL cells and MM plasma cells, respectively. Binding of EPO-digoxigenin could be detected on UT-7 cells significantly above the background staining level with EPO-digoxigenin concentrations in the range of 1 to 10 nmol/L (Figs. 2 and 3). The mean ± SD increase of UT-7–specific EPO-digoxigenin binding relative to the digoxigenin-control (set as 100%) was 216 ± 50% (1 nmol/L EPO-digoxigenin), 255 ± 50% (3 nmol/L EPO-digoxigenin), and 281 ± 81% (10 nmol/L EPO-digoxigenin). The specificity of UT-7 expression was evident by competitive blockage of EPO-digoxigenin in the presence of an excess (×100) of unlabeled epoetin (Figs. 2 and 3), and the relative fluorescence intensities decreased to 112 ± 2%, 116 ± 3%, and 131 ± 9%, respectively. Student’s t test analysis of the absolute values revealed statistical significance of the increase of all EPO-digoxigenin labels on UT-7–positive control cells over the digoxigenin-control (P = 0.001-0.008). In the presence of a 100× excess of unlabeled EPO, none of the EPO-digoxigenin–labeled UT-7 samples reached significance over the digoxigenin-control (P = 0.235-0.955), indicating specificity and robustness of the technique. Contrary to the results obtained with the UT-7 cells, EPO-R protein could not be detected on the cell surface of enriched tumor cells from any of the nine tested B-CLL patients (Fig. 2; five of these patients were EPO-R positive at the mRNA level and four were mRNA negative). Similarly, enriched plasma cells from four EPO-R mRNA–positive MM patients stained negative for surface EPO-R (Fig. 3), irrespective of the EPO-digoxigenin concentration used and with or without epoetin competition. A somewhat higher and more variable unspecific (background) staining was observed in the MM patients (Fig. 3) than in CLL patients (Fig. 2), which may be explained by a higher and variable proportion of preapoptotic/apoptotic cells in the MM samples as revealed by propidium iodide/Annexin V staining (data not shown).

**Intracellular staining.** Intracellular EPO-R protein expression was assessed by flow cytometry using commercially available rabbit polyclonal anti–EPO-R antibodies (see Materials and Methods). The results of a representative double-staining experiment on B-CLL cells from one EPO-R mRNA–positive and one EPO-R mRNA–negative patient are shown in Fig. 4A.
and B, respectively. Weak positive staining was obtained in the EPO-RmRNA–positive patient and an almost identical staining pattern was seen in the EPO-R mRNA–negative patient. Additional experiments, using extensive washing and stepwise serial dilutions of the antibody (from 8.00 to 0.02 ng), could not eliminate unspecific binding. Similar results were obtained using the cytospin technique (data not shown). Testing of additional patients (eight with B-CLL and four with MCL) revealed identical results.

Functional effects of epoetins on tumor cells

We tested the ability of epoetin α, epoetin β, and darbepoetin α to induce tumor cell proliferation ([3H]thymidine incorporation) in enriched tumor cells from eight patients with B-CLL (Fig. 5A) and four patients with MCL (Fig. 5B). BMNC from four MM patients were also tested for epoetin-induced proliferation (Fig. 5C). No significant epoetin-induced proliferation was observed after in vitro stimulation of tumor cells of any of the three epoetin preparations. As a positive control, all three epoetin preparations were also tested together with the EPO-R–positive cell line K562 and a significant stimulatory effect was observed (P < 0.05; Fig. 5D).

The viability of CLL cells in routine culture was reduced according to the length of the culture period. The average viability was reduced from 98% at day 1 to 44 ± 5.7% after 96 h culture, as revealed by Annexin V staining (data not shown). Addition of epoetin did not alter this pattern.

Additional experiments were done in tumor cells from B-CLL and MCL patients by coculturing cells with irradiated CD40L-transfected fibroblasts. No additional stimulatory effect was observed with the addition of epoetin to cell cultures (data not shown).

We then analyzed the expression of CD69 (cellular activation marker) in eight patients with B-CLL and four patients with MCL before and after stimulation of tumor cells with one of the three epoetin preparations alone, or in combination with irradiated CD40L-transfected fibroblasts as costimulators. No epoetin-induced activation was observed with epoetin alone or on top of the marked (and significant) activation induced by CD40L (Fig. 6A and B).
Discussion

Apart from its well-known central role in erythropoiesis, emerging data now seem to indicate that EPO also has pleiotropic functions in various normal cell types (6, 37). EPO-R expression has recently been identified in many nonerythroid tissue types, including the central nervous system, retina, heart, vascular endothelium, kidney, lung, liver, and gastrointestinal and reproductive tracts (5, 6, 13, 37). Clinically beneficial effects have been claimed for EPO signaling through up-regulation of various pathways. These include not only stimulation of erythropoiesis, but also antiapoptotic, anti-inflammatory, immunomodulatory, angiogenic, neurotrophic, and neuroprotective mechanisms (5, 6, 13, 37). EPO generally protects cells from injury, most notably after ischemic insult (7, 10, 11, 38). Hypoxia-driven EPO and EPO-R overexpression enhance ischemic and hypoxic neural cell survival (39, 40). EPO signaling might also protect against light-induced retinal degeneration (41), enhance fibrin-induced wound healing (12), and increase chances of recovery from ischemic myocardial injuries (5).

However, some recent reports indicated that EPO-R may be expressed by various tumor cell types and that EPO signaling might also be implicated in promoting the growth and survival of cancer cells in solid tumors and malignant cell lines (2, 4, 18, 20, 21). These include prostate (42), head and neck (43, 44), melanoma (45), female reproductive organs (19, 46, 47), breast (3, 48), and non–small cell lung cancers (49). EPO signaling is also reported to correlate positively with tumor hypoxia (50) and induce cancer cell resistance to ionizing radiation and chemotherapy (44, 51). Further concerns were raised in vivo from clinical observations in patients, as two recently published randomized trials that prospectively analyzed survival effects of epoetin therapy in patients with breast cancer (15) and head and neck tumors (17) both reported an inferior outcome in patients treated with epoetin. In patients with head and neck cancer, the negative survival effect seemed to be confined to patients with EPO-R–positive tumors only (25). Furthermore, another large randomized trial on epoetin therapy in patients with head and neck tumors receiving radiotherapy was closed early due to inferior outcome in the epoetin group at an interim analysis (26). Other recently published in vitro studies reported that epoetin did not affect tumor growth or angiogenesis in tumors (22, 24), and there are reports of epoetin-induced sensitization of malignant cells to chemotherapy and/or radiotherapy (52, 53) or no interference with the antiproliferative and cytotoxic effects of antitumor drugs (24).

Although epoetin is used frequently to correct anemia not only in solid but also in lymphoproliferative malignancies (54, 55), there are no published data on EPO-R expression and the functional in vitro effects of epoetin on tumor cells from patients with lymphoid cancers. There are, however, reports suggesting that epoetin may stimulate myeloma cell lines in vitro (30). Our study is the first to analyze the genomic and protein expression of EPO-R in various B-cell tumors and the in vitro functional effect of various epoetins preparations on such tumor cells. We also analyzed genomic EPO-R expression in normal B and T cells in a limited number of patients and controls. PBMC/BMMNC or purified tumor cells from most, but not all, B-CLL, MCL, and MM patients (70-100%) expressed EPO-R mRNA as assessed by RT-PCR (Table 2; Fig. 1A-C). In the lymphoid cell–enriched subset analyses, measures were taken to obtain highly purified cell subsets to minimize the risk of false-positive results caused by contamination with the remaining nonenriched (PBMC/BMMC) cell fraction. Due to the sensitivity of the PCR analysis, such contamination could not be fully excluded; however, this is unlikely to explain the reproducible findings of completely negative PCR results in the cell subset analysis.

Despite frequent genomic EPO-R expression, the corresponding EPO-R protein could not be detected on the surface of any of the tumor cells from B-CLL and MM patients assessed by a sensitive EPO-digoxigenin + antidigoxigenin FITC–labeled surface staining flow cytometry technique. No functional effects were observed in vitro because high concentrations of three different epoetins (epoetin α, epoetin β, and darbepoetin α) failed to induce activation and/or proliferation of tumor cells during a 5-day culture period, with or without CD40L-transfected fibroblast costimulation. These results suggest that EPO-R is frequently expressed at the gene level, but not as a...
surface protein on malignant B cells, and that exogenously added epoetin does not induce tumor growth or activation under the experimental conditions used.

An unexpected finding in our study was that normal lymphoid (B and T) cells may also express EPO-R at the gene level (Fig. 1C). These results need to be confirmed in extended in vitro studies to understand the frequency and biological implication of normal B- and T-cell EPO-R gene expression. It could be speculated that EPO gene expression in lymphoid cells is related to a broader, yet to be identified, physiological function.

It is not clear from this study why EPO-R mRNA should fail to translate into functional protein. It is possible that genes may be suppressed or even silenced by cell cycle control mechanisms when their product protein is deemed adversely to affect normal cellular homeostasis (56–58). We used a sensitive flow cytometry technique for surface EPO-R detection, including the positive control cell line UT-7, which is known to express a low or intermediate copy number of EPO-R (35). Therefore, the negative flow-cytometry data are unlikely to be due to the detection limits of the technique used. In addition, we failed to obtain reliable and specific staining results with one of the commercially available polyclonal rabbit antibodies designed for intracellular EPO-R protein detection (Fig. 4). This is in line with another recent report showing that most of these antibodies seem to bind unspecifically and cross-react with non–EPO-R proteins (59). Moreover, in a further antibody study, EPO-R localization was exclusively cytosolic and no specific epitope binding was observed in in vivo xenografts (60). Thus, the findings of Elliott et al. (59), LaMontagne et al. (60), and our data indicate that the results from previously published articles, which only used antibodies for analysis of EPO-R expression in tumors, should be viewed with caution.

Acknowledgments

We thank Dr. Hoesel (Roche Diagnostics GmbH, Mannheim, Germany) for providing EPO-dioxigenin and Leila Relander for secretarial help.

References


Expression of Erythropoietin Receptor and *In vitro* Functional Effects of Epoetins in B-Cell Malignancies
