

Erythropoietin Restores the Anemia-induced Reduction in Cyclophosphamide Cytotoxicity in Rat Tumors

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Abstract

The aim of this study was to examine the impact of anemia prevention by recombinant human erythropoietin (rHuEPO) treatment on the cytotoxicity of cyclophosphamide in solid experimental tumors. Anemia was induced using a single dose of carboplatin (50 mg/kg i.v.) resulting in a long-lasting reduction (30%) of the hemoglobin concentration. In a second group, the development of anemia was prevented by rHuEPO (1000 IU/kg) administered s.c. three times/week starting 7 days before carboplatin application. Four days after carboplatin treatment, tumors (DS-sarcoma of the rat) were implanted s.c. onto the hind food dorsum.

Neither carboplatin nor rHuEPO treatment influenced tumor growth rate *per se*. When tumors were treated with a single dose of cyclophosphamide (60 mg/kg i.p.) 5 days after implantation, a growth delay with a subsequent regrowth of the tumors was observed. In the anemia group, the growth delay was significantly shorter compared with nonanemic controls (13.3 days *versus* 8.6 days). In the group where anemia was prevented by rHuEPO treatment, growth delay was comparable with that of nonanemic controls (13.3 days). These results suggest that chemotherapy-induced anemia reduces cytotoxicity of cyclophosphamide in tumors, whereas correction of anemia by rHuEPO treatment (epoetin α) increases the sensitivity, probably as a result of an improved oxygen supply to tumor tissue.

Introduction

Anemia is a frequent phenomenon in clinical oncology that can be caused by the malignant disease itself (*e.g.*, because of deficiency of erythropoietic factors, myelosuppression by inflammatory cytokines, hemolysis, bone marrow infiltration, or paraneoplastic syndromes; for a review see Ref. 1), by chemotherapy, or by acute or chronic bleeding of the tumor. Tumor-associated anemia can severely affect the general well being of the patient (*e.g.*, development of fatigue syndrome) and may limit the applicability and efficacy of several antitumor therapy modalities. Numerous studies (for a review, see Ref. 2) have shown a strong relationship between the therapeutic outcome of radiotherapy and cHb^2 indicating that anemic patients have a poorer prognosis after standard radiotherapy.

The diminished therapeutic effect of radiotherapy in anemic patients might be a result of the reduced oxygen-carrying capacity of the blood, which in turn decreases the arterial O_2 -supply to the tumor. Thus, severe anemia will result in a poorer oxygenation status further increasing the hypoxia already present in many tumors (3). Kelleher *et al.* (4) found that anemia leads to a significantly lower median tumor- pO_2 and a higher fraction of hypoxic pO_2 values ($\text{pO}_2 \leq 2.5$

mm Hg). The correction of anemia by rHuEPO treatment (or by RBC transfusion) has been shown to increase the oxygen-carrying capacity of blood and thus improves tumor oxygenation (4, 5).

Tumor hypoxia or anoxia protects tumor cells from sparsely ionizing radiation and thus reduces the efficacy of radiotherapy. In anemic animals, therefore, a radioresistance has been observed that could be significantly alleviated when anemia was corrected by rHuEPO (6).

From other studies (7–10) there is evidence that the efficacy of several chemotherapeutic agents (*e.g.*, cyclophosphamide) may also depend on tissue oxygenation. If these differences in chemosensitivity are indeed the result of tumor hypoxia, the cytotoxic effect of cyclophosphamide would be expected to be reduced in anemic patients as a result of a compromised O_2 -supply to the tissue. On the other hand, correcting anemia should lead to an improved efficacy of O_2 -sensitive chemotherapeutic agents, although this has not been conclusively confirmed in *in vivo* studies to date. The present study thus explored the effect of anemia prevention or correction with rHuEPO treatment on tumor sensitivity to chemotherapy.

Materials and Methods

Animals. Male Sprague Dawley rats (Charles River Wiga, Sulzfeld, Germany; body weight, 100 to 160 g) housed in our animal care facility were used in the study. Animals were allowed access to food and acidified water *ad libitum* before and throughout the investigation. All of the experiments had been approved previously by the regional animal ethics committee and were conducted in accordance with the German Law for Animal Protection and the United Kingdom Coordinating Committee on Cancer Research Guidelines (11).

Tumors. Solid DS-sarcomas were induced by injecting DS-sarcoma cells ($0.4 \text{ ml of } \approx 10^4 \text{ cells}/\mu\text{l}$) s.c. into the hind foot dorsum. Tumors grew as flat, spherical segments and replaced the subcutis and corium completely. Volumes were determined by measuring the three orthogonal diameters of the tumors and using an ellipsoid approximation with the formula: $V = d_1 \times d_2 \times d_3 \times \pi/6$. From the volume growth curves, the volume-doubling time was calculated during exponential tumor growth.

Anemia Induction. Prolonged anemia was induced by a single injection of carboplatin (Sigma-Aldrich, Steinheim, Germany) at a dose of 50 mg/kg body weight (dissolved in isotonic saline at a concentration of 20 mg/ml) into the tail vein 4 days before tumor implantation. The resulting anemia has been shown to last for at least 10 days and to be sensitive to rHuEPO treatment (6). This carboplatin treatment regime does not affect the growth of DS-sarcomas (6).

rHuEPO Treatment. rHuEPO (ERYPO 4000; Janssen-Cilag, Neuss, Germany) in isotonic NaCl solution was administered (1000 IU/kg) three times/week over 16 days by s.c. injection starting 11 days before tumor implantation. Control animals received equivalent volumes of the solvent (0.5 ml/kg).

Cyclophosphamide Treatment. Tumors were treated on day 5 after implantation when they had reached a volume of $\sim 0.5 \text{ ml}$ with a single dose of cyclophosphamide (Endoxan; Asta, Frankfurt/Main, Germany; 60 mg/kg i.p.). This dose results in a growth delay of approximately 8 to 10 days but not to be curative. Animals treated with equivalent volumes of the solvent (isotonic saline, 6 ml/kg) served as controls.

Experimental Groups. The experimental groups can be described as follows: Group 1 (nonanemic, $n = 42$ tumors), animals received cyclophospha-

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² The abbreviations used are: cHb, hemoglobin concentration; rHuEPO, recombinant human erythropoietin, epoetin α ; pO_2 , oxygen partial pressure; RBC, red blood cells, erythrocytes; MCV, mean cell volume; MCH, mean corpuscular hemoglobin content; MCHC, mean corpuscular hemoglobin concentration.

mide on day 5 after tumor implantation; Group 2 (anemic, $n = 44$ tumors), animals treated 4 days before tumor implantation with carboplatin for anemia induction. Cyclophosphamide was administered on day 5 after implantation; Group 3 (anemic, rHuEPO-treated, $n = 40$ tumors), animals treated with rHuEPO three times/week from 11 days before tumor implantation and up until the day of cyclophosphamide application. Carboplatin (for anemia induction) was administered on day -4, and cyclophosphamide was applied on day 5.

Controls for all of the three experimental groups received equivalent volumes of the solvent instead of cyclophosphamide on day 5 after tumor implantation.

Measurements. Besides tumor growth, blood cell parameters were assessed using a multiparameter, automated hematology analyzer (Ac-T₈; Beckman-Coulter, Krefeld, Germany) whereby erythrocyte, WBC, and platelet counts together with the MCV were measured by an impedance technique and the hemoglobin concentration by a photometric method. In addition, the analyzer uses the measured values to calculate several other parameters (e.g., hematocrit, MCH, and MCHC). All of the measurements were performed using a sample of venous blood (20 μ l) taken from a tail vein.

Statistical Analysis. Results are expressed as means \pm SE. Differences between the groups were assessed by the two-tailed Wilcoxon test for unpaired samples. The significance level was set at $\alpha = 5\%$ for all of the comparisons. For characterizing the effect of chemotherapy on tumor growth, the growth delay induced by cyclophosphamide was calculated.

Results

Carboplatin application 4 days before tumor implantation significantly reduced the cHb. Starting at a mean cHb of ≈ 130 g/liter at day -4, a single dose of carboplatin of 50 mg/kg body weight i.v. resulted in a moderate anemia in rats with a mean cHb of about 100 g/liter 9 days after application. The hemoglobin content remained at these lower levels for at least 8 days (Fig. 1). Continuous treatment with rHuEPO in otherwise untreated rats increased the cHb within 1 week to 153 g/liter (Fig. 1). A subsequent application of carboplatin 7 days after commencement of rHuEPO therapy reduced the hemoglobin level within 9 days to values comparable with the cHb of untreated control animals. Withdrawal of further rHuEPO application led to the continued development of anemia (Fig. 1). Thus, rHuEPO therapy for approximately 1 week before carboplatin application resulted in prevention (or reversal) of the carboplatin-induced anemia at the time of chemotherapy of the tumor.

The growth curves of tumors not treated with cyclophosphamide chemotherapy were more or less identical in the nonanemic and anemic group as well as in the group where anemia was prevented by rHuEPO (Fig. 2). The volume-doubling time was approximately 1.8

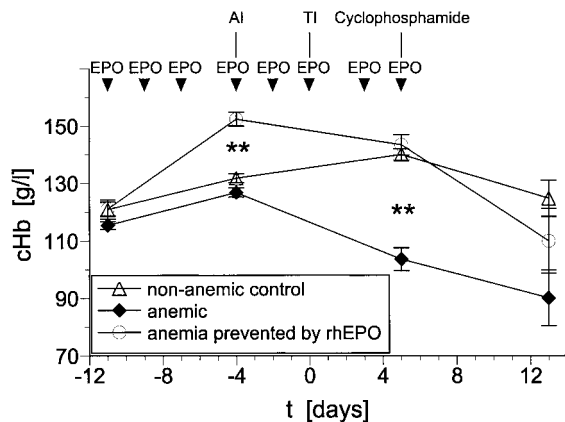


Fig. 1. Time course of cHb in animals treated only with a single dose of carboplatin on day -4 (\blacklozenge), in animals additionally treated with rHuEPO (from day -11 until day +5, three times a week; \circ), and in nonanemic control animals (\triangle). Day 0 is the day of tumor implantation. Each point represents data from at least 22 animals. Arrows indicate the times of rHuEPO application (EPO), anemia induction (AI) by carboplatin injection, as well as tumor implantation (TI) and cyclophosphamide treatment. **, $P < 0.01$.

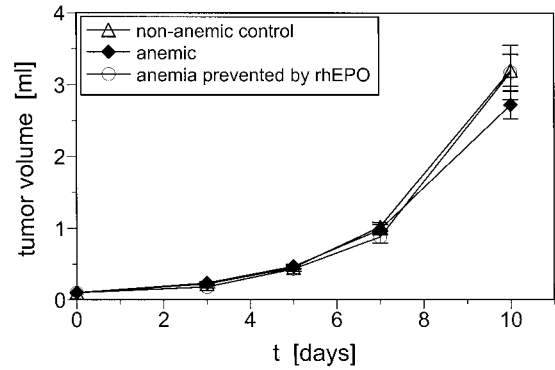


Fig. 2. Tumor volume in animals treated only with a single dose of carboplatin for anemia induction 4 days before tumor implantation (\blacklozenge), in animals additionally treated with rHuEPO (\circ), and in nonanemic control animals (\triangle). Each data point represents the mean tumor volume of at least 14 tumors.

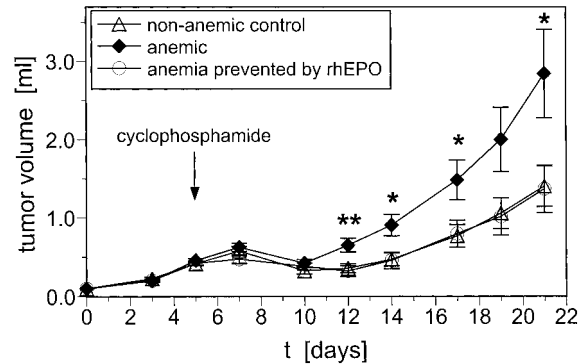


Fig. 3. Tumor volume in animals with carboplatin-induced anemia (\blacklozenge), in animals where the development of anemia was prevented by rHuEPO treatment (\circ), and in nonanemic controls (\triangle). All of the tumors were treated with a single dose of cyclophosphamide (60 mg/kg, i.p.) on day 5. Each data point represents the mean tumor volume of at least 10 tumors. *, $P < 0.05$; **, $P < 0.01$ for the comparison of anemic animals with the group where anemia was prevented by rHuEPO treatment.

days independent of the actual cHb or treatment with rHuEPO for 16 days (Fig. 2). From these results, which are in good accordance with data obtained in previous studies (4, 6), it can also be concluded that anemia induction by carboplatin (4 days before tumor implantation) as well as rHuEPO treatment have no impact *per se* on the growth rate of the tumors under investigation.

Fig. 3 shows the tumor growth rate in the three groups when tumors were treated with a single dose of cyclophosphamide on day 5 after tumor implantation. After cyclophosphamide treatment, tumor growth was temporarily arrested and in some cases a slight shrinkage of the tumor was observed. After a period of 6 to 9 days, the tumors started to regrow. On the day of cyclophosphamide treatment, RBC-related parameters were significantly different between the two groups where anemia was induced by carboplatin (Table 1), with the anemic group showing a mean cHb of 98 g/liter and the group in which anemia development was prevented by rHuEPO administration showing a cHb of 137 g/liter (comparable with the cHb in the nonanemic control group with a cHb of 141 g/liter). In both anemia groups, the indices of the RBCs (MCV, MCH, and MCHC) showed only minor differences and were within the normal range, indicating a normocytic, normochromic anemia induced by carboplatin (Table 1).

Fig. 3 also shows the regrowth characteristics after cyclophosphamide treatment in the anemic group, the group where anemia was reversed by rHuEPO, and the nonanemic group. The growth delay after chemotherapy (at the 1.3-ml tumor volume level) was approximately 13.3 days for both the nonanemic and the rHuEPO-treated

Table 1 RBC-related parameters on the day of cyclophosphamide treatment in the anemic (carboplatin-treated) group, in the carboplatin group where anemia was prevented by rHuEPO treatment, and in nonanemic otherwise untreated animals

	Carboplatin-treated <i>n</i> = 12	Carboplatin + rHuEPO-treated <i>n</i> = 12	Untreated controls <i>n</i> = 11
cHb (g/liter)	98 ± 3	137 ± 5	141 ± 3
Hematocrit (%)	28 ± 1	42 ± 2	43 ± 1
RBC count (10 ⁶ /μl)	4.5 ± 0.2	6.2 ± 0.2	6.8 ± 0.1
MCV (fl)	63 ± 1	67 ± 1	63 ± 1
MCH (pg)	22 ± 1	22 ± 1	21 ± 1
MCHC (g/liter)	348 ± 5	331 ± 3	331 ± 5

^a *P* for the comparison of anemic control versus rHuEPO group.

^b n.s., not significant.

group but was only 8.6 days in the anemic group. During the regrowth period, the tumor growth rate in the anemic group was slightly higher than in rHuEPO-treated or nonanemic animals (volume-doubling time during the regrowth period was 4.0 days in the anemic group, 4.6 days in the nonanemic group, and 4.3 days in rHuEPO-treated animals). All of the results were confirmed in two independent experimental series.

Discussion

In the present study, i.v. application of carboplatin at a dose of 50 mg/kg body weight was used to induce a moderate anemia (National Cancer Institute-scale) with a mean hemoglobin concentration of approximately 100 g/liter. This treatment results in a normocytic, normochromic anemia lasting for more than 10 days (6). The major mechanism of anemia induction by carboplatin seems to be myelosuppression. For this reason, rHuEPO treatment was only able to prevent anemia development if it was administered before carboplatin. In experiments where carboplatin was given first, rHuEPO treatment could only slightly restore RBC-related parameters (6). Because the growth rate of DS-sarcomas in carboplatin-treated control animals (not treated with cyclophosphamide) was almost identical to that of untreated animals (Fig. 2), it can be concluded that administration of carboplatin at this dose 4 days before tumor implantation has no effect on the growth rate of DS-sarcomas, which might be the result of the relatively short biological half-life of carboplatin (approximately 3–4 h in humans; Ref. 12). The growth curves of tumors in control animals also show that rHuEPO (epoetin α) applied for 2 weeks three times/week at a dose of 1000 IU/kg body weight has no impact on the tumor growth *per se*. These results are in good accordance with previous studies (4, 6) using epoetin β treatment in the same animal and tumor model.

Anemia with a comparable hemoglobin concentration as in the present study has been shown to result in a worsening of the oxygenation status of experimental tumors. Kelleher *et al.* (4) found, using the same tumor model, that the fraction of hypoxic pO₂ values between 0 and 2.5 mm Hg increased significantly (from 21 to 76%) upon induction of a moderate anemia. These results have recently been confirmed for human squamous cell carcinomas of the head and neck region (13). Kelleher *et al.* (4) also demonstrated that correcting anemia by rHuEPO treatment could improve the oxygenation status as indicated by a reduction in the fraction of hypoxic pO₂ values \leq 2.5 mm Hg to 55%. Previous studies (6) have demonstrated that an anemia-related worsening of tumor oxygenation seems to be at least partially responsible for the reduced efficacy of standard radiotherapy. In turn, correction of anemia by rHuEPO was able to improve the

radiosensitivity (6). These results might explain the poorer prognosis of anemic patients after standard radiotherapy (2).

Hypoxia in solid tumors is one major reason for limited efficacy of several nonsurgical treatment modalities such as sparsely ionizing radiation (14) or photodynamic therapy (15). In addition, in cell culture experiments several studies demonstrated that various chemotherapeutic agents were also more effective in the presence of oxygen (7, 8, 16). Teicher *et al.* (10) also showed *in vivo* that the antineoplastic activity of several drugs in experimental sarcomas of mice was correlated to tumor perfusion. The authors assumed that well-perfused tissue areas correspond to normoxic regions, whereas poorly perfused regions indicate hypoxic tissue. Teicher *et al.* (10) found that several chemotherapeutic agents (such as cyclophosphamide, carboplatin, and melphalan) induced a more pronounced cell kill in “oxygenated” tumor areas than in “hypoxic” regions, indicating a possible O₂-sensitivity of these drugs. Various reasons for these differences have been discussed (for reviews, see Refs. 17, 18): (a) oxygen might directly influence the mechanism of action (pharmacodynamics) of antineoplastic drugs (*e.g.*, alkylating agents; Ref. 9); (b) hypoxia can cause a cell-cycle arrest and, in turn, reduce the efficacy of agents acting only on proliferating cells (9, 19); (c) hypoxic tissues show a pronounced extracellular acidosis (3) that might influence the intracellular/extracellular drug distribution (19); and (d) an increase in multidrug resistance gene expression (7, 8).

In the present study, the sensitivity of experimental tumors to cyclophosphamide was studied. Cyclophosphamide was chosen because Teicher *et al.* (10) demonstrated a pronounced oxygen sensitivity of this drug and because the DS-sarcoma has been shown to be sensitive to treatment with this agent (20). The results of the present study clearly indicate that clinically relevant anemia (cHb = 98 g/liter) results in a worsening of the sensitivity of DS-sarcomas to cyclophosphamide treatment. In turn, reversing anemia by rHuEPO treatment significantly increases the cytotoxicity of this chemotherapy (Fig. 3).

Silver and Piver (21) found in an animal study that the efficacy of cisplatin treatment on xenotransplanted tumors was increased (as indicated by a lower tumor volume) when mice were simultaneously treated with erythropoietin for 3 to 4 weeks. The authors attributed the improved chemosensitivity to a better oxygenation status of the tumor as a result of the higher hemoglobin concentration. However, in their study, animals were not anemic on the days of cisplatin treatment, and no oxygen tension measurements have been performed in the tumor to clearly demonstrate an “oxygen effect” of epoetin treatment.

In the present study, anemia (which is correlated with a worsening of the oxygenation status; Ref. 4) resulted in a reduced efficacy of cyclophosphamide, whereas reversal of anemia by rHuEPO improved the effectiveness of this treatment. For this reason, it seems probable that the changes in cyclophosphamide sensitivity can be at least partially attributed to the local oxygenation status of the tumor tissue, which might directly (O₂-dependency of the cytotoxic mechanism) or indirectly (*e.g.*, by altering the cell cycle distribution, the extracellular/intracellular drug distribution, the drug resistance gene expression, the extracellular/intracellular pH gradient, and the development of an aggressive tumor phenotype upon anemic hypoxia) affect the antitumor effect of cyclophosphamide (17, 18). However, other mechanisms, by which changes of the hemoglobin concentration or the RBC count can alter the efficacy of chemotherapy, must also be discussed. It has been demonstrated that several chemotherapeutic drugs are transported preferentially in the erythrocyte rather than in the plasma (22). In this case, anemia with a reduced RBC volume might lead to a worsening of drug transport to the tumor, and increasing the RBC count by rHuEPO treatment might be responsible for a more effective chemotherapy treatment. Our results could form a basis for the per-

formance of additional studies on improving the outcome of chemotherapy in anemic patients by rHuEPO treatment, especially in patients where tumors are known to be hypoxic.

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