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ERYTHROPOIETIN RESTORES THE ANEMIA-INDUCED REDUCTION IN RADIOSENSITIVITY OF EXPERIMENTAL HUMAN TUMORS IN NUDE MICE

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Purpose: The effect of recombinant human erythropoietin (rhEPO) on the radiosensitivity of human tumor xenografts growing in anemic and nonanemic nude mice was studied.

Methods and Materials: Anemia was induced by total body irradiation ([TBI], 2 × 4 Gy) of mice before tumor implantation into the subcutis of the hind leg. The development of anemia was prevented by rhEPO (750 U/kg s.c.) given 3 times weekly starting 2 weeks before TBI. Fourteen days after fractionated TBI (tumor volume of approx. 40 mm^3), single-dose irradiation of the tumor with varying doses was performed so that in full dose–response relationship for the probability of tumor cure was obtained.

Results: Radiation-induced anemia (hemoglobin concentration [cHb] = 9.9 g/dl) led to a reduced radiosensitivity compared to controls [49.4 vs. 40.1 Gy radiation dose to control 50% of the tumors (TCD50)]. Upon rhEPO treatment for anemia prevention (cHb = 13.3 g/dl), the TCD50 was 39.8 Gy, illustrating restored radiosensitivity compared to anemic mice.

Conclusion: These data provide further experimental evidence for restored radiosensitivity upon prevention of anemia with rhEPO.

Radiosensitivity, Anemia, Erythropoietin, Human sarcoma xenografts, Hypoxia.

INTRODUCTION

A major factor contributing to radioresistance is tumor hypoxia. Over the last two decades, it has become clear that experimental tumors contain significant amounts of hypoxic cells (1–3), and increasing evidence is accumulating suggesting that hypoxia is also relevant in the clinical situation (4–8). For several pathophysiologic reasons, anemia is common in clinical oncology (9), and its incidence also increases with the use of modern neoadjuvant protocols. In this context, chemotherapy-induced anemia might reduce the efficacy of radiation treatment and vice versa.

Historical observations have already implied a reduced efficacy of radiation treatments in anemic patients (10). This is thought to result from a reduced oxygen-carrying capacity of the blood, leading to a decreased arterial O_2 supply to the tumor. If pronounced anemia is present, one way of reducing tumor hypoxia might be the application of recombinant human erythropoietin (rhEPO) to overcome or prevent anemia-associated radioresistance. A previous experimental study showed that correcting anemia by rhEPO treatment can improve tumor oxygenation substantially (11). Although a number of clinical trials are currently being carried out, experimental data on the effects of rhEPO on radiosensitivity of tumors in anemic animals are still scarce. In contrast to previous studies (12, 13), which used tumor growth delay as the biologic end point, this study examined the effect of rhEPO treatment on the tumor cure end point with the full dose–response relationship being investigated.

METHODS AND MATERIALS

Animals

Nude mice (nu/nu of NMRI inbred background, body weight: 27–32 g) were used in this study. The mice were obtained from the central animal care facility of Essen University, where breeding was performed under pathogen-free conditions. Mice were housed in the radiation oncology department in laminar air flow units and had unlimited

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access to water (supplemented with chlortetracycline [10 g/L] and K^+-sorbate [1.35 g/L] acidified to a pH = 3.0) and a high-calorie laboratory diet. The mice entered the experiment at the age of 6 to 9 weeks. All experimentation had been previously approved by the regional animal ethics committee.

Tumor and transplantation

The rapidly growing human neurogenic sarcoma ENE2, a human tumor cell line established from a biopsy of a local recurrence of the primary with a tumor volume doubling time of 2.9 ± 0.2 (mean ± SEM) days, was used for the current investigation. Tumor pieces of 2–3 mm were implanted into the subcutis of the right hind leg of the mice. Tumors were repeatedly characterized according to DNA content, volume doubling time, and isoenzyme pattern of LDH and GPD (14). During the experimental period, no changes in these parameters were observed, confirming the human origin of this tumor.

Tumor irradiation

Based on pilot experiments that showed the nadir of the hemoglobin content 12–14 days after total body irradiation (TBI), single-dose irradiation of the tumor was given 2 weeks after TBI. At that time, tumors had reached a volume of 41 ± 3.6 mm^3. The tumor-bearing legs were irradiated with 15-MeV photons generated by a linear accelerator at a dose rate of 2.5 Gy/min. The focus isocenter distance was 100 cm with field sizes of 3 × 2 cm^2 at the isocenter. The remainder of the mouse's body was shielded by a 12-cm-thick Lipowitz's metal shielding block, such that the whole body dose to mice was only 8% of the total tumor absorbed dose. Field homogeneity was repeatedly checked with lithium fluoride thermoluminescent dosimeters. The dose variation was found to be less than 4% over a length of 6 mm in the center of the collimated fields, which corresponds to the size and position of the tumors at the time of treatment. The radiation dose ranged from 18 to 60 Gy. Ten to 16 mice were used for each radiation dose point. A total of 279 mice entered the experiments. After irradiation, the tumors were observed 2 to 3 times weekly for up to 180 days after treatment. Local tumor control was defined as being achieved when, after this period, tumor volume was less than twice the volume on the day of irradiation. Because the tumor model used does not metastasize, none of the mice have to be eliminated from further statistical evaluation. For statistical analysis, the percentage of mice showing local tumor control was calculated, and full radiation dose–response curves were constructed. The doses yielding 50% local control (TCD50) with correction for censored data were then estimated by fitting a logistic regression to the entire dose–response data (15). Values in brackets represent 95% confidence intervals.

Anesthesia

Details of the experimental setting used for the irradiation treatments have been described earlier (16). Briefly, mice were positioned concentrically to the midpoint of the experimental setup. They spontaneously breathed an anesthetic gas mixture through openings in the distributor. Enflurane (Ethrane) was circulated by a membrane pump and was mixed with air. For further details of the anesthetic procedure, see Ang *et al.* (17). A decrease in body temperature during anesthesia was avoided by surrounding the mouse gently with a Perspex tube. In addition, two thermostatically controlled fan heaters were positioned at a distance of 40 cm to the experimental setting during irradiation.

Induction of anemia

The mice received a fractionated TBI at a dose of 2 × 4 Gy (5 MeV photons generated by a linear accelerator at a dose rate of 2.5 Gy/min), on 2 consecutive days (24 h interfraction interval). The focus isocenter distance was 100 cm with field sizes of 20 × 20 cm^2 at the isocenter. The tumor transplantation took place 6 h after the second TBI fraction.

Blood cell count

Erythrocyte and leukocyte parameters were assessed using a multiparameter automated hematology analyzer (Coulter MD II, Coulter Corp., Miami, FL). All measurements were performed using a sample of venous blood (100 μl) from the retrobulbar plexus.

Erythropoietin treatment

The development of anemia was prevented by rhEPO (750 U/kg s.c. ERYPO, Ortho Biotech, Neuss, Germany) given 3 times weekly starting 2 weeks before the total body irradiation. EPO treatment was continued for 4 weeks (12 injections). EPO treatment was suspended 3 days before irradiation to prevent polyglobulia on the day of radiation treatment.

RESULTS

Total body irradiation resulted in a substantial anemia in nude mice, as illustrated in Fig. 1. With a TBI dose of 2 × 4 Gy, the initial hemoglobin concentration (cHb) of mice not receiving rhEPO dropped, typically reaching a nadir 12–14 days after TBI (circles). All mice recovered from the radiation-induced anemia. As a consequence of 12 s.c. injections with 750 U/kg rhEPO, the anemia induction of TBI was prevented, and the cHb of rhEPO-treated mice was maintained >13 g/dl (triangles). Hemoglobin concentrations and hematocrit values measured at the time of tumor irradiation are given in Table 1.

Figure 2 illustrates the radiation response of tumors when the irradiation was administered at different hemoglobin concentrations. Anemia (induced by fractionated TBI) leads to a significantly reduced efficacy of radiation treatment, compared to controls. The TCD50 derived under anemic conditions (squares, cHb = 9.9 g/dl) was 49.4 (47.3–53.1) Gy, whereas the TCD50 for control (no TBI, no rhEPO)

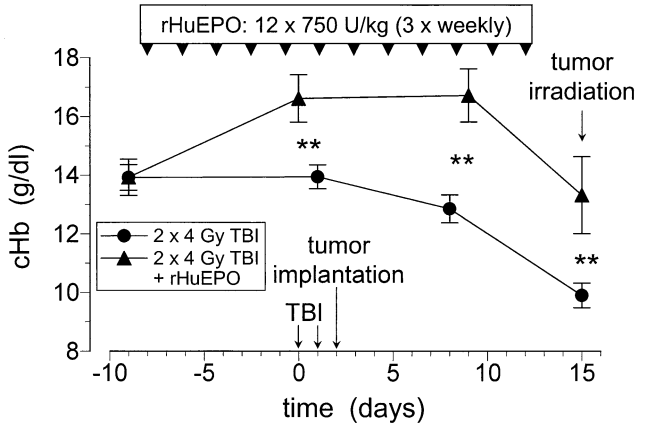

Fig. 1. Time course of hemoglobin concentration (cHb) in animals treated with fractionated total body irradiation ([TBI], 2 × 4 Gy). Circles represent total body–irradiated (non EPO-treated) animals; triangles show the cHb course of TBI animals treated with rhEPO (12 × 750 U/kg s.c. injections). Day 0 is the day of the first TBI fraction. Tumor transplantation was performed one day after the second TBI fraction.

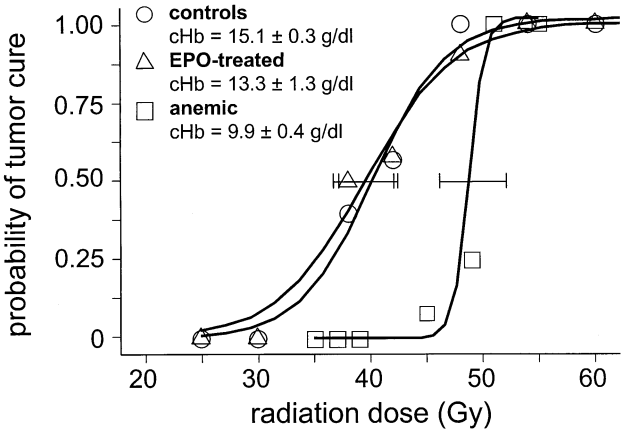

Fig. 2. Effect of different hemoglobin concentrations (cHb) and anemia prevention on the radiation response of xenografted ENE2 sarcomas. Tumors were locally irradiated with increasing single radiation doses. Tumors were treated in control animals (no TBI, no rhEPO [circles]) after TBI-induced anemia (squares) and prevention of anemia (triangles) by repeated rhEPO injections. Results show local tumor control as a function of radiation dose. Lines were fitted by probit analysis, and the bars show 95% confidence intervals of the TCD50 values.

mice (circles, cHb = 15.1 g/dl) was 40.1 (37.2–42.1) Gy. This difference between the radiation response of the tumors grown in control and anemic mice corresponds to a dose-modifying factor of 1.2. Prevention of anemia by EPO (triangles, cHb = 13.3 g/dl) shifted the dose–response curve (compared to anemic mice) to the left, with a TCD50 of 39.8 (36.7–42.4) Gy.

DISCUSSION

Fractionated whole-body irradiation of mice at a dose of 2 × 4 Gy resulted in a pronounced and prolonged anemia. At this dose, the mean cHb was reduced by 34% of the control level with a nadir on Days 12–14. This decrease in cHb was the result of a radiation-induced myelosuppression comparable to that found in the clinical situation after large-field irradiation such as TBI or abdominal bath. In the present study, rhEPO was able to prevent anemia if administered before whole body irradiation, which per se has no effect on the growth rate of tumors when delivered 6 h before tumor implantation, as shown earlier (13).

Based on the observation that some tumors tend to grow more slowly under hypoxic and/or anemic conditions (18, 19), a clinical concern is the possibility of enhanced tumor growth when improved oxygenation is obtained after EPO treatment. In addition, recent studies have demonstrated that human breast cancers express erythropoietin receptors *in vivo*, whereas in normal breast cells or benign papilloma and fibrocystic tissue, these receptors are not found (20, 21). The authors of these studies reported that the expression of the EPO receptor was more pronounced in hypoxic regions and postulated from these findings that erythropoietin and EPO receptors may play a role in carcinogenesis or promotion of human tumors (20, 21). However, it should be noted that in these studies, only the expression of the receptors was analyzed and not the impact of receptor stimulation on tumor growth. Westphal *et al.* (22) recently demonstrated the expression of EPO receptors in numerous human tumor cell lines; however, in these cells EPO application did not modulate the growth rate. Previous experimental studies both in rat and xenotransplanted human tumors also clearly showed that EPO per se had no effect on the growth rate of tumors *in vivo* (12, 13) and are therefore in accordance with the *in vitro* experiments of Westphal *et al.* (22). These findings taken together suggest that the mere expression of EPO receptors does not necessarily present the tumor cell with a mechanism for growth stimulation upon application

Table 1. Hemoglobin concentrations (cHb), hematocrit values, and tumor volumes on the day of tumor irradiation

Group	cHb (g dl^{-1})	Hematocrit (%)	Number of animals	Mean volume (mm^3)
Controls	15.1 ± 0.3	44 ± 0.6	78	41 ± 6
Anemic	9.9 ± 0.4	29 ± 0.6	127	42 ± 6
EPO treatment for anemia prevention	13.3 ± 0.4	39 ± 0.8	74	41 ± 5

Notes: For comparison, data of non–total body irradiated (control) animals are also given. Values are means ± SEM. Hemoglobin concentrations and hematocrit values were statistically significant ($P<0.0001$) between all groups.

of EPO. Certainly, the significance of these receptors in tumor cells warrants further investigation.

Several studies have analyzed the impact of anemia on the radiosensitivity of solid tumors, describing either an increase in radioresistance (12, 13, 23–26) or no effect on the outcome of radiotherapy (25, 27). One major factor for radioresistance during anemia seems to be the period of time over which anemia occurs. Pronounced differences were seen between studies where anemia was acutely (23, 25) or chronically (25, 27, 28) induced. In a meta-analysis of more than 50 clinical studies including 14,000 patients, the hemoglobin level was found to be an important parameter affecting the clinical outcome of radiotherapy for different tumor entities (10).

One possible reason for the impact of anemia on radiosensitivity might be the presence of hypoxic or even anoxic regions within the tumor, which are known to increase radioresistance of the tissue (29). At a tissue Po_2 of approx. 3–4 mm Hg, the radiosensitivity of the tumor is reduced by 50%. The poor oxygenation status in many tumors results mostly from an insufficient oxygen delivery to the tissue via perfusion and/or diffusion. Under anemic conditions, the O_2 transport capacity is reduced, leading to a further worsening of the O_2 supply and thus to an increase in tumor hypoxia. This has been demonstrated in experimental sarcomas where the fraction of measured Po_2 values $\leq$2.5 mm Hg increased from 20% under nonanemic control conditions to 76% in anemic animals with a mean hemoglobin concentration of 9.5 g/dl (11). In contrast, correcting anemia either by EPO treatment or by red blood cell transfusion reduced tumor hypoxia significantly (11, 19), although a complete reduction of hypoxia to the level found in tumors of nonanemic animals could not be reached. The tumor model used in the present study is rather hypoxic with a fraction of hypoxic Po_2 values of more than 50%, even in nonanemic mice. In anemic mice, hypoxia became even more pronounced, although not to the same extent as in the rat tumor model mentioned above. For this reason, other mechanisms by which anemia correction with EPO may improve radiosensitivity have to be taken into account. Because anemia was induced in these mice by TBI, not only red blood cells are affected, but also leukocytes and platelets, effects that can lead to a number of systemic disorders. Because erythropoietin application leads to a faster reconstitution of blood parameters (either directly by stimulating blood cell proliferation or indirectly by improving oxygen transport), EPO-treated animals show better general health, an aspect that might affect tumor cure. Even so, an impact of EPO on the immune system can be ruled out, because in the present study xenotransplanted tumors in nude mice were used.

Despite other factors that may be involved, the available literature suggests a predominant role of EPO-induced tumor oxygenation improvement in increasing radiosensitivity (12, 13, 23, 25, 28). Only a few studies have been performed to examine radiosensitivity after EPO correction/prevention of anemia. Joiner *et al.* (27) used EPO to correct tumor-associated anemia and measured radiosensitivity in anemic mice as well as in mice where anemia was treated with different doses of EPO. The authors could not demonstrate an improvement in radiosensitivity after "normalization" of the hemoglobin level. However, the "correction" of anemia in this latter study led to a hematocrit of 65%, a clear "overtreatment," which can be interpreted as an EPO-induced polyglobulia. At a hematocrit of 65%, the rheological properties of blood change considerably, resulting in an increase in the viscous resistance to flow and thus in a decrease in tumor perfusion, which can even lead to a reduction of the oxygen supply to the tumor. Thus the overcompensation of anemia in the study by Joiner *et al.* (27) could be the reason for a lack of improvement in radiosensitivity. Thews *et al.* (12) demonstrated that correction of a clinically relevant anemia (cHb approx. 9 g/dl) by EPO treatment significantly increased the radiosensitivity of experimental rat sarcomas. The results obtained in the present study using a xenotransplanted human sarcoma model in nude mice are in good accordance with those of Thews *et al.* (12). The present experiments, however, not only confirm previously obtained data, but also indicate the capacity of EPO to increase radiosensitivity in a human tumor cell line as measured with the most relevant biologic end point: tumor cure.

In conclusion, the present study revealed a significant impact of a clinically relevant anemia and anemia correction by EPO treatment on the radiosensitivity of tumors. In the tumor model used, moderate anemia increased radioresistance (as indicated by an increased TCD50). Normalizing the hemoglobin level resulted in an improvement in radiosensitivity, leading to a TCD50 comparable to that obtained in nonanemic mice. Because anemia is a common problem in neoadjuvant treatment protocols using myelosuppressive chemotherapeutic agents (down to a cHb level of 10 g/dl), the results of the present study illustrate the impact that reduced hemoglobin levels can have on the efficacy of nonsurgical treatment modalities (radiotherapy with X-rays and γ-rays, O_2-dependent chemotherapy [9]). However, further experimental and clinical studies are necessary to elucidate the role of anemia and anemia correction during fractionated irradiation schedules.

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