Impact of Anemia Prevention by Recombinant Human Erythropoietin on the Sensitivity of Xenografted Glioblastomas to Fractionated Irradiation

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Background: Pronounced oxygen deficiency in tumors which might be caused by a diminished oxygen transport capacity of the blood (e.g., in anemia) reduces the efficacy of ionizing radiation. The aim of this study was to analyze whether anemia prevention by recombinant human erythropoietin (rHuEPO) affects the radiosensitivity of human glioblastoma xenografts during fractionated irradiation.

Material and Methods: Anemia was induced by total body irradiation (TBI, 2 × 4 Gy) of mice prior to tumor implantation into the subcutis of the hind leg. In one experimental group, the development of anemia was prevented by rHuEPO (750 U/kg s.c.) given three times weekly starting 10 days prior to TBI. 13 days after tumor implantation (tumor volume approx. 40 mm^3), fractionated irradiation (4 × 7 Gy, one daily fraction) of the glioblastomas was performed resulting in a growth delay with subsequent regrowth of the tumors.

Results: Compared to nonanemic control animals (hemoglobin concentration cHb = 14.7 g/dl), the growth delay in anemic mice (cHb = 9.9 g/dl) was significantly shorter (49 ± 5 days vs. 79 ± 4 days to reach four times the initial tumor volume) upon fractionated radiation. The prevention of anemia by rHuEPO treatment (cHb = 13.3 g/dl) resulted in a significantly prolonged growth delay (61 ± 5 days) compared to the anemia group, even though the growth inhibition found in control animals was not completely achieved.

Conclusions: These data indicate that moderate anemia significantly reduces the efficacy of radiotherapy. Prevention of anemia with rHuEPO partially restores the radiosensitivity of xenografted glioblastomas to fractionated irradiation.

Key Words: Radiosensitivity · Anemia · Hypoxia · Erythropoietin · Fractionated irradiation

Einfluss der Anämieprävention mit rekombinantem humanem Erythropoietin auf die Sensitivität xenotransplantierter Glioblastome gegenüber fraktionierter Bestrahlung

Hintergrund: Ein ausgeprägter Sauerstoffmangel im Tumorgewebe, der durch eine Verminderung der O_2-Transportkapazität des Blutes (z.B. bei Anämie) verursacht sein kann, schränkt die Wirksamkeit ionisierender Strahlen ein. Ziel der Studie war, den Einfluss einer Anämieprävention mit rekombinantem humanem Erythropoietin (rHuEPO) auf die Strahlensensibilität von humanen xenotransplantierten Glioblastomen bei fraktionierter Bestrahlung zu untersuchen.

Material und Methodik: In Mäusen wurde eine Anämie durch Ganzkörperbestrahlung (TBI, 2 × 4 Gy) unmittelbar vor Tumorimplantation in die Subkutis des Hinterlaufs erzeugt. Ein Teil der Versuchstiere erhielt zur Anämieprävention rHuEPO (750 U/kg s.c., dreimal wöchentlich, beginnend 10 Tage vor TBI). 13 Tage nach Implantation (Tumorvolumen ca. 40 mm^3) wurden die Glioblastome fraktioniert bestrahlt (4 × 7 Gy, tägliche Fraktion), was zu einer Wachstumsverzögerung mit anschließendem Nachwachsen der Tumoren führte.

Ergebnisse: Im Vergleich zu nichtanämischen Kontrolltieren (Hämoglobinkonzentration cHb = 14,7 g/dl) war die Wachstumsverzögerung durch fraktionierte Bestrahlung in anämischen Mäusen (cHb = 9,9 g/dl) signifikant kürzer (49 ± 5 Tage vs. 79 ± 4 Tage bis zum Erreichen des vierfachen Tumorvolumens). Die Anämieprävention mit rHuEPO (cHb = 13,3 g/dl) führte wieder zu einer signifikanten Zunahme der Wachstumsverzögerung (61 ± 5 Tage) im Vergleich zur Anämiegruppe, wobei jedoch die Wachstumshemmung bei den Kontrolltieren nicht vollständig erreicht wurde.

Schlussfolgerungen: Die Ergebnisse der Untersuchung belegen, dass eine moderate Anämie die Effektivität einer Bestrahlung signifikant verschlechtert. Eine Anämieprävention mit rHuEPO erhöht die Sensitivität von Glioblastomen gegenüber fraktionierter Bestrahlung wieder, ohne jedoch die Wachstumshemmung von nichtanämischen Kontrolltieren zu erzielen.

Schlüsselwörter: Strahlensensibilität · Anämie · Hypoxie · Erythropoietin · Fraktionierte Bestrahlung

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Introduction

The oxygenation status of malignancies is known to substantially affect their radiosensitivity. The sensitivity to sparsely ionizing radiation is approximately three times higher in well-oxygenated tumors compared to anoxic tissues [12]. One possible cause for the development of an oxygen deficiency (hypoxia) is a reduced oxygen transport capacity of the blood due to anemia [18]. Animal studies have clearly demonstrated that while anemia worsens already existing hypoxia, anemia correction either by erythrocyte transfusion or stimulation of erythropoiesis upon erythropoietin treatment leads to an improvement of the O_2-status in experimental tumors [21, 22]. The impact of hemoglobin level on tumor oxygenation has also been demonstrated in human tumors [33, 34, 36], even though in some cases a worsening of the O_2-status was found only with pronounced anemia [2, 8].

Anemia is a common phenomenon in clinical oncology which can be caused by the neoplastic disorder itself (due to e.g., deficiency of erythropoietic factors, bone marrow inhibition by inflammatory cytokines, hemolysis, bone marrow infiltration, or paraneoplastic syndromes), by myelosuppressive therapy modalities, by cisplatin-induced nephrotoxicity leading to a reduced erythropoietin production, or by acute or chronic tumor bleeding [19, 24]. Since anemia worsens the O_2-status of the tumor tissue, radiotherapy in anemic patients cannot be expected to show its optimal efficacy. Many clinical studies have demonstrated a significant impact of the hemoglobin level on the radiotherapy outcome with anemic patients showing a poor long-term prognosis following irradiation (for a review see [11]).

Previous studies analyzed the impact of anemia and anemia correction using recombinant human erythropoietin (rHuEPO) on the sensitivity of experimental tumors to ionizing radiation. These studies clearly demonstrated that anemia reduces the antitumoral effect of radiotherapy, whereas restoring the hemoglobin concentration (cHb) to normal levels renders the radiotherapy more effective [27, 31]. However, these studies were performed only with a single irradiation dose (10 or 12 Gy) and therefore the question remains as to whether these results were transferable to the clinical setting, where the total dose is applied in multiple fractions. For this reason, the aim of the present study was to analyze whether anemia plays a role for radiosensitivity in a fractionated irradiation schedule and whether anemia correction or prevention by rHuEPO prior to irradiation can restore the efficacy of fractionated irradiation. The study was performed in a well-defined tumor model (xenografted HTZ11 glioblastoma s.c. implanted in mice) chosen in order to minimize the intrinsic variability of radiosensitivity (which may occur in human tumors in the clinical setting) which may mask a possible impact of hemoglobin level.

Material and Methods

Animals

Nude mice (nu/nu of NMRI-inbred background) were used in this study. The mice were obtained from the central animal care facility of the Essen University, Germany, where breeding was performed under pathogen-free conditions. Animals were housed in laminar air flow units at the Department of Radiation Oncology and had unlimited 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 animals entered the experiment at an age of 6–9 weeks. All experimentation had previously been approved by the regional animal ethics committee.

Tumor and Transplantation

The rapidly growing glioblastoma HTZ11 cell line established from a biopsy of the primary tumor with a volume-doubling time of 1.7 ± 0.3 days, was used for the investigation. Tumor pieces of 2–3 mm were implanted into the subcutis of the right hind leg of the mice. Tumors were repeatedly characterized by means of DNA content, volume-doubling time, and isoenzyme pattern of lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (GPD) [3]. During the experimental period, no changes in these parameters were observed, confirming the human origin of the tumor.

Tumor Growth

Tumor size was measured using two perpendicular diameters twice to three times a week, and tumor volume was calculated by $V = a \times b^2/2$ (where a and b are the long and short axes, respectively).

Anesthesia

Details of the experimental setting used for the irradiation treatments have been described previously [25]. Briefly, mice were positioned concentrically to the midpoint of the experimental setup, spontaneously breathing 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. [1]. A decrease in body temperature during anesthesia was avoided by surrounding the animal 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

Animals received total body irradiation (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), 6 and 30 h prior to tumor implantation. The focus-isocenter distance was 100 cm with field sizes of 20 × 20 cm at the isocenter.

Tumor Irradiation

The tumor-bearing mouse legs were irradiated with 5-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 at the isocenter. The remainder of the animal

body was shielded from the direct beam such that the animals were mainly exposed to scattered radiation. The whole body dose of mice during tumor irradiation was 8% of the total tumor-absorbed dose.

Based on previous experiments which showed the nadir of the hemoglobin content 12–14 days after TBI, the fractionated irradiation of the tumor was applied 14 days after first TBI. At the time of the first fraction, the tumors reached a volume of 40.7 ± 3.4 mm^3. The radiation dose delivered per fraction was 7 Gy. Four fractions were given, one fraction per day (24-h interfraction interval).

Blood Cell Count

Erythrocyte and leukocyte parameters were assessed using a multiparameter, automated hematology analyzer (Coulter MD II, Coulter, Miami, FL, USA). 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 application of rHuEpo (epoetin alfa, ERYPO®, Ortho Biotech, Neuss, Germany; 750 U/kg s.c.) given three times weekly starting 10 days prior to the TBI. rHuEPO treatment was continued over 4 weeks (13 injections; Figure 1).

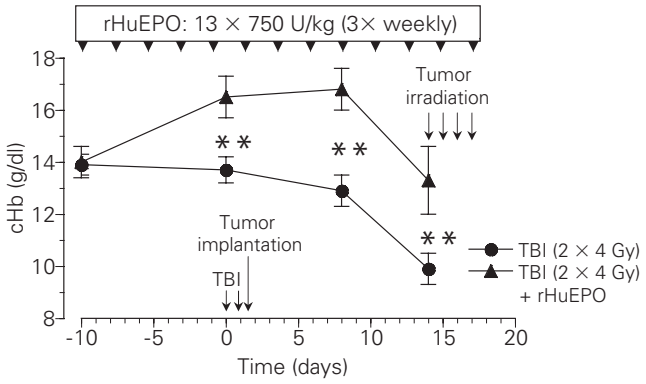

Figure 1. Hemoglobin concentration (cHb) as a function of time in animals treated only with total body irradiation (TBI, 2 × 4 Gy on days 0 and 1) for anemia induction (dots) and animals additionally treated with recombinant human erythropoietin (rHuEPO, from day –10 until day +17, three times a week; triangles). Tumors were implanted 6 h after the second TBI. Arrow heads indicate the times of rHuEPO treatment, arrows mark TBI, tumor implantation and tumor irradiation (4 × 7 Gy given daily, starting 14 days after first TBI). Values are means ± SEM of at least 20 animals; **p < 0.001.

Abbildung 1. Verlauf der Hämoglobinkonzentration (cHb) in Tieren, die zur Anämieinduktion nur Ganzkörperbestrahlung (TBI, 2 × 4 Gy an den Tagen 0 und 1) erhielten (Punkte), und in Mäusen, die zusätzlich mit rekombinantem humanem Erythropoietin (rHuEPO, von Tag –10 bis Tag +17, dreimal wöchentlich) behandelt wurden (Dreiecke). Die Pfeilspitzen bezeichnen die Zeitpunkte der rHuEPO-Gabe, Pfeile markieren die TBI, Tumorimplantation und Tumorbestrahlung (4 × 7 Gy täglich, beginnend 14 Tage nach der ersten TBI). Angegeben sind jeweils Mittelwert ± SEM von mindestens 20 Tieren; **p < 0,001.

Table 1. Hemoglobin concentrations (cHb), hematocrit values, and tumor volumes on the day of the first fraction of tumor irradiation in animals not receiving total body irradiation (TBI; Control), animals treated with TBI alone (Anemic), and mice receiving TBI and recombinant human erythropoietin (EPO-treated). Values are means ± SEM. n: number of animals.

Tabelle 1. Hämoglobinkonzentrationen (cHb), Hämatokritwerte und Tumorvolumina am Tag der ersten Tumorbestrahlung in unbehandelten Kontrolltieren (Control), in Tieren, die nur Ganzkörperbestrahlung (TBI) erhielten (Anemic), und Mäusen, die sowohl mit TBI als auch mit rekombinantem humanem Erythropoietin behandelt wurden (EPO-treated). Angegeben sind jeweils Mittelwert ± SEM. n: Anzahl der untersuchten Tiere.

	n	cHb (g/dl)	Hematocrit (%)	Tumor volume (mm^3)
Control	21	14.7 ± 0.2	43.8 ± 0.5	41 ± 5
Anemic	20	9.9 ± 0.3	28.9 ± 0.5	42 ± 6
EPO-treated	22	13.3 ± 0.4*	38.8 ± 0.9*	41 ± 5

Statistical Analysis

Results are expressed as means ± standard error of the mean (SEM). Differences between the groups were assessed by two-tailed Wilcoxon test for unpaired samples. The significance level was set at $\alpha = 5\%$ for all comparisons.

Results

TBI applied shortly prior to tumor implantation significantly reduced the cHb. Starting at a mean cHb of 13.9 ± 0.4 g/dl 10 days before TBI, a dose of 2 × 4 Gy resulted in a moderate anemia in the animals with a mean cHb of 9.9 ± 0.6 g/dl, 14 days after TBI (Figure 1). All animals recovered from the radiation-induced anemia spontaneously within 30 days (data not shown). Treatment with rHuEPO for 10 days prior to TBI increased the hemoglobin level to 16.5 ± 0.8 g/dl. After TBI in this group (10 days after commencement of rHuEPO therapy), cHb remained constant for approximately 10 days and then reached a value slightly lower than prior to rHuEPO application (13.3 ± 1.3 g/dl; Figure 1). Thus, rHuEPO therapy for 10 days prior to TBI resulted in prevention (or reversal) of the radiation-induced anemia by the time of the fractionated tumor irradiation (13 days after tumor implantation). Table 1 shows red blood cell-(RBC-)related parameters at the time of the first fraction of tumor irradiation.

The fractionated irradiation of tumors with a dose of 4 × 7 Gy resulted in a significant tumor growth delay with a subsequent regrowth of the tumors in all experimental groups. In nonanemic control animals (without rHuEPO treatment), the time period needed to reach four times the initial tumor volume was 79 ± 4 days (Figure 2). Tumors growing in anemic animals were significantly less sensitive to irradiation (49 ± 5 days to reach four times the initial tumor volume). The prevention of anemia by rHuEPO treatment resulted in a significantly slower regrowth (61 ± 5 days to reach four times the initial volume; Figure 2) compared to the anemic animals. How-

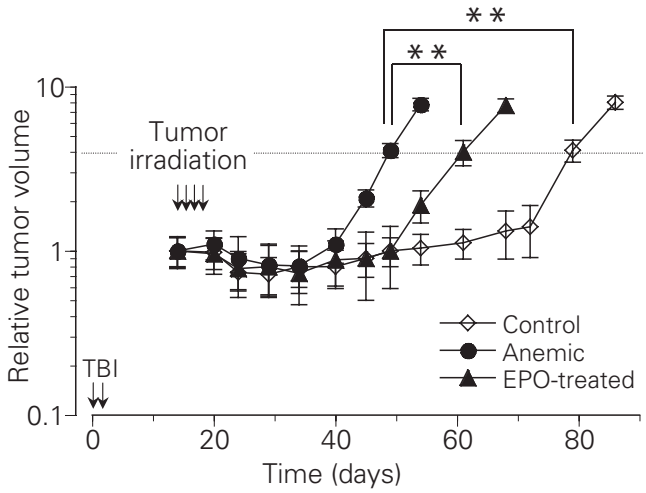

Figure 2. Relative tumor volume (normalized to the mean volume on the 1st day of tumor irradiation) as a function of time in control animals (without TBI), animals treated with TBI alone (Anemic) and mice receiving TBI and rHuEPO (EPO-treated). Tumors were implanted 6 h after the second TBI. Values are means ± SEM of at least 20 animals; **p < 0.001. Abbreviations see Figure 1.

Abbildung 2. Verlauf des relativen Tumorvolumens (normiert auf das mittlere Volumen am 1. Tag der Bestrahlung) in unbehandelten Kontrolltieren (Control), in Tieren, die nur TBI erhielten (Anemic), und Mäusen, die sowohl mit TBI als auch mit rHuEPO behandelt wurden (EPO-treated). Die Tumoren wurden 6 h nach der zweiten TBI implantiert. Angegeben sind jeweils Mittelwert ± SEM von mindestens 20 Tieren; **p < 0,001. Abkürzungen s. Abbildung 1.

ever, the growth delay seen in nonanemic control animals was not reached in the rHuEPO-treated group coinciding with a somewhat lower cHb in the latter group. The growth rate during the regrowth period was comparable in all three experimental groups (Figure 2).

Since previous studies clearly showed that neither TBI nor rHuEPO treatment affect tumor growth per se [27], the differences in growth delay have to be attributed to differences in radiosensitivity upon fractionated irradiation.

Discussion

TBI of mice at a dose of 2 × 4 Gy induces a long-lasting reduction in the hemoglobin level of approximately 30% with a nadir cHb being reached 14 days after TBI. In comparison to previous studies where anemia was induced by a single dose of 5 Gy during TBI [27], the schedule using 2 × 4 Gy resulted in a slightly longer duration of the reduction in cHb necessary for the fractionated tumor irradiation scheme. The cHb on the day of the first fraction of tumor irradiation was 9.9 g/dl which corresponds to a clinically relevant moderate anemia (NCI scale). For this reason, the model of radiation-induced anemia used in the present study seems to be comparable to the clinical situation found in large-field irradiation. Primarily, the mechanism by which TBI induces anemia seems to be destruction of myelopoietic stem cells. For this reason, rHuEPO treatment was able to prevent anemia development only if it was administered prior to TBI. In experiments where TBI was applied first, rHuEPO treatment could only partially restore RBC-related parameters (data not shown). Previous data with the same tumor and animal model and almost the same procedure for anemia induction clearly showed that neither TBI applied immediately prior to tumor implantation nor the application of rHuEPO over 4 weeks (in the same schedule as used in the present study) had a marked impact on tumor growth per se [27]. As has been demonstrated in another tumor system [30, 31], erythropoietin had no promoting impact on tumor growth since neither a direct stimulation of cell proliferation by the pleiotropic growth factor EPO [37] nor by an improvement in tumor oxygenation [21] which might induce a faster cell division [29] was seen. This also applied to human tumors of neurogenic origin transplanted into nude mice (HTZ11 glioblastoma included [26, 27]).

Irradiation of the HTZ11 glioblastoma tumors in a fractionated schedule of 4 × 7 Gy induced a pronounced growth inhibition of approximately 60 days followed by an exponential regrowth (Figure 2). As might have been expected, the growth delay induced by this fractionated scheme was found to be significantly longer compared to single irradiation dose of 12 Gy [27]. However, it was unclear what impact anemia might have during such a fractionated irradiation schedule. Several experimental studies have clearly shown that tumor oxygenation is slightly worsened during fractionated irradiation only if the total dose exceeds 45 Gy [40]. The intensified tumor hypoxia in this case is most probably the result of vascular destruction [38, 39] followed by a deterioration of nutrient supply [32]. These data may indicate that during fractionated irradiation, the impact of moderate anemia on the oxygenation status of the tumor and, by this, on the radiosensitivity plays only a minor role due to the overwhelming destruction of tumor vasculature. However, the data of the present study reveal that the hemoglobin level is of importance even during fractionated radiotherapy. In anemic animals with a cHb of 9.9 g/dl, the growth delay was 30 days less than in the controls. Anemia prevention, however, improved the efficacy of radiation as indicated by a significantly longer growth retardation of approximately 12 days compared to anemic mice. At present, it is unclear whether the oxygenation status is indeed significantly improved by rHuEPO during fractionated irradiation in anemic subjects. A pronounced worsening of the O_2-status has been described in fractionated schedules only at high total doses (> 45 Gy) applied over > 3 weeks [40]. In the present study, the dose of 28 Gy was applied in four fractions within 1 week. In a previous study, it has been shown that after fractionated irradiation using six fractions of 6 Gy applied within 11 days, the oxygenation status changed only slightly over 4 weeks [28]. Since the regrowth rate of the tumors after the growth delay was comparable in all three groups, it is unclear whether differences in tumor composition (e.g., fraction of apoptotic or necrotic cells) after irradiation play a role in

the differences seen in growth delay. In addition, a further analysis of whether the site of tumor implantation (orthotopic vs. heterotopic) plays a role in the results obtained in the present study may be necessary.

Since the irradiation schedule used in the present study is only loosely based on that used in clinical radiotherapy, the results obtained in the present study can only be transferred to the clinical setting with some reservation. Further studies need to elucidate whether the hemoglobin level also plays a role at the end of a "regular" irradiation schedule (e.g., after several weeks) when the tumor vasculature is severely injured resulting in a pronounced deterioration of the O_2-status. The present study, however, clearly shows that at least during the early period of fractionation, anemia worsens the efficacy of radiation significantly.

The mechanism behind the impact of hemoglobin levels on radiosensitivity seems to involve variations in tumor oxygenation. Animal experiments in a well-defined tumor model showed that anemia reduced the oxygen supply to the tumor and, by this, worsened the O_2-status [21] which may induce tumor neovascularization by increased vascular endothelial growth factor (VEGF) production [7]. In turn, anemia correction or prevention led to a reduction in hypoxia, even though the values seen in control animals were not restored [20, 21]. In some human tumors, it has also been shown that anemia with a cHb < 11 g/dl reduced tumor oxygenation [33, 34, 36], whereas in others with only mild anemia, no substantial changes in the O_2-status were seen [2]. Obviously, compared to the well-defined tumor models in animal experiments, solid tumors in the clinical setting show a more pronounced heterogeneity of the O_2-status, which could mask the impact of cHb [2, 8]. The anemia-induced increase in tumor hypoxia (which, in turn, is known to reduce radiosensitivity) has been discussed as a prominent mechanism by which a lower cHb may influence the outcome of radiotherapy in the clinical setting [4–6, 10, 13, 15, 17, 35]. However, clinical studies concerning the impact of anemia correction (e.g., by rHuEPO application) on the efficacy of radiotherapy are rare [9, 13, 23]. In a preliminary study on head and neck cancers undergoing preoperative chemoradiotherapy, it has been shown that patients with a reduced cHb had a worse locoregional control and a shorter overall survival [9]. Erythropoietin treatment of anemia led to a long-term outcome which was comparable to that found in nonanemic patients. However, since this is the only finalized clinical study and the results are only preliminary, presently ongoing clinical studies [14, 16] have to show whether anemia correction by rHuEPO can indeed improve radiosensitivity and the long-term prognosis of patients upon fractionated irradiation in the clinical setting.

Conclusions

The present study demonstrated, in a well-defined tumor model, that clinically relevant anemia (cHb = 9.9 g/dl) limits the efficacy of radiotherapy in a fractionated schedule. The prevention of anemia by rHuEPO application significantly increased radiosensitivity, even though the level found in nonanemic controls was not reached. Further studies are needed to elucidate the role of the fractionation scheme used in animal experiments, since this may not be fully transferable to the clinical situation. The results, however, clearly show that at least during the early period of fractionation, anemia worsens radiosensitivity and anemia prevention by rHuEPO partially restores the efficacy of fractionated irradiation.

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