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Recombinant human erythropoietin increases the radiosensitivity of xenografted human tumours in anaemic nude mice

Abstract ***Purpose:*** The effect of recombinant human erythropoietin (Epo) on the radiosensitivity of human tumour xenografts growing in anaemic nude mice was studied. ***Methods and materials:*** Anaemia was induced by total body irradiation (TBI) of mice prior to tumour transplantation. The development of anaemia was prevented by Epo (1000 U/kg s.c.) given 3 times weekly starting 2 weeks prior to TBI (5 Gy). Epo treatment did not influence the growth rate of the tumours, which were transplanted into the subcutis of the hind leg of mice. Thirteen days after TBI (tumour volume of approx. 40 mm^3), a single-dose irradiation (12 Gy) of the tumour was performed resulting in a growth delay with subsequent regrowth of the tumours. ***Results:*** In Epo-treated animals the tumour growth delay was significantly longer compared to anaemic mice. However, the radiosensitivity of tumours in non-anaemic animals' (non-Epo-treated) tumours could not fully be restored. ***Conclusion:*** These data give evidence for restored radiosensitivity after correction of anaemia with Epo.

Key words Radiosensitivity · Anaemia · Hypoxia · Erythropoietin · Human tumour xenografts

Introduction

A major factor contributing to radioresistance is tumour hypoxia. Over the last few years it has become clear that experimental tumours contain significant amounts of hypoxic cells (Moulder and Rockwell 1984; Rockwell and Moulder 1990; Stüben et al. 1998) and more and more evidence is accumulating which suggests that hypoxia is relevant in the clinical situation (Overgaard 1989; Nordsmark et al. 1996; Höckel et al. 1993, 1996; Kaanders et al. 1998). For several pathophysiological reasons, anaemia is common in clinical oncology and its incidence also increases with the use of modern neoadjuvant protocols. In this context, chemotherapy-induced anaemia might reduce the efficacy of radiation treatment.

Historical observations have already implicated reduced efficacy of radiation treatments in anaemic patients. This is thought to result from a reduced oxygen-carrying capacity of the blood leading to a decreased arterial O_2 supply to the tumour. If pronounced anaemia is present, one way of reducing tumour hypoxia might be the application of recombinant human erythropoietin (Epo) in order to overcome anaemia-associated radioresistance. A previous experimental study showed that correcting anaemia by Epo treatment can improve tumour oxygenation substantially (Kelleher et al. 1996). Despite the fact that there are several ongoing clinical trials, experimental data on the effects of Epo on radiosensitivity in anaemic tumour models are still scarce. In contrast to previous studies (Thews et al. 1998), we evaluated Epo treatments on the radiation response of human tumour xenografts in mice with radiation-induced anaemia.

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Materials 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 Essen University, where breeding was performed under pathogen-free conditions. Animals were housed in the Dept. of Radiation Oncology in laminar air-flow units and had unlimited access to water [supplemented with chlortetracycline (1.35 g/l) and potassium sorbate (10 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.

Tumours and transplantation

The rapidly growing glioblastoma HTZ11 cell line established from a biopsy of the primary tumour with a volume doubling time of 1.7 ± 0.3 days, was used for the investigation. Tumour pieces of 2–3 mm were transplanted into the subcutis of the right hind leg of the mice. Tumours were repeatedly characterised by means of DNA content, volume doubling time, and isoenzyme pattern of LDH and GPD (Budach et al. 1989). During the experimental period, no changes in these parameters were observed, confirming the human origin of the tumour.

Tumour growth

Animals were assigned to treatment groups when tumours reached a volume of approximately 40 mm^3. Tumour size was measured using two perpendicular diameters 2–3 times a week and tumour volume was calculated as

$$V = \frac{a \times b^2}{2}$$

where a and b are the long and the short axes, respectively.

Anaesthesia

Details of the experimental setting used for the irradiation treatments have been described previously (Stüben et al. 1994). Briefly, mice were positioned concentrically to the midpoint of the experimental set-up, spontaneously breathing an anaesthetic 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 anaesthetic procedure see Ang et al. (1982). A decrease in body temperature during anaesthesia 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 anaemia

Animals received total body irradiation (TBI) at a dose of 5 Gy (5 MeV photons generated by a linear accelerator at a dose rate of 2.5 Gy/min), 6 h prior to tumour transplantation. The focus isocenter distance was 100 cm with field sizes of 20×20 cm^2 at the isocenter.

Tumour irradiation

The tumour-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^2 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 was 8% of the total tumour-absorbed dose.

Based on previous experiments which showed the nadir of the haemoglobin content 12–14 days after TBI, the single-dose irradiation of the tumour was applied 13 days after transplantation. At that time, the tumours reached a volume of approx. 40 mm^3 (40.7 ± 3.4) mm^3. The single dose of radiation delivered was 12 Gy.

Blood cell count

Erythrocyte and leukocyte parameters were assessed using a multiparameter, automated haematology analyser (Coulter MD II, Coulter, Fla., USA). All measurements were performed using a sample of venous blood (100 μl) from the retrobulbar plexus.

Erythropoietin treatment

The development of anaemia was prevented by Epo (1000 U/kg s.c.) given 3 times weekly starting 2 weeks prior to the TBI. Epo treatment was continued for 4 weeks (13 injections).

Experimental design

The experiments consisted of six groups, as detailed in Table 1.

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

Total body irradiation resulted in a substantial anaemia in the nude mice. With a TBI dose of 5 Gy the initial haemoglobin level dropped, typically reaching a nadir at days 12–14 after TBI. All animals recovered from the radiation-induced anaemia. Haemoglobin concentrations (cHb) and haematocrit values measured at the time of tumour irradiation are given in Table 2.

Figure 1 illustrates the volume growth curves of non-irradiated tumours in control, anaemic, and Epo-treated animals, respectively. Regardless of the haemoglobin concentration of animals all tumours had comparable growth characteristics.

The single-dose irradiation of the tumour with a dose of 12 Gy resulted in a significant tumour growth delay in all experimental groups (see Fig. 2). Tumours growing in anaemic animals were significantly less sensitive to irradiation compared to control animals (24 ± 3 days to reach 4 times the initial tumour volume in anaemic mice compared to 42 ± 2 days in control animals). The prevention of anaemia by Epo-treatment resulted in a significantly improved radiosensitivity compared to tumours of anaemic mice (36 ± 3 days to reach 4 times the initial tumour volume in Epo-treated mice compared to 23 ± 3 days in anaemic animals). However, the radiosensitivity of tumours growing in animals in which

Table 1 Characteristics of the experimental groups (*n* number of mice per group)

Group number	*n*	TBI	Tumour irradiation	Epo	Anaemic
I	9	No	No	No	No
II	8	Yes	No	Yes	No
III	7	Yes	No	No	Yes
IV	7	Yes	Yes	No	Yes
V	6	Yes	Yes	Yes	No
VI	9	No	Yes	No	No

Table 2 Haemoglobin concentrations (cHb), haematocrit values, and tumour volume on the day of tumour irradiation. For comparison, data of non-irradiated control animals are presented. Values are means ± SEM

Group	cHb (g/dl^{-1})	Haematocrit (%)	Number of animals	Volume (mm^3)
Controls	14.7 ± 0.2	43.8 ± 0.6	9	40 ± 7
Anaemic	11.4 ± 0.2	33.6 ± 0.4	7	42 ± 6
Anaemia prevention	15.9 ± 0.3	45.7 ± 1.0	6	41 ± 5

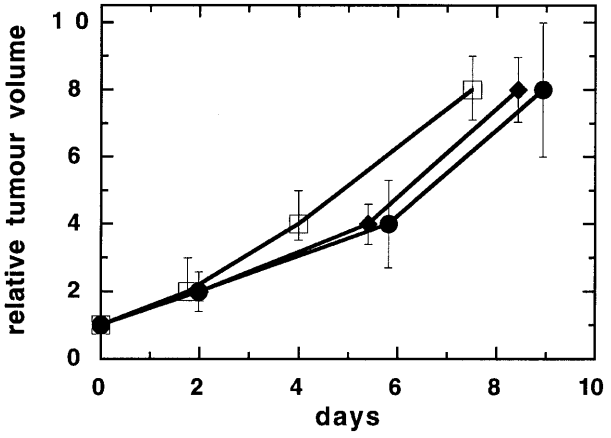

Fig. 1 Relative volume growth in non-irradiated tumours. Growth characteristics of control mice (□ group I), Epo-treated animals (◆ group II), and growth characteristics of tumours in anaemic animals (● group II), respectively

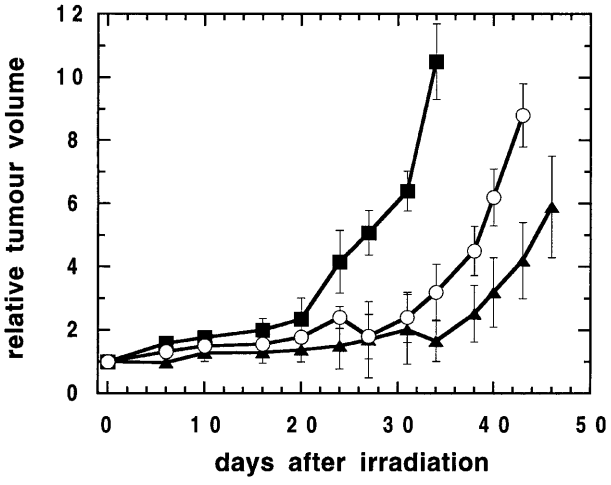

Fig. 2 Relative volume growth in tumours treated with single-dose irradiation (12 Gy). Growth characteristics of anaemic animals (■ group IV) Epo-treated animals (○ group V), and growth characteristics of control mice (▲ group VI), respectively

anaemia development had been prevented by Epo-treatment could not fully be restored to the level found in control animals.

Discussion

Total body irradiation of mice at a dose of 5 Gy resulted in a pronounced and prolonged anaemia. At this dose the mean haemoglobin concentration (cHb) is reduced by 22% 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 the clinical situation found in large field irradiation such as TBI or abdominal bath. In the present study, recombinant human erythropoietin was able to prevent anaemia if it was administered prior to whole body irradiation. As shown in Fig. 1, TBI 6 h prior to tumour implantation had no effect on the growth kinetics of the glioblastoma cell line used in the present study.

Based on the observation that some tumours tend to grow slower under hypoxic and/or anaemic conditions (Tannock and Steel 1970; Kelleher et al. 1996), a clinical concern is the possibility of enhanced tumour growth when improved oxygenation is obtained following Epo-treatment. In the present study, the growth curves of non-irradiated tumours were almost identical in the Epo-treated group compared with non-anaemic controls as well as with the group where anaemia was induced by whole body irradiation. From these data it can be concluded that Epo per se has no effect on the growth rate of tumours. These results are in good accordance with a previous study where tumour growth of an experimental rat sarcoma was neither affected by a chemotherapy-induced anaemia nor the prevention of this anaemia by Epo treatment (Thews et al. 1998).

Several studies have analysed the impact of anaemia on the radiosensitivity of solid tumours describing either an increase in radioresistance (Thews et al. 1998; Hewitt and Blake 1971; Hill et al. 1971; Hirst et al. 1984; McCormack et al. 1990) or no effect on the outcome of radiotherapy (Hirst et al. 1984; Joiner et al. 1993). One major factor for radioresistance during anaemia seems to be the period of time over which anaemia occurred. Pronounced differences were seen between studies where anaemia was acutely (Hewitt and Blake 1971; Hirst et al. 1984) or chronically (Hirst et al. 1984; Joiner et al. 1993; Rojas et al. 1987) induced. In a meta-analysis of more than 50 clinical studies including 14,000 patients the haemoglobin level was found to be an important parameter affecting the clinical outcome of radiotherapy for different tumour entities (Grau and Overgaard 1998).

One possible reason for the impact of anaemia on radiosensitivity might be the presence of hypoxic or even anoxic regions within the tumour which are known to increase the radioresistance of the tissue (Bush et al. 1978). Below a tissue pO_2 of approx. 3-4 mmHg the radiosensitivity of the tumour is reduced by 50%. The

poor oxygenation status in many tumours results mostly from an insufficient oxygen transport to the tissue. Under anaemic conditions, the O_2 transport capacity is reduced leading to a further worsening of the O_2 supply and thus to an increase in tumour hypoxia. This has been demonstrated in experimental sarcomas where the fraction of measured pO_2 values <2.5 mmHg increased from 20% under non-anaemic control conditions to 76% in anaemic animals, with a mean haemoglobin concentration of 9.5 g/dl (Kelleher et al. 1996). In contrast, correcting anaemia either by Epo treatment or by RBC transfusion reduced tumour hypoxia significantly (Kelleher et al. 1995, 1996), although a complete reduction of hypoxia to the level found in tumours of non-anaemic animals could not be reached.

An improvement of tumour oxygenation following anaemia correction might be the reason for an increase in radiosensitivity (Hewitt and Blake 1971; Hirst et al. 1984; Rojas et al. 1987; Thews et al. 1998). Only two studies have been performed to examine radiosensitivity following Epo-correction/prevention of anaemia. Joiner et al. (1993) used Epo to correct a tumour-associated anaemia and measured radiosensitivity in anaemic animals as well as in mice where anaemia was treated with different doses of Epo. The authors could not demonstrate an improvement in radiosensitivity following "normalisation" of the haemoglobin level. However, the correction of the anaemia led to a haematocrit of 65%, a clear overcompensation which can be interpreted as an Epo-induced polyglobulia. At a haematocrit of 65% the rheological properties of blood worsen, resulting in an increase in the viscous resistance to flow and, thus, in a decrease in tumour perfusion which might even lead to a reduction in the oxygen supply to the tumour. Thus, the overcompensation of anaemia in Joiner's study could be the reason for a lack of improvement in radiosensitivity. Thews et al. (1998) demonstrated that correction of a clinically relevant anaemia (cHb approx. 9 g/dl) by treatment with Epo significantly increased the radiosensitivity of experimental rat sarcomas. The results obtained in the present study using a xenotransplanted human tumour model in nude mice are in good accordance with those of Thews et al. (1998). The present experiments, however, not only confirm previously obtained data but also indicate the capacity of Epo to increase radiosensitivity: (i) in a different species; (ii) following a different mode of anaemia induction; and (iii) in a human tumour cell line.

In conclusion, the present study revealed a significant impact of a clinically relevant anaemia and anaemia correction by Epo treatment on the radiosensitivity of tumours. In the tumour model used, moderate anaemia increased radioresistance (as indicated by a reduction in the radiation-induced growth delay). Normalising the haemoglobin level resulted in an improvement in radiosensitivity leading to a growth delay comparable to that obtained in non-anaemic animals. Since in neoadjuvant treatment protocols using myelosuppressive chemotherapeutic agents, anaemia down to a cHb level of 10 g/dl is a common problem, the results of the present study illustrate the importance of reduced haemoglobin levels for the efficacy of non-surgical treatment modalities. However, further experimental and clinical studies are necessary to elucidate the role of anaemia and anaemia correction during fractionated irradiation schedules.

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References

1. Ang KK, van der Kogel AJ, van der Schueren E (1982) Inhalation anesthesia in experimental radiotherapy: a reliable and time-saving system for multifractionation studies in a clinical department. Int J Radiat Oncol Biol Phys 8: 145–148
2. Budach V, Bamberg M, Streffer C, Budach W, Stuschke M, Fabry W (1989) Establishment and characterization of human tumours in nu/nu-mice. Strahlenther Onkol 165: 500–501
3. Bush RS, Jenkin RDT, Allt WEC, Beale FA, Bean H, Demko AJ, Prigle JF (1978) Definitive evidence for hypoxic cells influencing cure in cancer therapy. Br J Cancer 37: 302–306
4. Grau C, Overgaard J (1998) Significance of hemoglobin concentration for treatment outcome. In: Molls M, Vaupel P (eds) Blood perfusion and microenvironment of human tumours – implications for clinical radiology. Springer, Berlin Heidelberg New York, pp 101–112
5. Hewitt HB, Blake ER (1971) Effect of induced host anaemia on the viability and radiosensitivity of murine malignant cells in vivo. Br J Cancer 25: 323–336
6. Hill RP, Bush RS, Yeung P (1971) The effect of anemia on the fraction of hypoxic cells in an experimental tumor. Br J Radiol 44: 299–304
7. Hirst DG, Hazlehurst JL, Brown JM (1984) The effect of alterations in haematocrit on tumour sensitivity to X-rays. Int J Radiat Biol Relat Stud Phys Chem Med 46: 345–354
8. Höckel M, Knoop C, Schlenger K, Vorndran B, Baussmann E, Mitze M, Knapstein PG, Vaupel P (1993) Intratumoral pO_2 predicts survival in advanced cancer of the uterine cervix. Radiother Oncol 26: 45–50
9. Höckel M, Schlenger K, Aral B, Mitze M, Schäffer U, Vaupel P (1996) Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 56: 4509–4515
10. Joiner B, Hirst VK, McKeown SR, McAleer JJ, Hirst DG (1993) The effect of recombinant human erythropoietin treatment on tumour radiosensitivity and cancer-associated anaemia in the mouse. Br J Cancer 68: 720–726
11. Kaanders JH, Pop LA, Marres HA, van den Hoogen FJ, van Daal WA, van der Kogel AJ (1998) Accelerated radiotherapy with carbogen and nicotinamide (ARCON) for laryngeal cancer. Radiother Oncol 48: 115–122
12. Kelleher DK, Matthiensen U, Thews O, Vaupel P (1995) Tumor oxygenation in anemic rats: effects of erythropoietin treatment versus red blood cell transfusion. Acta Oncol 34: 379–384
13. Kelleher DK, Matthiensen U, Thews O, Vaupel P (1996) Blood flow, oxygenation, and bioenergetic status of tumors after erythropoietin treatment in normal and anemic rats. Cancer Res 56: 4728–4734
14. McCormack M, Nias AH, Smith E (1990) Chronic anaemia, hyperbaric oxygen and tumour radiosensitivity. Br J Radiol 63: 752–759
15. Moulder JE, Rockwell S (1984) Hypoxic fractions of solid tumors: experimental techniques, methods of analysis, and a survey of existing data. Int J Radiat Oncol Biol Phys 10: 695–712

16. Nordsmark M, Overgaard M, Overgaard J (1996) Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol 41: 31–39
17. Overgaard J (1989) Sensitization of hypoxic tumour cells-clinical experience. Int J Radiat Biol 56: 801–811
18. Rockwell S, Moulder JE (1990) Hypoxic fractions of human tumors xenografted into mice: a review. Int J Radiat Oncol Biol Phys 19: 197–202
19. Rojas A, Stewart FA, Smith KA, Soranson JA, Randhawa VS, Stratford MR, Denekamp J (1987) Effect of anemia on tumor radiosensitivity under normo- and hyperbaric conditions. Int J Radiat Oncol Biol Phys 13: 1681–1689
20. Stüben G, Budach W, Schick KH, Stuschke M, Stapper N, Müller S, Feldmann HJ (1994) A time-saving system for irradiation of experimental tumors. Strahlenther Onkol 170: 36–41
21. Stüben G, Stuschke M, Knühmann K, Horsman MR, Sack H (1998) The effect of combined nicotinamide and carbogen treatments in human tumour xenografts: oxygenation and tumour control studies. Radiother Oncol 48: 143–148
22. Tannock IF, Steel GG (1970) Tumor growth and cell kinetics in chronically hypoxic animals. J Natl Cancer Inst 45: 123–133
23. Thews O, Koenig R, Kelleher DK, Kutzner J, Vaupel P (1998) Enhanced radiosensitivity in experimental tumours following erythropoietin treatment of chemotherapy-induced anaemia. Br J Cancer 78: 752–756