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Tumour Oxygenation During Fractionated Radiotherapy

Comparison with Size-matched Controls

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The effect of fractionated irradiation on the oxygenation status of experimental tumours was investigated using polarographic assessment of the pO_2 distribution. Since an improvement in tumour oxygenation could simply be the result of tumour shrinkage, a comparison of pO_2 readings of untreated size-matched control tumours was performed. Irradiation was carried out using 6 fractions of 6 Gy applied within 11 days. A comparison of polarographic pO_2 data with size-matched untreated tumours revealed a significant improvement in oxygenation after the irradiation. The median pO_2 was 0.9 ± 0.1 mmHg for unirradiated tumours at a volume of 180 mm^3, while the corresponding data for irradiated tumours of comparable size were 2.3 ± 0.5 mmHg on day 21 and 4.8 ± 0.9 mmHg on day 28 after start of irradiation. From these results it can be concluded that the improvement of oxygenation after fractionated irradiation is not solely the result of a reduced tumour volume.

Reoxygenation is thought to be a major factor contributing to the efficacy of fractionated irradiation in hypoxic tumours. However, because of complex alterations occurring in the tumour microenvironment following irradiation, a quantification of the cellular processes leading to a decrease in the radiobiological hypoxic fraction during a fractionated radiation regime is difficult to achieve. Modifications of tumour blood flow (1, 2), changes in the tumour vasculature architecture, reduced intratumoral pressure (3) and a decrease in cellular oxygen consumption (4) have been discussed as being the most important factors leading to the reduced hypoxic fraction observed in some studies after fractionated irradiation.

It is, however, broadly accepted that in many experimental tumours as well in some human malignancies, oxygenation worsens with enlarging tumour volume (5–7). Therefore, an improvement in the oxygenation status by cytotoxic treatments might simply be a result of tumour shrinkage (8).

The aim of the present investigation was to evaluate the effects of fractionated irradiation on the oxygenation of human tumour xenografts by means of invasive polarographic oxygen tension measurements. A comparison with size-matched unirradiated control tumours was carried out in order to distinguish between treatment-related tumour shrinkage effects and volume-independent factors contributing to the improvement of oxygenation seen during a course of fractionated irradiation.

MATERIAL AND METHODS

Animals

Nude mice (nu/nu of NMRI inbred background) were used for this study. The mice were obtained from the central animal facilities of Essen University, where the breeding was performed under pathogen-free conditions. During the experiments the animals were housed in laminar air-flow units in the laboratory of the Department of Radiation Oncology. The animals had unlimited access to water (supplemented with chlortetracycline (10g/L) and K-sorbate (1.35 g/L) acidified to a pH of 3.0) and a high caloric diet. Experiments were performed when the mice were 6–9 weeks of age; the regional animal ethics committee had previously approved all experimentation.

Tumours and transplantation

A human neurogenic sarcoma (ENE2), established from a biopsy of a local recurrence of the primary, with a tumour volume doubling time of 2.9 days was used for this investigation. Further details of the tumour model have been described previously (9).

Tumour chunks of 2 to 3 mm were transplanted into the s.c. tissue of the right hind leg of the mice. Tumours were repeatedly characterized by means of DNA content, volume doubling time, and isoenzyme pattern of LDH and GPD (10). During the experimental period, no changes in these parameters were observed, confirming the human origin of the tumour.

Assessment

Animals were assigned to treatment when tumours reached a volume of approximately 170 mm^3. Tumour size was measured in two perpendicular diameters 2 to 3 times a week and tumour volume calculated as

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

where a and b are the long and the short axes, respectively. Forty-two mice were irradiated, whereas 36 animals were used as controls.

Anaesthesia

Details of the experimental setting used for both irradiations and pO_2 measurements have been described previously (11). 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 narcotic procedure, see Ang et al. (12). A decrease in body temperature during anaesthesia was avoided by gently enclosing the animal body in a perspex tube. In addition, two thermostatically controlled fan heaters were positioned at a distance of 40 cm to the experimental setting during irradiation.

Polarographic pO_2 measurements

Polarographic intratumoural oxygen partial pressure (pO_2) measurements and irradiation were carried out under identical conditions (e.g. positioning of animals, depth and duration of anaesthesia, avoidance of decreases in body temperature). The invasive pO_2-measurements were performed using a computerized system (KIMOC 6650, Eppendorf, Hamburg, Germany) on days 7, 14, 21, 28 relative to the start of irradiation and in size-matched control animals (see Fig. 1). The details of this technique have been described previously (13). Briefly, the intratumoural pO_2 was assessed using polarographic needle electrodes with a diameter of 300 μm. The O_2 sensitive gold cathode in the steel shaft needle had a diameter of 16 μm resulting in a hemispherical measuring volume with a diameter of approx. 50 μm around the tip of the electrode. With this technique the microregional oxygenation of approx. 50 to 200 tumour cells was assessed. The needle electrode was moved through the tumour tissue in steps of 0.5 mm. Each forward movement was immediately followed by a backward step of 0.2 mm (in order to minimize tissue compression artefacts (14)) resulting in an effective step size of 0.3 mm. Tumour oxygenation measurements were performed along three tracks within each individual tumour so that approximately 36 individual readings were obtained per tumour. Data obtained at the same time after irradiation were pooled for analysis. At least 360 individual pO_2 data points (360–468) per time point were collected for statistical evaluation. Negative values in the range -1 to 0 mmHg, which may occur because of the principal of the polarographic measurements, were treated as belonging to the lowest class reading (0–2.5 mmHg). Values below that range (i.e., < -1 mmHg) were not observed in our investigation. The drift of the electrode was $< 0.4\%$/min. The raw data of the pO_2 measurements were exported from the KIMOC device to a personal computer and analysed using standard statistical software. The oxygenation status of each individual tumour was described by the median pO_2 and the fraction of hypoxic pO_2 values ≤ 2.5 mmHg. The results for the different treatment groups were described by the mean $\pm$ SEM of the oxygenation parameters. The comparison of the median pO_2 as well as of the fraction of hypoxic pO_2 values between the groups was performed with the two-tailed Wilcoxon rank-sum test for unpaired samples. In order to avoid possible artefactual effects on the oxygenation status resulting from repeated measurements in damaged tissue, pO_2 measurements performed at different time intervals after irradiation were carried out in different animals.

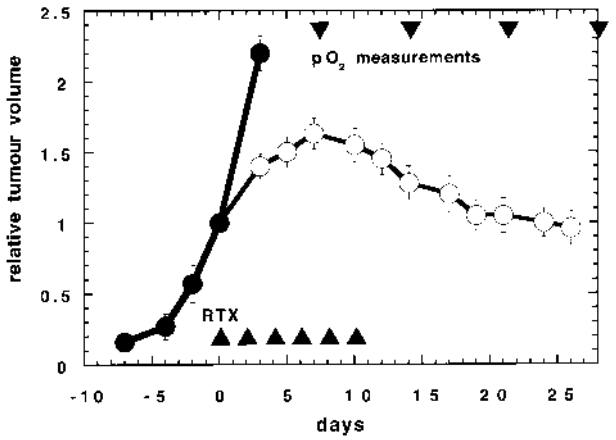

Fig. 1. Relative tumour growth in tumours treated with fractionated irradiation (○; 6 × 6 gy every second day) and in untreated controls (●). Arrows indicate the days of irradiation (RTX) and of pO_2 measurements. The relative volume of 1 corresponds to 178 mm^3. Each point represents a minimum of 36 tumours.

Table 1

Oxygenation of the ENE2 sarcoma in a course of fractionated irradiation with six fractions of 6 Gy. Data are given as mean ± SEM

Time of pO_2 measurement	Tumour volume (mm^3)	pO_2 (mmHg)	Percentage ≤ 2.5 mmHg	No. of readings	No. of tumours
Controls	123 ± 18	1.9 ± 0.2	58 ± 3	428	12
Controls	178 ± 10	0.9 ± 0.1	69 ± 2	468	13
Controls	241 ± 26	0.8 ± 0.1	73 ± 3	468	13
Day 7	281 ± 18	0.9 ± 0.1	76 ± 5	360	10
Day 14	207 ± 23	0.9 ± 0.2	73 ± 3	432	12
Day 21	187 ± 21	2.3 ± 0.5*	53 ± 7*	360	10
Day 28	186 ± 20	4.8 ± 0.9**	41 ± 6**	360	10

** $p < 0.01$ level compared with unirradiated control tumours of comparable size (178 ± 2.0 mm^3).
* $p < 0.05$ level compared with unirradiated control tumours of comparable size (178 ± 2.0 mm^3).

Irradiation

Tumour-bearing legs of mice were irradiated with 15 MeV photons generated by a linear accelerator (Mevatron) at a dose rate of 2.5 Gy/min. The focus isocentre distance was 100 cm with field sizes of 3×2 cm^2 at the isocentre. The remainder of the animals' bodies 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.

The fractionated irradiation consisted of six fractions given on alternate days. The first fraction was applied, when the tumour reached a volume of approx. 170 mm^3. Previous experiments showed that this schedule results in a characteristic growth curve, where the initial tumour volume (at the start of irradiation) is reached approximately three weeks after the first fraction (see Fig. 1).

RESULTS

Irradiation

The growth curve of the ENE2 tumour during a course of fractionated irradiation with six times 6 Gy given over 11 days is shown in Fig. 1. During the early irradiation period an initial growth up to a relative volume of 1.6 ± 0.1 at day 7 was seen, followed by tumour shrinkage towards the end of the fractionated schedule. From day 18 onwards, the mean tumour volume was comparable to the volume measured at the beginning of the irradiation schedule.

PO_2 measurements

The xenografted neurogenic sarcoma (ENE2) investigated showed a significant fraction of hypoxic pO_2 values ≤ 2.5 mmHg. Details of the results of pO_2 measurements are shown in Table 1. At a tumour volume of approx. 120 mm^3, 60% of the pO_2 values were in the range 0 to 2.5 mmHg. With increasing volume up to 240 mm^3 a worsening of the oxygenation status was observed (70% $pO_2 \leq 2.5$ mmHg). Under ongoing irradiation, tumour volume increased up to 280 mm^3 on day 7 although no significant worsening of the oxygenation status occurred as compared to tumours with volumes of 180 mm^3 (i.e., the volume on the first days of irradiation). From day 21 to day 28, a significant improvement in the oxygenation status compared to unirradiated control tumours of comparable size (mean volume 178 ± 10 mm^3) was observed.

DISCUSSION

It is broadly accepted that the tissue oxygenation status in many experimental tumours deteriorates with enlarging tumour mass (7, 8). Therefore, an improvement in oxygenation ('reoxygenation') seen during or after cytotoxic treatment may occur solely as a result of tumour shrinkage. Indeed, Busse & Vaupel (8) found identical oxygenation profiles in cyclophosphamide-treated rat sarcomas and size-matched controls.

In our experimental system a size-matched comparison of tumours exposed to fractionated irradiation with control tumours showed a significant improvement in the oxygenation status. This finding therefore suggests that a fractionated irradiation leads to de facto improvement in tumour oxygenation, rather than to a volume-related increase.

Tumour hypoxia might be the result of either diffusion-limited oxygen supply to the cells (chronic hypoxia) or fluctuations in the microregional tumour perfusion (acute hypoxia). With the polarographic needle electrodes tissue hypoxia per se is assessed without differentiation between these different origins of O_2 deficiency. The electrodes solely measure the actual oxygenation status in a small tissue region. One additional problem might be the determination of tumour oxygenation in necrotic tissue areas where the oxygenation status is of no interest. These areas have to be excluded from pO_2.analyses in order to avoid misleading interpretation of the mean overall oxygenation status. However, in the present study histological staining showed that there were no necrotic areas present in the tumour during fractionated irradiation where the tumour volume was less than 300 mm^3. For this reason, only the oxygenation of viable tumour tissue was assessed in our investigation.

Only a few reports describe oxygenation status during fractionated irradiation. In a multifractionated study with relevant fraction sizes, Zywietz et al. found a complex pattern of changes in oxygenation in the R1h tumour (15). Improved oxygenation was found only in the early phase of fractionated irradiation, while doses above 45 Gy resulted in a considerable decrease in the median pO_2. When large fraction sizes were used however, an improvement in tumour oxygenation was observed in a mouse mammary carcinoma (16, 17). Following single-dose irradiation a two-phase change in oxygenation in transplantable murine tumours was described (18). An initial decrease in pO_2 with a nadir at about 6 h was followed by a slow reoxygenation reaching its maximum at 48 h after radiation. In contrast, Teicher et al. (19) observed an increase in hypoxia 24 and 48 h after a daily irradiation with 3 Gy over 5 days in a rat mammary carcinoma. In summary, these data illustrate the heterogeneity of the kinetics of reoxygenation of different tumours. The fact that the oxygenation status at the beginning of the irradiation period differed considerably in these studies should also be taken into account. The possible impact of the applied anaesthesia on the oxygenation status of xenografted tumours has been discussed previously (9).

The few available clinical data on oxygenation during a course of fractionated irradiation are not conclusive (20–22). Different fractionation schedules were used in these studies. A tendency towards an improved oxygenation could be shown when oxygenation was assessed by means of PET-imaging (positron emission tomography) of non-small-cell lung cancer (23). However, again, a marked heterogeneity of the serial changes in tumour oxygenation was observed. A comparison of size-matched tumours during a course of treatment, which is associated with tumour shrinkage, cannot be performed in the clinical setting. Our data illustrate, however, that factors other than tumour shrinkage do indeed lead to improved oxygenation after a course of fractionated irradiation.

The microenvironmental reasons for the improved oxygenation of the ENE2 tumour after fractionated irradiation remain unclear at present, but several possible mechanisms have to be taken into account. First, in a murine tumour model Olive et al. (4) found evidence of a decrease in oxygen consumption 6 hours after a single dose of 10 Gy. Since tumour hypoxia results from the disparity between the oxygen supply to the tumour and the cellular O_2 demand, a reduced O_2 consumption after irradiation might improve tumour oxygenation (24). Another possible mechanism seems to be the induction of angiogenesis by fractionated irradiation. Murata et al. (25) demonstrated that simultaneous administration of fractionated irradiation combined with an inhibitor of angiogenesis worsened the outcome of radiotherapy. The authors attribute these findings to inhibition of reoxygenation by the anti-angiogenetic agent. At the same time, several in vitro as well as in vivo studies have shown inhibition of neovascularization following tumour or surrounding normal tissue irradiation (26–30). This inhibition occurred not only when irradiation was applied as a single, large dose (30) but also when a fractionated schedule was used (27), so that a stimulating effect of low doses of irradiation does not seem likely either. Zywietz et al. (31) showed that when during fractionated irradiation the cumulative dose exceeds 45 Gy, the tumour microvasculature is irreversibly destroyed, resulting in a severe worsening of tumour oxygenation. Znati et al. (32) demonstrated that in experiments where a total dose of fractionated irradiation exceeding 10 Gy was given, the interstitial pressure decreased significantly and resulted in an improvement in tumour oxygenation. Finally, following irradiation an imbalance occurs between the tumour cell population and the stromal cells, so that the polarographic measurements might be dominated by oxygenation of stromal cells. However, histological sections of the irradiated ENE2 tumours showed a significant proportion of vital tumour cells on day 21 after irradiation. The relevance of these cells is proven by the fact that all tumours irradiated with six times 6 Gy lead to recurrences within longer follow-up periods.

In conclusion, the exact mechanisms responsible for improving tumour oxygenation during fractionated irradiation are still unknown. Even so, knowledge of changes in oxygenation during a course of radiation could allow optimization of the radiation time schedule in order to obtain a maximum therapeutic effect.

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