Radiobiological Hypoxia, Oxygen Tension, Interstitial Fluid Pressure and Relative Viable Tumour Area in Two Human Squamous Cell Carcinomas in Nude Mice During Fractionated Radiotherapy

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Acta Oncologica Vol. 40, No. 4, pp. 519–528, 2001

Very little is known about the correlation between the radiobiological hypoxic fraction (rHF) and other measures of tumour oxygenation during fractionated irradiation. In the present study the rHF is determined in untreated human FaDu and GL squamous cell carcinoma in nude mice and in tumours irradiated with 10 fractions in 2 weeks and 20 fractions in 4 weeks, using tumour control as the experimental endpoint. The results were compared with measurements of the pO_2, the interstitial fluid pressure (IFP) and the relative viable tumour area. In FaDu tumours the radiobiological hypoxic fractions (rHFs) before and during irradiation were not statistically different from 100%. Depending on the assumptions made for D_0, the rHFs of GL tumours were between 0.2 and 4% or 30 and 53%. The median pO_2 values were 2.8 mmHg for untreated FaDu tumours and 0.2 mmHg for GL tumours ($p < 0.001$). The median IFP values were 2.6 mmHg in FaDu and 5.3 mmHg in GL tumours ($p = 0.01$). No important changes in the pO_2 and IFP values were observed during fractionated irradiation. The relative viable tumour area during irradiation decreased by 83% in FaDu tumours ($p = 0.002$) and by 54% in GL tumours ($p = 0.003$). It is concluded that differences in rHF exist between FaDu and GL tumours before and during fractionated irradiation and that these differences are not reflected by pO_2 and IFP values and the relative viable tumour area.

Received 30 June 2000
Accepted 29 November 2000

It is generally accepted that the presence of hypoxic clonogenic cells in tumours and the capacity of these cells to reoxygenate during fractionated irradiation are important parameters in determining the outcome of radiation therapy (1). In experimental tumours the proportion of severely hypoxic clonogenic tumour cells, the so-called radiobiological hypoxic fraction, can be determined by the paired survival curve assay, or by comparison of tumour growth delay or tumour control doses after irradiation under ambient and clamped hypoxic conditions (1, 2). These assays are not applicable in the clinical situation. Important research efforts have therefore been directed to develop techniques that are suitable for identification of tumour hypoxia or related parameters in patients (3, 4). Currently, the largest database is available for determination of the intratumoral oxygen partial pressure (pO_2) using microelectrodes. Several clinical studies have shown that the oxygenation status of tumours determined by microelectrodes before radiotherapy is a prognostic factor for local treatment outcome (5–9) but also for the rate of distant metastases (10, 11), which suggests that tumour hypoxia is correlated not only with radioresistance but also with a more malignant tumour phenotype.

Animal experiments and clinical investigations using the pO_2 histograph have shown that the oxygenation status of tumours may change substantially during fractionated radiation therapy (12–19). Increased as well as decreased oxygenation can occur. So far, very little is known about

 ISSN 0284-186X

the influence of these changes on radiation response and about the correlation of radiobiological hypoxia with other measures of tumour oxygenation during fractionated radiotherapy. The present study addresses this question in two human squamous cell carcinomas in nude mice.

MATERIAL AND METHODS

Animals

The experiments were performed using specific pathogen-free 7–14-week-old male and female NMRI (nu/nu) mice and were approved in accordance with the German animal welfare regulations. Details of the experimental facilities have been given previously (20). To immunosuppress the nude mice further, they were given whole-body irradiation 1–2 days before tumour transplantation with 4 Gy using 200 kV x-rays (0.5 mm Cu; 1.3 Gy/min).

Tumours and transplantation

The FaDu and GL tumour lines have been described in detail elsewhere (20, 21). In brief, FaDu is a human hypopharyngeal squamous cell carcinoma line that in nude mice grows as an undifferentiated carcinoma. The median volume doubling time (VDT) of FaDu tumours between 100 mm^3 and 400 mm^3 was 3.6 days (10–90 percentiles 2.9; 4.7) in the experiments reported here. GL was established from a laryngeal carcinoma (21). Histologically, the tumours are moderately well differentiated keratinizing squamous cell carcinomas. In the present experiments the median VDT of GL tumours between 100 mm^3 and 400 mm^3 was 6.7 days (4.8; 8.8). LDH electrophoresis of FaDu and GL tumours showed a typical human isoenzyme pattern. Both tumour lines have been shown to evoke no or only a very low level of residual immune reactivity in nude mice (21, 22). For the experiments, source tumours of FaDu and GL were cut into small pieces and transplanted s.c. into the right hind leg of the anaesthetized mice (intraperitoneal (i.p.) injection of 120 mg/kg bodyweight ketamine and 16 mg/kg xylazine).

Determination of tumour volumes

Tumour diameters were measured twice weekly. Tumour volumes were determined by the formula of a rotational ellipsoid $\pi/6 \times a \times b^2$, where a is the longer and b the perpendicular shorter tumour axis.

Local tumour irradiation

Local irradiations were given under ambient or clamp hypoxic conditions using 200 kV x-rays (0.5 mm Cu; 1.1–1.2 Gy/min). Irradiation under ambient blood flow conditions was given without anaesthesia to air-breathing animals. During treatments, the tumour-bearing leg was positioned in the irradiation field using a foot-holder, distal to the tumour. For irradiation under homogeneous hypoxia, a heavy clamp was placed over the proximal thigh of the anaesthetized mice (120 mg/kg bodyweight ketamine i.p. and 16 mg/kg xylazine i.p.) 2 min before and during irradiation. The effectiveness of the clamp to interrupt the blood flow to the tumour-bearing hind leg has previously been assured by scintigraphy (23).

Irradiation protocols were started either on Mondays or Thursdays. The tumour volumes at start of irradiation were 100–250 mm^3. Seven experimental arms were used in both of the tumour models: graded single doses under (i) ambient blood flow and under (ii) clamp hypoxia; 10 equal fractions under ambient blood flow conditions applied within 2 weeks followed by graded top-up doses under (iii) ambient blood flow and under (iv) clamp hypoxia; 20 equal fractions under ambient blood flow conditions applied within 4 weeks followed by graded top-up doses under (v) ambient blood flow and (vi) clamp hypoxia; (vii) 30 equal fractions at graded dose levels applied under ambient blood flow within 6 weeks. The top-up doses in experimental arms 3-6 were applied 24 h (range 12–32) after the last ambient fraction. In previous experiments TCD50 (tumour control dose 50%) values of about 60 Gy for FaDu and 45 Gy for GL were obtained after irradiation with ambient 30 fractions within 6 weeks (20, 21). Based on these results the per fraction doses of 2.0 Gy for FaDu and 1.5 Gy for GL tumours were chosen for the present experiments.

The tumour control assays include data obtained from 989 irradiated and 84 untreated control tumours. The animals were randomized over the experimental matrix in groups of 3–4, aiming for about 10 animals (range 7–15 animals) for each of the 6–8 dose groups in each experimental arm. A further 184 animals were randomly allocated to measurements of pO_2 and IFP and to histology.

Determination of the TCD50 values, dose modifying factors and the radiobiological hypoxic fraction

The animals were observed for 120 days (FaDu) or 180 days (GL) after the end of treatment. This is sufficient time to detect virtually all regrowing tumours (20, 21). Dose-response curves for local tumour control, TCD_{50} values, i.e. the radiation dose necessary to control 50% of the tumours locally, and dose-modifying factors (DMF) for irradiation under ambient compared with clamp hypoxic conditions were calculated from the local control rates using a Poisson-based inactivation model and maximum likelihood analysis (24). For a total dose (D) the tumour control probability is

$$TCP = e^{-N_0 \times e^{-\rho \times D \times DMF}} \quad [1]$$

where N_0 is the number of tumour clonogens, ρ is their effective cellular radiosensitivity and DMF is the dose modifying factor for irradiation under ambient conditions compared with irradiation under clamp hypoxia. Censored animals were taken into consideration according to the method described by Walker & Suit (25). Determinations

of 95% confidence limits and comparisons of dose-response curves were performed using likelihood ratio statistics (24). All calculations were performed using the AlphaBetter v. 1.0 software (H.H. Dubben, University of Hamburg).

The rHF was estimated using the results of the maximum likelihood analysis and adapting the method described by Moulder & Rockwell (2):

$$rHF = e^{(TCD_{ambient} - TCD_{clamp})/D_{0,hypoxic}} \quad [2]$$

According to Equation [1] the tumour control doses obtained under ambient and clamp hypoxic conditions are related by

$$TCD_{clamp} = DMF \times TCD_{ambient} \quad [3]$$

Combination of Equation [2] and Equation [3] gives

$$rHF = e^{(TCD_{ambient} - DMF \times TCD_{ambient})/D_{0,hypoxic}} = e^{(1 - DMF) \times TCD_{ambient}/D_{0,hypoxic}} \quad [4]$$

The effective $D_{0,\ hypoxic}$ value can be derived from Equation [1] as

$$D_{0eff,hypoxic} = \frac{1}{\rho_{single\ dose,\ clamp}} = \frac{1}{(\rho_{single\ dose,\ ambient}/DMF_{single\ dose})} = \frac{DMF_{single\ dose}}{\rho_{single\ dose,\ ambient}} \quad [5]$$

Combination of Equation [4] and Equation [5] yields

$$rHF = e^{(1 - DMF) \times TCD_{ambient} \times \rho_{single\ dose\ ambient}/DMF} \quad [6]$$

and

$$rHF = e^{\frac{1 - DMF}{DMF_{single\ dose}} \times TCD_{ambient} \times \rho_{single\ dose\ ambient}} \quad [7]$$

For determination of the confidence interval of rHF, only the 95% confidence interval of DMF was considered.

pO_2 measurements

The oxygenation status was measured using a polarographic needle electrode (KIMOC-6650 pO_2-Histograph, Eppendorf, Germany) in 32 FaDu and 36 GL tumours. Unanaesthetized mice were placed in the irradiation room under the same set-up used for irradiation without control of haemodynamic parameters. The tip of the probe (0.3 mm diameter, diameter of the electrode 0.12 μm) was placed 0.5 to 1 mm below the surface of the tumour and allowed to equilibrate. The polarization cable was connected to an Ag/AgCl EKG anode, attached to the tail of the mice. The measurements were carried out with forward steps of 0.5 mm followed immediately by a retraction of 0.2 mm. A median of 6 parallel tracks (range 3–15) was measured in each tumour, resulting in a median of 121 (range 51–331) values. The pO_2 measurements in the tumours were followed by measurements in s.c. tissue of the ipsilateral leg in 44 animals. The probes were calibrated before and after each measurement. The values were corrected for temperature determined in the individual tumours and for air pressure. Histograms were generated using the pO_2-POOL v.1.2 software (Eppendorf, Hamburg, Germany). Negative pO_2 values are considered to be an indicator of the quality of measurements (26, 27) and have not been excluded in this study.

Measurement of the interstitial fluid pressure (IFP)

The wick-in-needle-technique (28) was used to measure the interstitial fluid pressure. A 24-gauge needle with a 2–3 mm-long sidehole 3 mm distant from the tip was filled with 3–4 nylon surgical sutures (6-0, Ethicon, Germany). The needle was connected to a piezoelectric pressure transducer (ST 3000 Smart Transducer, Honeywell, Germany) by a polyethylene tube filled with heparinized isotone saline. The system was calibrated using a water column. For measurements, the needle was placed in the centre of the tumour and the pressure was sampled in a time interval of 5 s. To test the correct connection between the interstitial fluid and the system, the tube was compressed after 6–10 min, when a stable fluid pressure was detected and decompressed after 12–20 min. The data evaluations were performed using an interactive code running under IDL (v. 3.6. 1c, Research Systems Inc., USA) which performs a non-linear least squares fit of the data in a user-defined time interval to the function

$$p(t) = p_0 + p_1 e^{-\lambda t},$$

where p_0 is the pressure to be measured, p_1 is the additional pressure due to compression ($p_1 > 0$) or decompression ($p_1 < 0$) and λ is the pressure relaxation coefficient. Tumours were excluded from analysis if the IFP value differed by more than 30% from the value after compression or after decompression. This occurred in 10 out of 32 FaDu tumours and in 25 out of 46 GL tumours.

Determinations of relative viable tumour area

The relative viable tumour area was determined in haematoxylin- and eosine-stained 5 μm central sections of 20 shock frozen FaDu and 18 GL tumours using a Zeiss Axioplan 2 microscope (Carl Zeiss, Jena, Germany) equipped with a Maerzhäuser scanning stage and

a colour video camera MC-3254 (Sony Corporation, Köln, Germany). The scanning process and the image analysis were performed using the KS300 image analysis software (Kontron Elektronik, Eching, Germany) and an objective with 10-fold magnification resulting in a composite image of 64 to 360 visual fields for each central tumour section. In the composite images, the total tumour area and the viable subareas were delineated for calculation of the relative viable area.

Statistics

PO_2 values, IFP values and relative viable areas in the different groups were compared using the Mann-Whitney U-test or the Kruskal-Wallis test and a commercial software package (SPSS for Windows 9.0, SPSS Inc., 1999).

RESULTS

Tumour volume during fractionated irradiation

The volumes of FaDu and GL tumours irradiated with 30 fractions within 6 weeks are shown in Fig. 1. The median volume increased during the initial 1–2 weeks of treatment in FaDu tumours and during the first 2–3 weeks in GL tumours. Regression rates varied widely in FaDu tumours and were more homogeneous in GL tumours. The volumes of tumours used for determination of pO_2, IFP and relative viable tumour area were in the same range as the those of tumours used for the irradiation assays (data not shown).

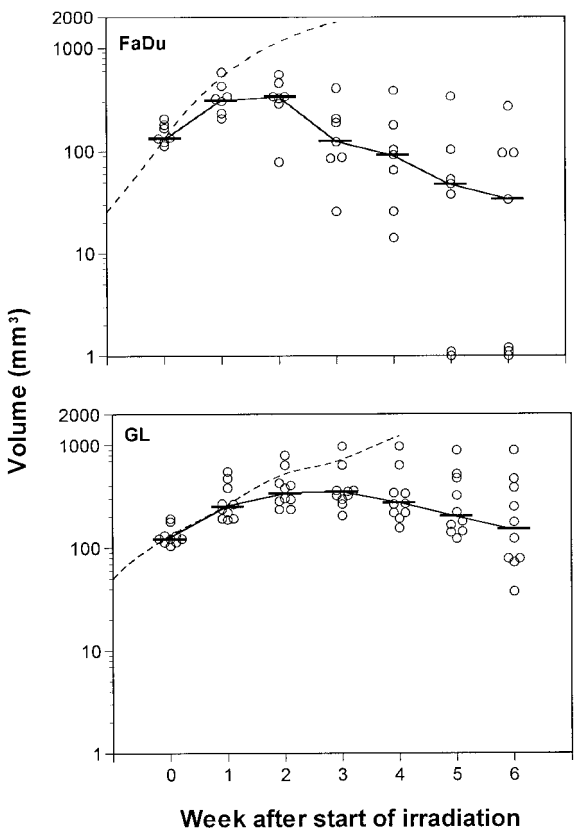

Fig. 1. Volume changes of FaDu tumours and GL tumours during irradiation with 30 fractions within 6 weeks. Doses per fraction were 2.0 Gy in FaDu tumours and 1.5 Gy in GL tumours. Each symbol represents an individual tumour, the horizontal bars indicate the median values, the dashed lines the median growth curve of untreated FaDu and GL tumours.

Radiation tumour control experiments

In Fig. 2 we present the observed local tumour control rates and the calculated tumour control probabilities for FaDu and GL tumours irradiated with graded single doses or top-up doses after 10 ambient fractions in 2 weeks or 20 fractions in 4 weeks. Single doses and top-up irradiation were applied under ambient conditions or under clamp hypoxia. In an additional experimental arm, both tumours were irradiated with 30 ambient fractions within 6 weeks. The TCD_{50} values and the DMFs obtained from the comparison of irradiation under both ambient and clamp hypoxic conditions are listed in Table 1. In FaDu tumours the DMFs were 1.1 in previously untreated tumours, 1.1 after 10 fractions and 0.7 after 20 fractions. The 95% confidence intervals of the DMFs overlapped widely and all included 1.0. In GL tumours the DMFs were 1.5, 1.3 and 1.8. In contrast to FaDu tumours, none of the 95% confidence intervals of the DMFs included 1.0, i.e. in all three comparisons GL tumours were controlled at significantly lower doses when irradiated under ambient conditions compared with irradiation under clamped hypoxia.

Measurements of pO_2

The pooled histograms of the oxygen distributions in normal s.c. tissues and in FaDu and GL tumours before and during fractionated irradiation are presented in Fig. 3. In normal s.c. tissues, a symmetric but relatively flat distribution with a median value of 42 mmHg was found (Table 2). Likely owing to pressure artefacts that can occur when the loose skin of mice is pushed forward by the probe, 11% of the values were below 10 mmHg. Compared with normal s.c. tissue, the histograms for FaDu and GL tumours were skewed and shifted to significantly lower pO_2 values ($p < 0.001$ for both tumours). The median pO_2 values were 2.8 mmHg in untreated FaDu tumours and 0.2 mmHg in untreated GL tumours, indicating severe hypoxia in both tumour models (Table 2). Also during fractionated irradiation, very low oxygenation was found in both tumour models (Fig. 3, Table 2). Owing to the large number of measurements in the pooled histograms, the small differences in the pO_2 values between FaDu and GL tumours and within the tumour models during fractionated irradiation were statistically significant ($p < 0.001$ for all comparisons). Comparing the fraction of values below 5 mmHg determined for individual tumours (Fig. 4), no significant differences were observed between untreated FaDu and GL

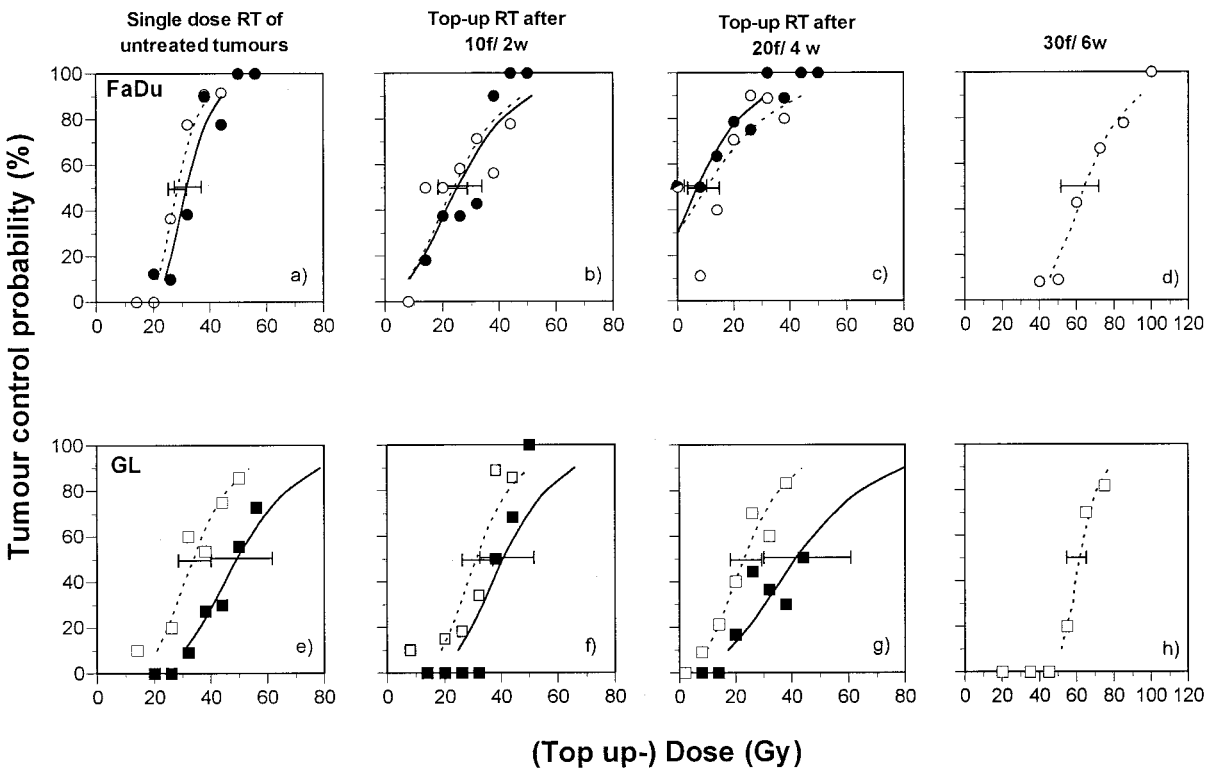

Fig. 2. Local control rates of FaDu and GL tumours irradiated with single doses or top-up doses under ambient (open symbols) or clamped hypoxic (closed symbols) conditions. Single doses or top-up doses were applied to previously untreated tumours (a, e), after 10 ambient fractions in 2 weeks (b, f) or after 20 ambient fractions in 4 weeks (c, g). Doses per fraction were 2.0 Gy in FaDu tumours and 1.5 Gy in GL tumours. Panels (d) and (h) show the results of irradiations with 30 ambient fractions in 6 weeks. Each symbol represents an average group of 10 animals. Tumour control probabilities were calculated by maximum likelihood analysis according to Equation [1]. Error bars represent 95% confidence intervals of TCD_{50}. Note: 7/14 FaDu tumours were locally controlled after 20 × 2 Gy without top-up irradiation (half-filled symbol, panel c). The data of these tumours were included in the analysis (compare Table 1).

Table 1

TCD_{50} and DMF values for irradiation of FaDu and GL tumours in the different treatment arms

Tumour	Irradiation schedule	TCD_{50} ambient [95% CI] (Gy)	TCD_{50} clamp hypoxia [95% CI] (Gy)	DMF [95% CI]
FaDu	Single dose	28.4 [25;32]	31.8 [27;37]	1.12 [0.97;1.30]
	Top-up after 10 fractions in 2 weeks	23.6 [18;29]	25.3 [18;34]	1.07 [0.79;1.43]
	Top-up after 20 fractions in 4 weeks	10.1 [5;17] [1]	7.3 [4;12] [1]	0.72 [0.40;1.18] [1]
	30 fractions in 6 weeks	64.0 [56;77]	–	–
GL	Single dose	33.5 [28;41]	49.2 [39;62]	1.47 [1.19;1.84]
	Top-up after 10 fractions in 2 weeks	30.9 [26;37]	40.8 [32;52]	1.32 [1.05;1.67]
	Top-up after 20 fractions in 4 weeks	22.8 [18;30]	41.5 [30;61]	1.82 [1.32;2.67]
	30 fractions in 6 weeks	61.7 [58;69]	–	–

[1] 7/14 tumours were locally controlled after 20 × 2 Gy without top-up irradiation (compare Fig. 2c). The data of these tumours were included in the analysis. When these animals were excluded from the analysis, TCD_{50} values of 14.9 Gy [8;21] for irradiation under ambient conditions and 11.5 Gy [7;17] for irradiation under clamp hypoxia were obtained. The DMF was 0.77 [0.51; 1.11].

tumours ($p = 0.08$). In FaDu tumours the fraction of pO_2 values below 5 mmHg was significantly higher after 10 fractions compared with untreated tumours and tumours irradiated with 20 fractions ($p = 0.005$), while no changes were observed in GL tumours ($p = 0.87$).

Measurements of IFP

The IFP values of FaDu and GL tumours are shown in Fig. 5. The median IFP values of 2.6 mmHg for untreated FaDu tumours and 5.3 mmHg for untreated GL tumours were significantly different ($p = 0.01$). In FaDu tumours no significant changes in IFP were observed during fractionated irradiation ($p = 0.39$), while in GL tumours the IFP values after 20 fractions were slightly but significantly lower than in untreated tumours and after 10 fractions ($p = 0.04$).

Determinations of the relative viable tumour area

The relative viable area in central sections of FaDu and GL tumours decreased significantly during fractionated irradiation (Fig. 6). For FaDu the median values were 94% in untreated tumours, 17% after 10 fractions and 11% after 20 fractions ($p = 0.002$). The corresponding values for GL tumours were 81%, 41% and 27% ($p = 0.003$). Whereas in FaDu tumours the decrease in the relative viable area was caused by an increase in the relative necrotic area, an increase in the large keratinization figures with little necrosis was observed in GL tumours.

DISCUSSION

The aim of our study was to compare the proportion of radiobiologically hypoxic cells in two human squamous cell carcinomas in nude mice before and during fractionated radiation therapy with measurements of pO_2, IFP and relative viable tumour area. Assuming an oxygen enhancement ratio of 2.5 to 3 for mammalian cells and tissue (29), about 2.5 to 3-fold lower TCD_{50} values for irradiation of fully oxygenated tumours are expected compared with irradiation under clamp hypoxia (2). In Fig. 2 it is shown that this was not the case for FaDu and GL tumours, demonstrating that radiobiologically hypoxic cells were present in both tumour models. For previously non-irradiated FaDu tumours and for tumours irradiated with 10 fractions within 2 weeks and 20 fractions within 4 weeks, the dose-response curves for local tumour control under ambient and clamp hypoxic conditions are almost superimposed (Fig. 2). The rHFs, estimated according to Equation [7] from the tumour control data, were 60% for untreated FaDu tumours, 78% after 10 fractions and 100% after 20 fractions (Table 3). These estimates suggest that radiobiological hypoxia in FaDu tumours increases during

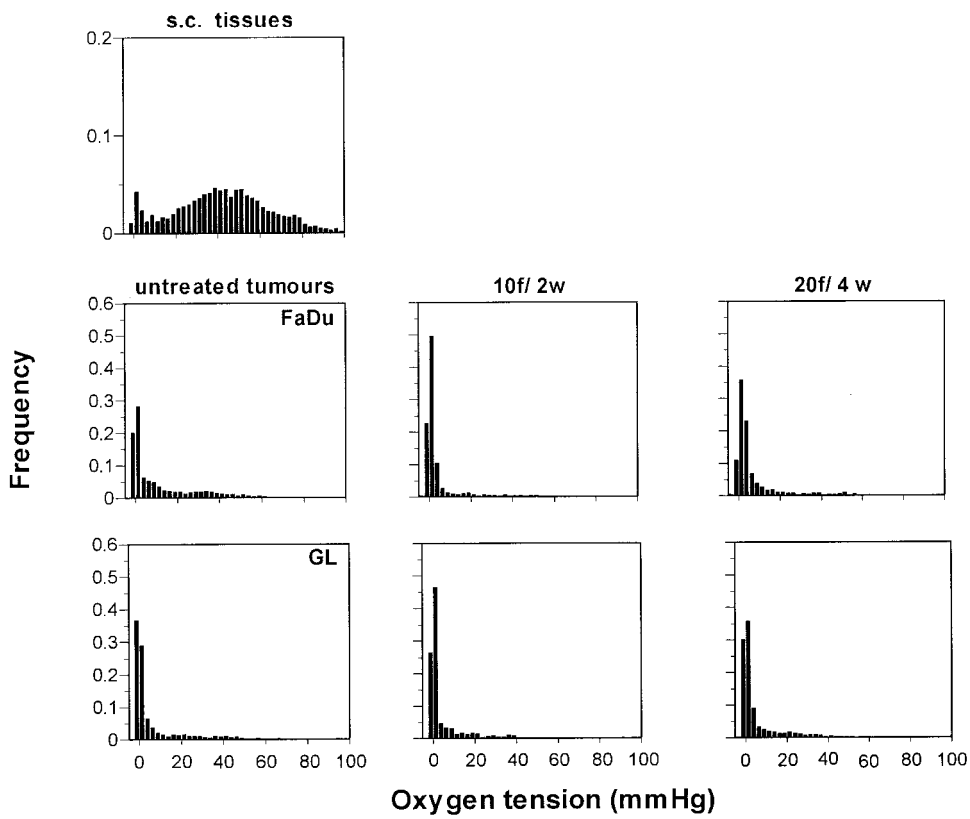

Fig. 3. Pooled pO_2 histograms of normal s.c. tissues, untreated FaDu and GL tumours and of tumours irradiated with 10 fractions in 2 weeks or 20 fractions in 4 weeks. Doses per fraction were 2.0 Gy in FaDu tumours and 1.5 Gy in GL tumours.

Table 2

Summary of the pooled pO_2 values obtained for untreated FaDu and GL tumours and for tumours treated with 10 fractions in 2 weeks or 20 fractions in 4 weeks

Tumour/Tissue	Irradiation schedule	No. of mice	No. of pO_2 values	pO_2 [mmHg]				Percent values		
				Median	Mean	10 percentile	90 percentile	<10 mmHg	<5 mmHg	<2.5 mmHg
S.c. tissue	Untreated	44	2940	42.3	42.6	9.0	73.4	11.0	7.8	5.4
FaDu	Untreated	11	1298	2.8	11.7	−0.5	38.7	65.7	55.3	48.9
	10 fractions	11	1455	0.7	4.3	−0.5	14.8	88.0	83.8	73.3
	20 fractions	10	917	2.7	7.1	−0.1	22.0	81.6	70.8	47.7
GL	Untreated	11	1457	0.2	6.7	−2.0	27.3	80.7	75.6	70.2
	10 fractions	12	1598	0.8	5.1	−1.0	19.5	83.4	76.8	72.1
	20 fractions	13	1672	1.1	6.4	−1.0	24.0	80.5	74.6	66.0

fractionated irradiation. However, since the confidence intervals overlap widely, this increase is not statistically significant. Furthermore, since the 95% confidence intervals of all three rHFs include 100%, it can not be excluded that all clonogenic cells in FaDu tumours before and during fractionated irradiation were radiobiologically hypoxic. It is difficult to conceive that tumour cells may survive for several weeks at the extremely low oxygen partial pressures necessary to induce complete radiobiological hypoxia. It seems more likely that although the proportion of radiobiologically hypoxic clonogenic cells in FaDu tumours was high, it was lower than 100%, and that the oxygenation status of the individual clonogenic cells tended to fluctuate, e.g. as a consequence of the reopening of transiently closed vessels (30) or decreased oxygen consumption (31). In contrast to FaDu tumours, all dose-response curves for GL tumours after irradiation under ambient conditions were significantly different from those obtained under clamp hypoxia. None of the DMFs included 1.0, indicating that a significant proportion of the clonogenic tumour cells before and during fractionated

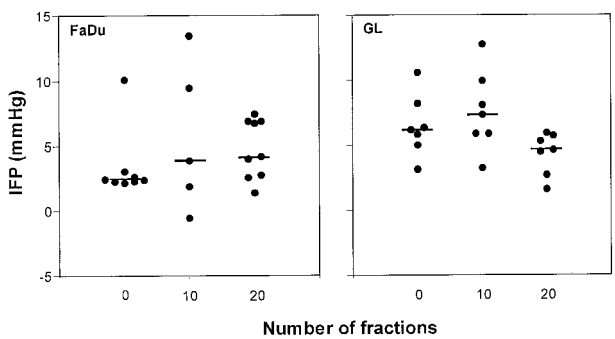

Fig. 5. Interstitial fluid pressure of untreated FaDu and GL tumours and of tumours irradiated with 10 fractions in 2 weeks or 20 fractions in 4 weeks. Doses per fraction were 2.0 Gy in FaDu tumours and 1.5 Gy in GL tumours. Each symbol represents an individual tumour, horizontal bars indicate the median values.

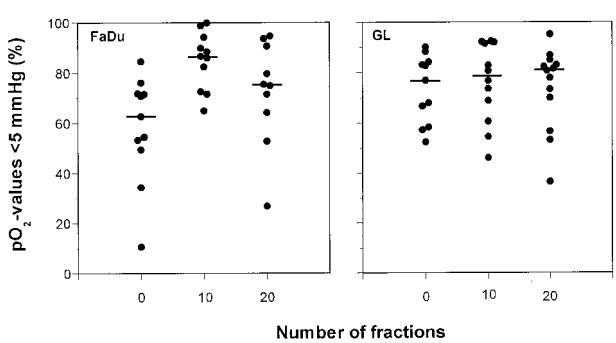

Fig. 4. Fraction of pO_2 values below 5 mmHg of untreated FaDu and GL tumours and of tumours irradiated with 10 fractions in 2 weeks or 20 fractions in 4 weeks. Doses per fraction were 2.0 Gy in FaDu tumours and 1.5 Gy in GL tumours. Each symbol represents an individual tumour; horizontal bars indicate the median values.

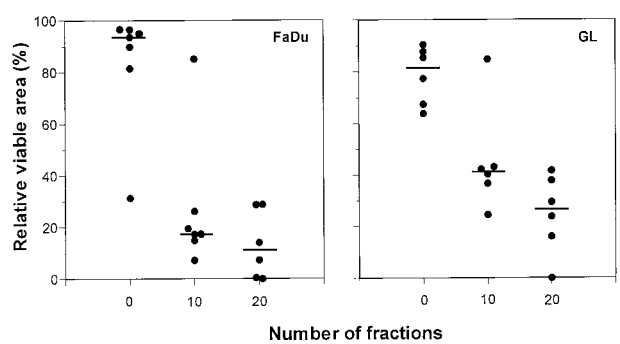

Fig. 6. Relative viable area determined in central sections of untreated FaDu and GL tumours and of tumours irradiated with 10 fractions in 2 weeks and 20 fractions in 4 weeks. Doses per fraction were 2.0 Gy in FaDu tumours and 1.5 Gy in GL tumours. Each symbol represents an individual tumour; horizontal bars indicate the median values.

irradiation were radiobiologically not hypoxic. The rHF was 36% for untreated GL tumours, 53% after 10 fractions and 30% after 20 fractions (Table 3), indicating that GL tumours reoxygenated during fractionated irradiation.

The estimates of the rHFs listed in Table 3 depend critically on the assumption that the term ρ determined from the tumour control data Equation [1] accurately describes the cellular radiation sensitivity, i.e. that $1/\rho$ corresponds to D_0 Equation [5]. However, this is likely not the case since determinations of the cellular radiation sensitivity from dose-response relationships for local tumour control are extremely sensitive to statistical influences and intertumoral heterogeneity (32). The D_0 value under hypoxic conditions (D_{0hyp}) determined in the present study according to Equation [5] for FaDu tumours was 6.6 Gy. This is about 2-fold higher than the D_{0hyp} value of 3 Gy determined for FaDu cells in vitro and in vivo (22), supporting that the values listed in Table 3 overestimate the true rHFs. To obtain an estimate of the low limit of the rHF in FaDu tumours, the data were recalculated according to Equation [2], assuming a D_{0hyp} value of 3 Gy. The resulting values were 32% for untreated FaDu tumours, 57% after 10 fractions and 100% after 20 fractions (Table 3). The D_{0hyp} value obtained according to Equation [5] for GL tumours was 16 Gy, which is unrealistically high. Unfortunately no independent value of D_{0hyp} for GL cells is available. Assuming that a D_{0hyp} value of 3 Gy also applies to GL, the rHF was estimated to be 0.5% in untreated GL tumours, 3.7% after 10 fractions and 0.2% after 20 fractions. Whatever the correct values of D_{0hyp} for FaDu and GL tumours are, comparison of the rHF within each tumour model is independent of the value chosen for the calculations. Furthermore, only the assumption of unreasonably high D_0 values for GL would overrule the conclusion that FaDu tumours are radiobiologically more hypoxic than GL tumours. It is interesting to note that the apparent differences in rHFs between FaDu and GL tumours do not translate into significantly different TCD_{50} values after irradiation with 30 fractions in 6 weeks (Table 1). This finding suggests that determination of rHF alone is not sufficient to fully explain the response of FaDu and GL tumours to fractionated irradiation.

The low oxygenation status of FaDu and GL tumours (Fig. 3, Table 2) is consistent with results obtained by others showing that murine tumours or human tumour xenografts in mice are markedly hypoxic (27, 33). Furthermore, the observation that the pO_2 values did not reflect the different rHFs in FaDu compared with GL tumours before and during a course of fractionated irradiation is in line with the overall conclusions from other studies. Clear-cut correlation between pO_2 measurements and radiobiological hypoxia was found in some (34, 35) but not all (36) experiments when the oxygenation level within one tumour model was systematically varied. No correlation was found when comparisons were made across different tumour models (37, 38) or between individual tumours of the same line (38, 39). In two other studies pO_2 was measured during fractionated irradiation in animal tumours (12, 16). Zywietz et al. (12) found a considerable decrease in oxygenation of R1H sarcomas beginning 3 weeks after start of irradiation when tumours were already regressing. In contrast, the median pO_2 values in OTS tumours decreased during a 2.5-week course of fractionated irradiation and recovered after the end of treatment (16). Whether these changes correlate with radiobiological hypoxia and with the outcome of radiotherapy is presently unknown but what makes this an interesting question is that changes in tumour oxygenation during radiotherapy have also been observed in patients (13–15, 17–19).

In agreement with findings in other experimental tumours and in patient tumours, positive IFP values were determined in untreated FaDu and GL tumours (40–42).

Table 3

Radiobiological hypoxic fraction estimated for untreated FaDu and GL tumours and for tumours irradiated with 10 fractions in 2 weeks and 20 fractions in 4 weeks

Tumour	Irradiation schedule	Radiobiological hypoxic fraction (%) [95% CI]	
		Calculated from Equation [7] [1]	Calculated from Equation [2] assuming $D_{0hyp} = 3$ Gy
FaDu	Untreated	60 [27;100]	32 [5;100]
	10 fraction in 2 weeks	78 [21;100]	57 [3;100]
	20 fractions in 4 weeks	100 [76;100]	100 [54;100]
GL	Untreated	36 [16;67]	0.5 [0.01; 12]
	10 fractions in 2 weeks	53 [26;91]	3.7 [0.1;60]
	20 fractions in 4 weeks	30 [8;63]	0.2 [<0.01;9]

[1] ρ values for irradiation with single dose under clamp hypoxia were 0.170 for FaDu tumours and 0.094 for GL tumours.

Initially, it has been suggested that an elevated IFP value may be inversely correlated with the oxygenation status of tumours (41, 43). However, later studies showed that no general correlation exists between IFP and pO_2 in solid tumours (42, 44). The present study found slightly, but significantly, higher IFP values for GL tumours which have a lower proportion of radiobiologically hypoxic cells compared with FaDu tumours, indicating that measurements of IFP in these tumour models did not correlate with rHF (Fig. 5, Table 3). Compared with untreated tumours, no changes in IFP were observed in FaDu and GL tumours during irradiation, with the exception of a small decrease in GL tumours after 20 fractions. These results do not correspond well to clinical observations in cervix carcinoma (41, Znati et al. cited in 45) and experimental studies on LS174T human colon carcinomas in nude mice, where the IFP decreased substantially after single doses and fractionated doses above 10 Gy (45). The reasons for these differences between different tumour models and the biological relevance of this phenomenon are presently unclear.

During fractionated irradiation the relative viable tumour area decreased significantly in FaDu and GL tumours (Fig. 5). These changes were not associated with significant changes of the rHF. This may well be due to the wide confidence intervals of the radiobiological data. However, it also seems possible that the microenvironments in the remaining viable tumour areas that are expected to host the surviving clonogenic cells did not change significantly during irradiation. An important caveat resulting from the histological findings is that, during fractionated irradiation, most of the pO_2 and IFP values were measured in necrotic areas of FaDu tumours and keratinizing areas of GL tumours. Based on the assumption that necrotic tissue contributes exclusively to the portion of low pO_2 values, it has been suggested that results of pO_2 measurements should be corrected for the histologically determined necrotic fraction (46). While this procedure has been shown to abrogate the volume dependence of pO_2 values in a C3H mammary carcinoma and to reduce this dependence in three other murine tumour models (47), it does not seem appropriate to use such a correction during irradiation of FaDu and GL tumours. Both tumours have a low oxygenation status already before the start of treatment when the necrotic or keratinizing fraction is small, and correction for necrotic fraction would result in an apparent improvement in oxygenation during treatment which does not correspond with the radiobiological data.

In conclusion, a different proportion of radiobiologically hypoxic cells before and during fractionated irradiation was demonstrated in two human squamous cell carcinomas in nude mice. This difference was not reflected in the pO_2 and IFP measurements and changes in the relative viable area of the tumours. An important caveat is that the pO_2 and IFP values during treatment were obtained mainly in necrotic areas of FaDu tumours and keratinizing areas of GL tumours and can therefore not be considered representative for the micromilieu of the surviving clonogenic cells. Furthermore, any extrapolation of the results obtained on rapidly growing, poorly oxygenated FaDu and GL tumours to better oxygenated rodent tumours or to slowly growing tumours in patients needs to be tempered with great caution.

ACKNOWLEDGEMENTS

The excellent technical assistance of Dorothea Pfitzmann and Carmen Hausmann is gratefully acknowledged. The authors thank Dr Vet. Peter Nelz and Dr Vet. Rainer Kumpf and their team for breeding and maintenance of high-quality nude mice. This study was carried out within the framework of the BIOMED II concerted action, *Development of Methods for Rapid Analysis of Tumor Oxygenation to Allow Treatment Stratification*, funded by the European Community. The work was supported by grants Ba 1433/1-2 and Ba 1433/1-3 awarded by the Deutsche Forschungsgemeinschaft.

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