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REOXYGENATION IN A C3H MOUSE MAMMARY CARCINOMA

The importance of chronic rather than acute hypoxia

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The role that chronic and acute hypoxia play in tumour reoxygenation after irradiation was investigated in a C3H mouse mammary carcinoma grown in the feet of female CDF1 mice. Tumours at 200 mm^3 in size were locally irradiated with a priming dose of 20 Gy and then at various times after given a range of radiation doses under normal or clamped conditions. Local tumour control was determined 90 days later from which the tumour hypoxic fractions were calculated. Untreated tumours contained 23% hypoxic cells. Immediately after 20 Gy this increased to 52% and by 24 h had fallen to 10%. These reoxygenation experiments were repeated, giving either nicotinamide (1000 mg/kg; i.p. injected 30 min before each irradiation) to remove acute hypoxia, or carbogen breathing (for 5 min before and during irradiation) to decrease chronic hypoxia. With nicotinamide the normal hypoxic fraction was reduced to 7%, but after irradiation it had risen to 46% and by 24 h there was full reoxygenation with a value of 5% being observed. Carbogen breathing also decreased the normal hypoxic fraction to 6%, and immediately after irradiation this was increased to 38%. However, by 24 h it was still elevated at around 23%. These results suggest that chronic rather than acute hypoxia is necessary for reoxygenation in this tumour.

Radiation resistant hypoxic cells have been directly identified in most animal solid tumours, with the values ranging from $< 1\%$ of the total viable cell population to well over 50% (1, 2). There is now good evidence to suggest that hypoxic cells exist in human tumours and that they may be one of the major reasons for failure to locally control certain tumour types with conventional radiation therapy (3). Originally, this hypoxia was considered to be chronic in nature arising from a diffusion limitation in oxygen (4), but later it was suggested that hypoxia could also be acute (5, 6). This has now been confirmed and shown to be the result of transient stoppages in tumour blood flow (7, 8).

Experimental studies have shown that immediately after irradiation there is actually an increase in the tumour hypoxic fraction, due to preferential killing of the aerobic cells, but that this increase is followed by a return to pretreatment levels generally within several hours (9–11). This so-called 'reoxygenation' of tumours has been reported to occur in both rodent tumours and human tumour xenografts (9–13), but not only have the mechanisms responsible not been established, it is also not known whether the effect occurs primarily in the chronic or acutely hypoxic population.

Previous studies have demonstrated that allowing mice to breathe carbogen gas, or injecting the animals with nicotinamide, substantially reduced the hypoxic fraction in a C3H mouse mammary carcinoma (14, 15). Moreover, while the effect of carbogen in this tumour model appeared to be essentially the result of a decrease in chronic hypoxia, with nicotinamide the effect was primarily due to a reduction in the level of acute hypoxia (16). In the current

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study, we have measured the 'reoxygenation profile' in this C3H mammary carcinoma under normal conditions and after treatment with carbogen breathing or nicotinamide, in an effort to determine whether it is the chronically or acutely hypoxic cells that play the major role in reoxygenation.

Material and Methods

Animal and tumour model. All experiments were performed using a C3H mouse mammary carcinoma, the derivation and maintenance of which has been previously described (17). The tumour was implanted in the right rear foot of 10- to 14-week-old female CDF1 mice and treated when it had reached 200 mm^3 in volume.

Drug and gas treatment. Nicotinamide (Sigma Chemical Co., St. Louis, MO) was dissolved at a concentration of 50 mg/ml in sterile saline (0.9% NaCl) immediately before each experiment and injected intraperitoneally (i.p.) at a constant injection volume of 0.02 ml/g body weight. The drug was generally injected 30 min prior to every irradiation, although for the 6-min interval between the priming dose and the second radiation treatment, the nicotinamide was only given 30 min before the priming radiation dose. For treatment with carbogen (95% O_2 + 5% CO_2) three non-anaesthetized mice were separately restrained in lucite jigs and then each jig individually gassed with carbogen, from a triple outlet nozzle, for 5 min before and during every irradiation. The flow rate into the nozzle was 2.5 l/min.

Radiation treatment. Irradiations to 3 animals at a time were given with a conventional therapeutic 250 kV x-ray machine at a dose rate of 2.3 Gy/min, dosimetry being achieved by use of an integrating chamber. Non-anaesthetized mice were restrained in lucite jigs and their tumour-bearing legs exposed and loosely attached to the jig with tape, without impairing the blood supply to the foot. Tumours only were irradiated, the remainder of the animal being shielded by 1 cm of lead. To secure homogeneity of the radiation dose, tumours were immersed in a water bath, maintained at 25°C, with about 5 cm of water between the x-ray source and the tumour. Irradiation of hypoxic tumours involved clamping the tumour-bearing leg for 5 min before and during irradiation, clamping being achieved by constriction of the blood flow using a rubber tube tightened around the leg.

Hypoxic fraction determination. Tumours were given a priming dose of 20 Gy and then either immediately, 6 min, 6 h or 24 h later irradiated again with different doses to produce full dose–response curves under normal or clamped conditions. Tumour response to treatment was assessed by observing mice at weekly intervals and estimating the percentage of animals in each treatment group showing local tumour control within 90 days. Hypoxic fractions were then calculated from the normal and clamped data, for each condition, by direct analysis as previously described (18).

Results

Examples of the radiation dose–response curves for this C3H mouse mammary carcinoma obtained after single irradiations under normal or clamped conditions are shown in Fig. 1. For air-breathing animals the TCD-50 dose (the radiation dose required to produce local tumour control in 50% of treated animals) was found to be 53 Gy (Fig. 1a). This was significantly increased to 63 Gy by clamping. Nicotinamide had no effect on the response of

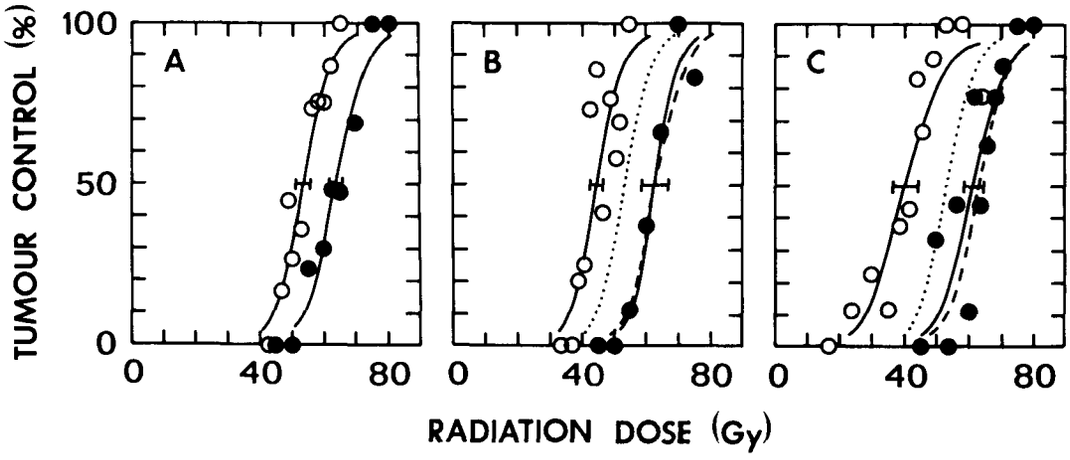

Fig. 1. The radiation response of a C3H mouse mammary carcinoma to single dose radiation treatments under normal (○) or clamped (●) conditions. A) Tumours irradiated under air-breathing conditions. B) Mice i.p. injected with 1 000 mg/kg nicotinamide 30 min before irradiation. C) Animals allowed to breathe carbogen gas for 5 min before and during irradiation. Each data point represents the average of 12 mice/group and the error bars show 95% confidence intervals on the TCD-50 values. The dotted and dashed curves in panels B and C represent the air and clamped curves respectively, from panel A. Lines fitted by logit analysis.

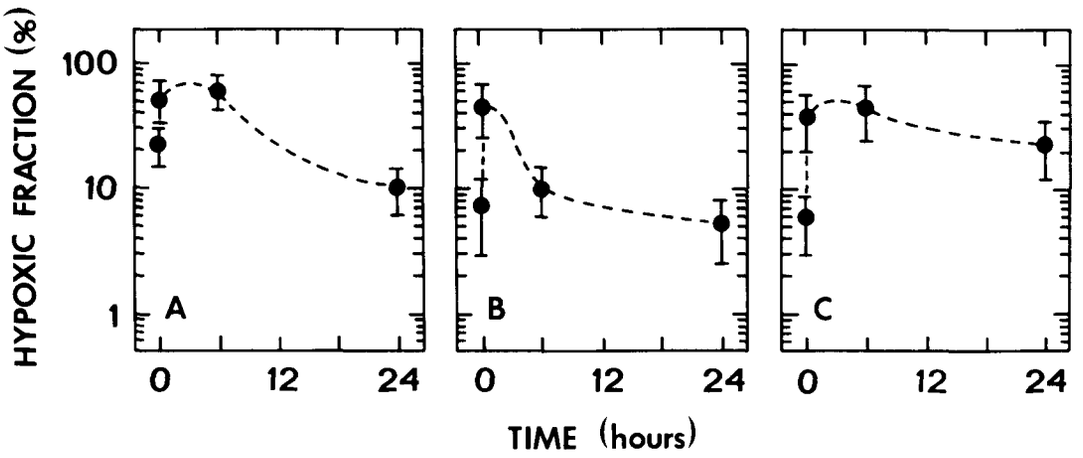

Fig. 2. Change in hypoxic fraction with time after irradiation. Tumours were locally irradiated with 20 Gy and then at different times given various radiation doses under normal and clamped conditions to produce full dose–response curves similar to those shown in Fig. 1. Hypoxic fractions were determined by direct analysis of the dose–response data. A) Tumours irradiated under air-breathing conditions. B) Mice i.p. injected with 1 000 mg/kg nicotinamide 30 min before each irradiation. C) Animals allowed to breathe carbogen gas for 5 min before and during each radiation treatment. Errors show 95% confidence intervals. Lines represent the best fit to the data by eye.

clamped tumours, but it decreased the air-breathing response, giving a TCD-50 value of 44 Gy (Fig. 1b). Similarly carbogen breathing had no effect under clamped conditions, but enhanced the response seen in air-breathing animals such that a TCD-50 value of 40 Gy was obtained (Fig. 1c).

The hypoxic fraction values calculated from the data of Fig. 1 and for all the other conditions in which a priming dose of 20 Gy was given at different times before graded radiation doses are shown in Fig. 2. For radiation alone the hypoxic fraction significantly increased immediately after irradiation and by 24 h had returned to slightly below the pretreatment value (Fig. 2a). Nicotinamide clearly reduced the starting hypoxic fraction, but the 'reoxygenation profile' basically paralleled that seen for radiation alone, with a substantial increase immediately after irradiating and then a return to the starting level by 24 h (Fig. 2b). Under carbogen-breathing conditions the starting hypoxic fraction was also reduced and a significant increase in hypoxia was seen within minutes of giving the radiation, but, by 24 h, little or no reoxygenation was observed (Fig. 2c).

Discussion

Reoxygenation was first demonstrated in the late 1960's (9, 10) and since then it has been shown to occur in a variety of rodent tumours and human tumour xenografts (11–13). However, the mechanisms responsible for this phenomenon have not been clearly established. Suggested mechanisms include reduced oxygen consumption by irradiated cells, rapid cell loss leading to tumour shrinkage, migration of cells from hypoxic to oxygenated areas and improvements in tumour blood flow (11). Most of these mechanisms will be expected to improve the oxygenation status of diffusion-limited chronically hypoxic cells, although if the improved tumour blood flow is really the result of reopening of transiently closed vessels then it would be the perfusion-limited acutely hypoxic cells that would be reoxygenated.

Recent studies in the SCCVII tumour suggested that it was a decrease in the level of acute hypoxia that primarily accounted for reoxygenation in this tumour (19). Indeed, additional studies in the same tumour model confirmed that after irradiation there was a decrease in transient blood vessel closure (20) which adds further support to the role of acutely hypoxic cells in the reoxygenation process. That latter study did, however, show an improvement in the diffusion distance of oxygen, an effect which would be expected to influence the chronically hypoxic cell population as well. The reoxygenation in the SCCVII was also very rapid (19), being almost complete within a few hours, which again is consistent with reoxygenation primarily occurring as a result of the reopening of transiently closed blood vessels because, at least in the SCCVII tumour, this transient closure is considered to be of relatively short duration (21). The time at which the hypoxic fraction returns to normal levels after irradiation in our C3H mammary carcinoma is not entirely clear, although the results from our current and previously published studies (22) seem to indicate that it does so at some time between 4 and 12 h after irradiation. This longer time interval than that seen with the SCCVII tumour would be more consistent with an effect on the chronic rather than acutely hypoxic population. Previous studies in this C3H tumour have also shown that not only can nicotinamide or carbogen breathing enhance radiation damage by decreasing the levels of radiation resistant hypoxic cells (14, 15) but, while

nicotinamide primarily affected the acutely hypoxic population, the principal action of carbogen was on the chronically hypoxic tumour cells (16). When we eliminated the acutely hypoxic cells with nicotinamide we observed a similar 'reoxygenation profile' as seen with radiation alone. Yet, removing the chronically hypoxic cells, by allowing the mice to breathe carbogen, resulted in little or no reoxygenation during the 24-h period after irradiation. These results suggest that the reoxygenation observed in this tumour model, following a large single dose of radiation, is primarily the result of improving the oxygenation status of the radiation resistant chronically, rather than acutely, hypoxic population.

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