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The effects of carbogen and nicotinamide on intravascular oxyhaemoglobin saturations in SCCVII and KHT murine tumours

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Summary Considerable effort has been focused on devising methods for manipulating tumour oxygenation and thereby improving tumour radiosensitivity. The combination of nicotinamide and carbogen has been proposed to oxygenate both chronically and acutely hypoxic cells in tumours. However, results have varied markedly with both tumour model and measurement technique. The current objectives were (1) to determine whether changes in radiosensitivity following oxygen manipulation correlated with changes in tumour oxygenation and (2) to assess whether oxygenation was preferentially improved in specific tumour microregions. Using two murine tumour lines, the SCCVII carcinoma and the KHT sarcoma, tumour intravascular HbO_2 saturations were measured cryospectrophotometrically following nicotinamide, carbogen or the combination. Generally, nicotinamide had minor effects on oxygenation, arguing against a substantial effect on acute hypoxia, while carbogen and the combination produced marked and equivalent improvements in oxygen availability. These results demonstrate that changes in tumour radiosensitivity may not agree with corresponding changes in oxygenation, even within a given tumour model, and that the efficacy of a given manipulative agent may vary substantially with tumour line. One possible explanation for these findings is that different subpopulations of clonogenic *vs* non-clonogenic cells may be oxygenated by alternative treatments.

Keywords: oxygen; radiosensitivity; tumour oxygenation; hypoxia; manipulation

Over the past few decades, numerous clinical and experimental studies have attempted to improve tumour radioresponse through the enhancement of tumour oxygenation. Several recent reports have indicated that either carbogen breathing (95% oxygen, 5% carbon dioxide) or nicotinamide (NIC) administration, alone or in combination, can effectively radiosensitise tumours in mice (Rojas, 1991; Chaplin *et al.*, 1993; Simon *et al.*, 1993; Siemann *et al.*, 1994; Dorie *et al.*, 1994; Martin *et al.*, 1994). It is generally believed that carbogen breathing improves response by increasing the amount of oxygen physically dissolved in the blood, thereby increasing the distance the oxygen is able to diffuse from the blood vessels to the tumour cells, and possibly by increasing tumour blood flow (Kruuv *et al.*, 1967). NIC, on the other hand, has been suggested to increase tumour oxygenation by reducing temporal fluctuations in tumour blood flow (Chaplin *et al.*, 1990, 1991; Horsman *et al.*, 1990) in addition to increasing tumour blood flow (Horsman *et al.*, 1989; Stone *et al.*, 1992; Kelleher and Vaupel, 1993).

Although previous studies have generally shown the greatest enhancement of radiosensitivity following the combination of NIC and carbogen, results are highly variable among different tumour models and laboratories. For example, in the CaNT mouse mammary carcinoma (Kjellen *et al.*, 1991), NIC demonstrated no significant effect, while carbogen and the combination showed equivalent enhancements. In the KHT sarcoma, the treatments had similar effects when delivered under optimum conditions (Siemann *et al.*, 1994). Finally, in the SCCVII carcinoma, carbogen and NIC were equivalent, while the combination was superior (Chaplin *et al.*, 1993). Measurements of alterations in tumour oxygenation following these agents have also varied markedly with tumour line (Lee and Song, 1992; Fenton and Boyce, 1993; Kelleher and Vaupel, 1993; Horsman *et al.*, 1995; Martin *et al.*, 1994).

A key question is whether a relationship exists between direct measures of tumour oxygenation and corresponding determinations of tumour radiosensitivity. Since tumour radiosensitivity is commonly calculated from the ratio of the fraction of anoxic clonogenic tumour cells to the fraction of total clonogenic cells (Moulder and Rockwell, 1984), it follows that if substantially different proportions of clonogenic and non-clonogenic tumour cells are oxygenated by the alternative treatments, direct measures of tumour oxygenation will not correlate with changes in radiosensitivity (Fenton *et al.*, 1995). Thus, although clear relationships may be demonstrated within specific tumour lines (Rofstad *et al.*, 1988; Horsman *et al.*, 1993), attempts to define similar correlations across tumour lines have proven unsuccessful (Rofstad *et al.*, 1988; Horsman *et al.*, 1995; Martin *et al.*, 1994).

The objective of the current study was to define further the physiological mechanisms responsible for the inter-tumour differences in response to these two agents. Using two murine tumour lines, the SCCVII carcinoma and the KHT sarcoma, changes in tumour intravascular oxyhaemoglobin (HbO_2) profiles were quantified following NIC, carbogen or the combination. The primary aims were (1) to determine whether previously reported changes in radiosensitivity, following growth and oxygen manipulation, could be explained solely on the basis of changes in oxygen availability and (2) to assess whether oxygen availability within specific regions of the tumour, i.e. interior *vs* periphery, is preferentially improved following the different treatments.

The major advantage of using tumour intravascular HbO_2 saturations as an index of tumour oxygenation is that microregional heterogeneities in oxygen availability can be spatially defined with an unequalled precision. While these measurements are not as direct a gauge of tumour radiosensitivity as electrode measurements of local oxygen pressures (pO_2), they may better reflect localised changes in tumour blood flow as well as the ability of manipulative agents to eliminate localised regions of anoxia. Neither anaesthesia nor physical restraint of the animals is required for these measurements.

Materials and methods

Mice and tumour models

The KHT tumour (Kallman *et al.*, 1967), a sarcoma maintained *in vivo*, and the SCCVII tumour, a squamous cell carcinoma maintained by alternate *in vivo/in vitro* passage (Olive *et al.*, 1985), were used in all experiments. Using 6- to 8-week-old female C_3H/HeJ mice (Jackson Laboratories, Bar

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Harbor, ME, USA), 2×10^5 KHT cells were inoculated intramuscularly into the hind limb or subcutaneously into the flank. For the SCCVII tumours, 2×10^5 cells were inoculated intramuscularly into the hind limb. Tumours were selected for cyrospectrophotometric analysis when they reached a volume of between 180 and 900 mm^3.

Drugs

Nicotinamide (Sigma, St Louis, MO, USA) was freshly prepared before each experiment in sterile phosphate-buffered saline and injected intraperitoneally at 1000 mg kg^{-1} 1 hr before tumour freezing.

Carbogen breathing

Mice were confined to plastic jigs (~50 cm^3 volume) and exposed to carbogen at a flow rate of ~4 l min^{-1} for 7 min before tumour freezing.

Tumour freezing and cryospectrophotometric determination of HbO_2 saturations

Approximately 2–3 h before freezing, tumours were first shaved and a depilatory agent applied to accelerate tumour freezing. Following each treatment, the mice were cervically dislocated and the tumours immediately quick frozen using a liquid nitrogen-cooled copper block and stored in cryotanks. Tumour sectioning and sampling procedures were as previously described (Fenton and Boyce, 1993). Four cross-sections of the tumour were exposed using a cooled scalpel blade in a dry ice–ethanol bath at −73°C. For HbO_2 determinations, the exposed tumour surfaces were analysed on a liquid nitrogen-cooled microscope stage. Approximately 95 blood vessels (diameter ≥ 6 μm) were systematically selected per tumour, the spatial positions of the blood vessels were recorded using stage micrometers and intravascular HbO_2 saturations were determined cryospectrophotometrically as previously described (Fenton and Gayeski, 1990). Briefly, HbO_2 saturations were calibrated as a function of reflected light intensity at three discrete wavelengths, based on the spectral differences between oxy- and deoxyhaemoglobin. Since optical density also varies as a function of haemoglobin concentration and light pathlength, the intensities at the three wavelengths were combined to normalise the measurement and to cancel out these dependencies, thus allowing vessels of widely varying haematocrit to be analysed using a single calibration curve.

Statistical considerations

The percentage of vessels containing $\geq 25\%$ HbO_2 was calculated for each of the tumours of a given treatment group, and the means at each distance class were compared using the unpaired Student *t*-test. Differences were considered significant for $P<0.05$.

Results

Tumour intravascular HbO_2 saturations vary as a function of both tumour size and spatial location within the tumour volume. In the figures to follow, results are presented in terms of the percentage of vessels $\geq 25\%$ HbO_2 saturation. Although this is a somewhat arbitrary HbO_2 cut-off, this type of index is expected to correlate more closely with corresponding changes in the radiobiological hypoxic fraction than mean or median HbO_2 levels, as has been discussed in a previous theoretical study (Fenton *et al.*, 1995).

Figure 1 illustrates the percentage of vessels with $\geq 25\%$ HbO_2 saturation as a function of distance of the vessels from the tumour surface for small (mean = 315 mm^3) and medium (mean = 733 mm^3) volume KHT tumours. The percentage with $\geq 25\%$ HbO_2 was significantly lower for the medium tumours than for the small tumours for the first two distance classes ($P = 0.002$ and $P = <0.001$). At distances of greater than 2 mm from the tumour surface, however, the percentage with $\geq 25\%$ HbO_2 was not significantly different between small and medium tumours. Figure 2 compares percentage with $\geq 25\%$ HbO_2 for KHT tumours implanted into two different sites. Tumour oxygen availability was significantly lower for the subcutaneous implantation site in the flank in relation to the intramuscular site in the hind leg at three of the four distance classes ($P = 0.002$, 0.007, 0.122 and 0.039).

Changes in the percentage with $\geq 25\%$ HbO_2 following NIC administration, carbogen breathing or the combination treatment compared with the untreated volume-matched KHT controls are summarised in Figure 3. The values obtained in NIC-treated animals were significantly different from those in untreated controls at only one of the four distance classes ($P = 0.962$, 0.018, 0.067 and 0.729). For carbogen breathing, the percentage with $\geq 25\%$ HbO_2 was significantly higher than untreated at each of the first three distance classes ($P = 0.003$, 0.006, 0.003 and 0.299). For the combination treatment, the percentage with $\geq 25\%$ HbO_2 was significantly higher at all distances ($P = 0.005$, 0.0003, 0.0005 and 0.022). Finally, the combination treatment was significantly higher than the NIC at two of the distances ($P = 0.023$, 0.163, 0.093 and 0.010) while not significantly different from the carbogen-breathing treatment at any distance.

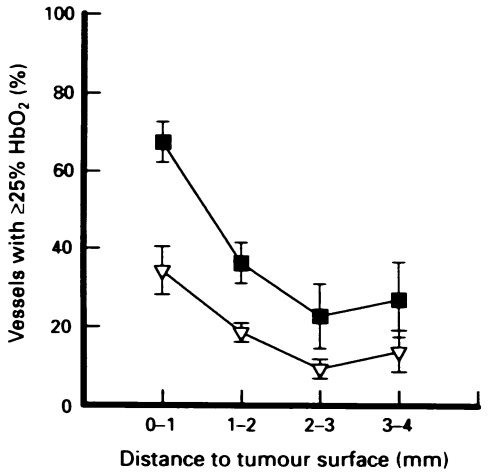

Figure 1 Percentage of vessels with $\geq 25\%$ HbO_2 (mean ± s.e.m.) as a function of distance from the tumour surface. Small-volume KHT tumours (■, 315 ± 46 mm^3, $n = 6$) are contrasted with medium volume tumours (▽, 733 ± 42 mm^3, $n = 6$).

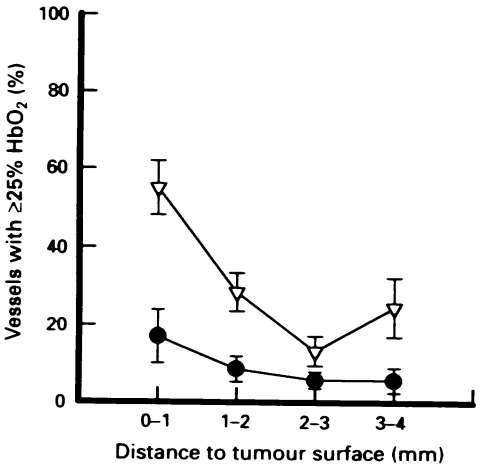

Figure 2 Percentage of vessels with $\geq 25\%$ HbO_2 (mean ± s.e.m.) as a function of distance from the tumour surface. Intramuscular KHT tumour implantations (▽, 529 ± 58 mm^3, $n = 8$) are contrasted with subcutaneous implantations (●, 591 ± 40 mm^3, $n = 7$).

Corresponding results for the SCCVII tumours are shown in Figure 4. For the SCCVII tumours, NIC produced a significant increase in percentage with $\geq 25\%$ HbO_2 only at the most peripheral distance class ($P = 0.019$). For carbogen breathing, the percentage with $\geq 25\%$ HbO_2 was again significantly increased for each of the first three distance classes ($P = 0.0001$, 0.0004, 0.0042 and 0.264). For the combination treatment, percentage with $\geq 25\%$ HbO_2 was significantly higher only for the first two distance classes ($P = 0.003$, 0.013, 0.060 and 0.842). As with the KHT, no significant differences were found between the carbogen breathing and the combination treatment. In contrast to the KHT, however, no significant differences were observed between the NIC and the combination treatment for the SCCVII tumours.

To illustrate more clearly overall variations between the two tumour lines, Figure 5 presents the mean percentage with $\geq 25\%$ HbO_2 averaged over the four distance classes. Overall the percentage of vessels with $\geq 25\%$ HbO_2 was calculated by taking the mean percentage with $\geq 25\%$ HbO_2 at each distance class, weighted by the corresponding tumour volume associated with that distance class for each tumour. This weighting compensates for the fact that the inner distance classes sample from smaller concentric shells of the tumour volume than at the outer distance classes. Trends for the KHT and SCCVII tumours generally parallel the previous figures. For the KHT, significant overall differences from untreated were found only for the carbogen and combination treatments ($P = 0.006$ and 0.0008 respectively). Carbogen and the combination were also significantly higher than NIC alone ($P = 0.080$ and 0.020). For the SCCVII, carbogen and the combination were again significantly higher than untreated ($P = 0.0003$ and 0.013), NIC was different from carbogen ($P = 0.015$) but not the combination ($P = 0.206$).

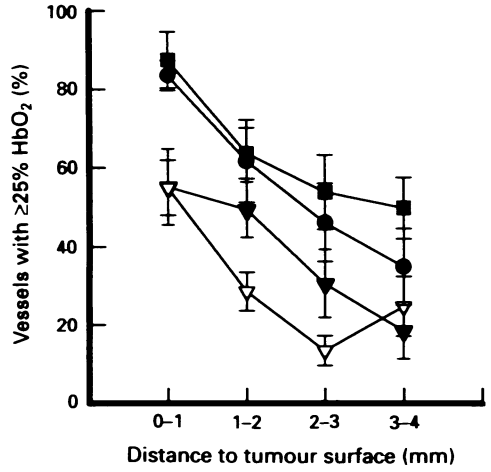

Figure 3 Percentage of vessels with $\geq 25\%$ HbO_2 (mean ± s.e.m.) as a function of distance from the tumour surface for KHT i.m. implanted tumours. Untreated controls (▽, 529 ± 58 mm^3, $n = 8$) are contrasted with NIC administration (▼, 530 ± 65 mm^3, $n = 9$), carbogen breathing (●, 483 ± 27 mm^3, $n = 7$) or the combination of both (■, 430 ± 25 mm^3, $n = 7$).

Discussion

As a rule, the radiobiological hypoxic fraction (HF) of experimental tumours increases with increasing tumour volume within a given tumour line (Rofstad *et al.*, 1988; Horsman *et al.*, 1995). For KHT tumours grown in the leg muscle, the HF increased from ~10% in 0.1–0.2 g tumours to 25–35% for 0.7–1.0 g tumours (Fenton and Siemann, 1994). As the HFs increased, the corresponding percentage with $\geq 25\%$ HbO_2 decreased, as expected. But this decrease in tumour oxygen availability was not uniformly distributed over the tumour volume. Significant changes in tumour oxygenation were confined to blood vessels within 2 mm of the tumour surface. For vessels closer to the centre of the tumour, HbO_2 levels were quite low to begin with and were not significantly decreased with growth.

HbO_2 levels also varied substantially with implantation site. Although the radiobiological HFs of leg- and flank-implanted KHT tumours were similar (Fenton and Siemann, 1994), the percentage with $\geq 25\%$ HbO_2 for the leg tumours was substantially higher. Despite essentially equal HFs for flank or leg KHT tumours, Horsman *et al.* (1995) also found 2–4 times higher median pO_2 values for leg than for flank tumours. This increase in oxygen availability in the leg tumours may relate to differences in the host vasculature or relative blood flow in the different sites, among other factors (Young *et al.*, 1979; Vaupel and Mueller-Klieser, 1986). Since the leg muscle would be expected to have higher oxygen requirements than the subcutaneous flank, it stands to reason that leg tumours should also tend to be better vascularised and oxygenated. It remains difficult to explain why the HbO_2 or pO_2 levels do not correlate with the hypoxic fractions between the two sites, although, if the tumour cells surrounding the low-HbO_2 vessels in the flank are predominantly non-clonogenic, the resultant oxygen profiles would be lower in this site than in the leg without a corresponding increase in the radiobiological hypoxic fraction (Fenton *et al.*, 1995).

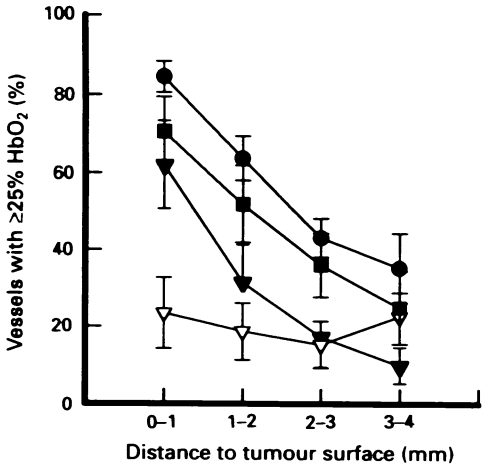

Figure 4 Percentage of vessels with $\geq 25\%$ HbO_2 (mean ± s.e.m.) as a function of distance from the tumour surface for SCCVII i.m. implanted tumours. Untreated controls (▽, 503 ± 62 mm^3, $n = 8$) are contrasted with NIC administration (▼, 456 ± 43 mm^3, $n = 7$), carbogen breathing (●, 423 ± 30 mm^3, $n = 7$) or the combination of both (■, 460 ± 18 mm^3, $n = 7$).

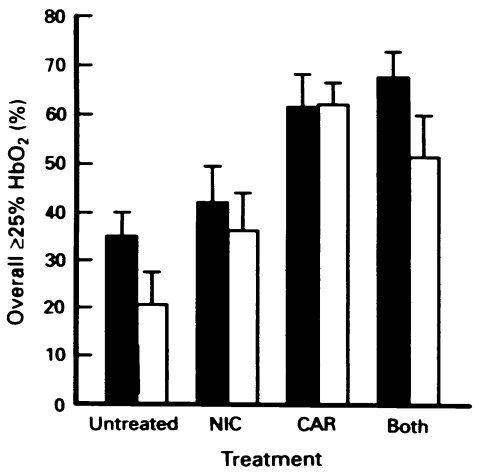

Figure 5 Overall percentage vessels with $\geq 25\%$ HbO_2 saturation (mean ± s.e.m.) as a function of treatment for KHT (■) and SCCVII (□) tumours. 'Both' denotes the combination of NIC and carbogen, and numbers of tumours are as described for Figures 4 and 5.

A number of additional mechanisms have also been suggested previously (Rofstad *et al.*, 1988).

Carbogen breathing and the combination of carbogen plus NIC provided the greatest enhancement of tumour oxygen availability for both the KHT and the SCCVII tumours, and this enhancement was not significantly different between the two treatments. HbO_2 levels were improved for only one distance class following NIC for either KHT or SCCVII tumours. Although these results suggest that tumour radiosensitivity should also tend to be highest following the carbogen and combination treatments, published changes in radioresponse following these agents do not support this conclusion. Siemann *et al.* (1994) found that NIC and carbogen were equally effective at radiosensitising KHT tumours. In addition, the radiosensitivity enhancement resulting from the combination of NIC and carbogen was equivalent to either agent alone if delivered under optimum conditions. These findings differ from the current results primarily in respect of the effect of NIC administration, in that tumour radiosensitivity is increased without a marked change in tumour oxygenation.

For subcutaneous SCCVII tumours, Chaplin *et al.* (1993) also reported that carbogen and NIC produced equivalent improvements in radioresponse. In contrast to the KHT tumours, however, the combination treatment produced a greater enhancement of radiation response than either agent alone. The radiosensitisation following either NIC or carbogen was essentially the same, despite markedly different HbO_2 profiles. Since the combination treatment produced the same effect on radiosensitivity as the fully aerobic response in the SCCVII tumours (Chaplin *et al.*, 1993), and since the interior of these tumours remains very poorly oxygenated following the combination treatment, it follows that these interior tumour cells must be non-clonogenic to begin with and therefore irrelevant in terms of radiotherapy.

In a study using C3H mouse mammary carcinomas (Horsman *et al.*, 1995), changes in the fraction of pO_2 readings ≤ 5 mmHg generally correlated with the corresponding HFs following different oxygen manipulations. However, changes in tumour pO_2 levels following NIC and carbogen were again inconsistent. Tumours were much better oxygenated following carbogen than NIC, in spite of equivalent radiosensitivities following either treatment. As was the case for the HbO_2 measurements, pO_2 levels following NIC were not different from the air-breathing controls. In the studies of Martin *et al.* (1994), the relationship between pO_2 profiles and surviving fraction varied markedly among tumour lines. In one, cell survival remained essentially constant between NIC and carbogen treatments, in spite of substantial differences in pO_2 profiles. Surprisingly, NIC increased tumour oxygenation more than carbogen in all three lines. Additional studies have shown similar variability between tumour lines in the radioresponse following NIC, carbogen or the combination (Kjellen *et al.*, 1991; Simon *et al.*, 1993; Dorie *et al.*, 1994).

The current disparity between HbO_2 results and HF changes can be rationalised if it is assumed that NIC and carbogen oxygenate different subpopulations of clonogenic *vs* non-clonogenic tumour cells. While direct measures of tumour oxygenation cannot distinguish between clonogenic and non-clonogenic cells, the radiobiological HF depends directly on the relative fractions of anoxic and oxygenated clonogenic cells contained in the tumour (Moulder and Rockwell, 1984; Fenton *et al.*, 1995). As described more fully in a previous theoretical study (Fenton *et al.*, 1995), HF determinations can vary independently of directly measured changes in tumour oxygenation within the non-clonogenic subpopulation. Thus, if a higher proportion of non-clonogenic *vs* clonogenic cells is oxygenated following a given treatment, higher oxygen levels will be observed in relation to the corresponding reduction in cell survival.

Carbogen breathing is believed to improve tumour oxygenation primarily by increasing the diffusion distance of the oxygen from the blood vessels – thus the clonogenic anoxic cells at the edge of the previously oxygenated regions will be the first cells oxygenated. As the oxygen diffuses further, anoxic cells may be reached that have been without oxygen long enough to become non-clonogenic while remaining viable. Increasing the diffusion distance enough to oxygenate these non-clonogenic cells results in an 'overkill' phenomenon in which no further enhancement of tumour radiosensitivity is realised in spite of the increased oxygen availability. Since the HF decreases in conjunction with the increase in tumour oxygenation following carbogen, an increase in oxygen delivery to some proportion of the clonogenic anoxic cells must also be occurring.

Previous work has suggested that NIC may act in part by reducing intermittent fluctuations in tumour blood flow (Chaplin *et al.*, 1990). If this is the case, some of the tumour cells that are oxygenated following NIC will be those that were previously exposed to intermittent flow. It is reasonable that such acutely hypoxic cells would more likely remain clonogenic than cells that have been beyond the diffusion distance of oxygen for extended periods of time (chronic hypoxia). This implies that differences in the frequency of intermittent blood flow between tumour lines could directly influence the correlation between tumour oxygenation and radioresponse for these same tumour lines. Other evidence that NIC may act by reducing intermittent flow is provided by Lee and Song (1992), who found that the effect of NIC administration was greater in large tumours than in small. They also attribute these differences to the fact that larger tumours are more likely to have intermittently opening blood vessels than small tumours (Chaplin *et al.*, 1986; Trotter *et al.*, 1989; Lord *et al.*, 1993).

But do such differences in intermittent flow exist between the KHT and SCCVII tumour lines? For SCCVII tumours, the number of blood vessels opening and closing over a 20 min period has been reported to be 10.3% (Chaplin *et al.*, 1990), based on dual-staining techniques. In the KHT tumours, only 4% intermittently flowing vessels were observed (Fenton and Siemann, 1994). Thus, in either case, a reduction in intermittent flow may have a relatively minor overall effect on tumour oxygenation. Although NIC-induced improvements in HbO_2 levels were observed only in the peripheral vessels, Chaplin *et al* (1990) report that flow intermittencies are, in contrast, more prevalent in central tumour regions. However, their dual-staining techniques are only capable of measuring whether a given blood vessel contains active blood flow – not whether this flow is functional in terms of oxygen delivery (Fenton and Boyce, 1993). Thus changes in dual-staining intermittency for blood vessels containing very low HbO_2 levels may or may not relate to either tumour oxygenation or radioresponse. Since overall HbO_2 levels were not substantially improved following NIC for either the KHT or the SCCVII, it appears unlikely that a NIC-induced decrease in acute hypoxia is the predominant mechanism for altering radioresponse in either tumour model. This suggests that the beneficial effects of combining NIC and carbogen may not involve a decrease in acutely hypoxic cells in all cases.

Finally, why are SCCVII and KHT HbO_2 levels increased so much more with carbogen than with NIC, despite similar effects on radiosensitivity? One possibility, is that, although both treatments may increase oxygen delivery to the clonogenic anoxic subpopulation, the carbogen may also tend to oxygenate some population of non-clonogenic anoxic cells. Following NIC, radioresponse increases with a minimal increase in oxygen availability, suggesting a redistribution of oxygen from non-clonogenic oxygenated cells to clonogenic anoxic cells. If NIC also reduces intermittent flow, then oxygen that was previously distributed to the non-clonogenic cells most distant from the previously open vessels will now be diverted to the clonogenic cells surrounding the newly opened vessels. Thus, less flow is now distributed among more vessels. This tends to decrease the oxygen diffusion distance while increasing oxygen delivery to the closest (and presumably clonogenic) tumour cells.

A final possible explanation for the NIC-induced radiosensitisation in the absence of significantly higher oxygen levels

is the possibility that NIC may act by inhibiting radiation-induced potentially lethal damage repair. While this result has been demonstrated *in vitro*, further studies have suggested that repair inhibition is not the principle mechanism responsible for *in vivo* tumour radiosensitisation (Horsman *et al.*, 1987).

In summary, it is clear that response to carbogen and NIC manipulation varies substantially with tumour line in terms of both tumour radiosensitivity and direct measures of tumour oxygenation. The dilemma is that no currently available method exists for predicting whether or not a correlation will exist between the two measures in a given tumour. Thus defining an 'optimal' manipulative agent solely on the basis of its ability to increase tumour oxygenation may lead to erroneous conclusions. Contrary to some previous findings (Martin *et al.*, 1994), alterations in tumour oxygenation within a given tumour line may not be reflective of corresponding changes in tumour radiosensitivity if significantly different fractions of non-clonogenic tumour cells are involved. Further work is needed both to describe the underlying physiological basis for the observed discrepancies and to discover more representative methods for estimating tumour radioresponse following oxygen manipulation. In addition, better methods for quantifying intermittencies in 'functional flow' are needed such that more subtle changes in tumour perfusion may be recognised.

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