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

BM Fenton

Department of Radiation Oncology, University of Rochester Medical Center, Rochester, New York 14642, USA.

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 oxygenation are related with changes in tumour oxygenation and (2) to assess whether oxygenation was preferentially improved in specific tumour micro-regions. Using two murine tumour lines, the SCCVII carcinoma and the KHT sarcoma, tumour intravascular HbO₂ 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 radiosensitivity 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., 1993). 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 (HbO₂) 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 HbO₂ saturations as an index of tumour oxygenation is that micro-regional 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 (pO₂), 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 C57/HeJ mice (Jackson Laboratories, Bar

Correspondence: BM Fenton, Box 704, University of Rochester Medical Center, Rochester, New York 14642, USA
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Harbor, ME, USA). 2 x 10^5 KHT cells were inoculated intra-
muscularly into the hind limb or subcutaneously into the
flank. For the SCCVII tumours, 2 x 10^5 cells were inoculated in
tramuscularly 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 pre-
bred 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^2 volume) and
exposed to carbogen at a flow rate of \~ 41 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 pre-
viously 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 \(-73^\circ\)C. For HbO_2 deter-
ninations, the exposed tumour surfaces were analysed on a
liquid nitrogen-cooled microscope stage. Approximately 95
blood vessels (diameter \~ 6 \mu m) 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 haemacrit to be analysed using a single
calibration curve.

Statistical considerations
The percentage of vessels containing \~ 25\% HbO_2 was cal-
culated 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 \~ 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
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 \~ 25\%
HbO_2 saturation as a function of distance from the tumour
surface. Figure 2 compares percentage with \~ 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 \~ 25\% HbO_2 following NIC
drugs administration, carbogen breathing or the combination
treatment as 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, 0.967 and 0.729). For
carbogen breathing, the percentage with \~ 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 \~ 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, 0.993 and 0.010) while not significantly
different from the carbogen-breathing treatment at any
distance.

Figure 1 Percentage of vessels with \~ 25\% HbO_2 (mean \pm
s.e.m.) as a function of distance from the tumour surface.
Small-volume KHT tumours \(\bullet, 315 \pm 46 \text{ mm}^3, \ n = 6\) are contrasted
with medium volume tumours \(\triangledown, 733 \pm 42 \text{ mm}^3, \ n = 6\).

Figure 2 Percentage of vessels with \~ 25\% HbO_2 (mean \pm
s.e.m.) as a function of distance from the tumour surface. Intramu-
scular KHT tumour implantations \(\triangledown, 529 \pm 58 \text{ mm}^3, \ n = 8\)
are contrasted with subcutaneous implantations \(\bullet, 591 \pm 40
\text{ mm}^3, \ n = 7\).
Corresponding results for the SCCVII tumours are shown in Figure 4. For the SCCVII tumours, NIC produced a significant increase in percentage with $\geq 25\%$ HbO₂ only at the most peripheral distance class ($P = 0.019$). For carbogen breathing, the percentage with $\geq 25\%$ HbO₂ 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 $\geq 25\%$ HbO₂ 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 $\geq 25\%$ HbO₂ averaged over the four distance classes. Overall the percentage of vessels with $\geq 25\%$ HbO₂ was calculated by taking the mean percentage with $\geq 25\%$ HbO₂ 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 ($P = 0.206$).

**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 $\sim 10\%$ 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 $\geq 25\%$ HbO₂ 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, HbO₂ levels were quite low to begin with and were not significantly decreased with growth.

HbO₂ 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 $\geq 25\%$ HbO₂ 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 pO₂ 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 Muller-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 HbO₂ or pO₂ levels do not correlate with the hypoxic fractions between the two sites, although, if the tumour cells surrounding the low-HbO₂ 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).
A number of additional mechanisms have also been suggested previously (Rofstad et al., 1988). Carboxen breathing and the combination of carboxen 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. HbO₂ levels were improved for only one distance class following NIC for either KHT or SCCVII tumours. These results suggest that tumour radio-sensitivity should also tend to be highest following the carboxen and combination treatments, published changes in radioreponse following these agents do not support this conclusion. Siemann et al. (1994) found that NIC and carboxen were equally effective at radiosensitising KHT tumours. In addition, the radiosensitivity enhancement resulting from the combination of NIC and carboxen was equivalent to either agent alone if distant under tumour margins. 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 carboxen and NIC produced equivalent improvements in radioreponse. 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 carboxen was essentially the same, despite markedly different HbO₂ 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 radiotolerance.

In a study using C3H mouse mammary carcinomas (Horsman et al., 1995), changes in the fraction of pO₂ readings of 5 mmHg generally correlated with the corresponding HFs following different oxygen manipulations. However, changes in tumour pO₂ levels following NIC and carboxen were again inconsistent. Tumours were much better oxygenated following carboxen than NIC, in spite of equivalent radiosensitivities following either treatment. As was the case for the HbO₂ measurements, pO₂ levels following NIC were not different from the air-breathing controls. In the studies of Martin et al. (1994), the relationship between pO₂ profiles and surviving fraction varied markedly among tumour lines. In one, cell survival remained essentially constant between NIC and carboxen treatments, in spite of substantial differences in pO₂ profiles. Surprisingly, NIC increased tumour oxygenation more than carboxen in all three lines. Additional studies have shown similar variations in the radioreponse following NIC, carboxen or the combination (Kjellen et al., 1991; Simon et al., 1993; Dorie et al., 1994).

The current disparity between HbO₂ results and HF changes can be rationalised if it is assumed that NIC and carboxen oxygenate different subpopulations of clonogenic vs non-clonogenic tumour cells. While direct measurements 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 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.

Carboxen 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 carboxen, 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 radioreponse 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 tumours. 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 HbO₂ 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 HbO₂ levels may or may not relate to either tumour oxygenation or radioreponse. Since overall HbO₂ 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 radioreponse in either tumour model. This suggests that the beneficial effects of combining NIC and carboxen may not involve a decrease in acutely hypoxic cells in all cases.

Finally, why are SCCVII and KHT HbO₂ levels increased so much more with carboxen than with NIC, despite similar effects on radiosensitivity? One possibility is that, although both treatments may increase oxygen delivery to the clonogenic vs anoxic subpopulations, carboxen may be able to oxygenate some population of non-clonogenic anoxic cells. Following NIC, radioreponse 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 and distant from the previously open vessels will now be diverted to the clonogenic cells in oxygenated open 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 radiosensitivity 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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Carbogen and nicotinamide effects on tumour oxygenation
BM Fenton