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ARE DIRECT MEASURES OF TUMOR OXYGENATION REFLECTIVE OF CHANGES IN TUMOR RADIOSENSITIVITY FOLLOWING OXYGEN MANIPULATION?

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The present study investigates the correlation between tumor oxygen availability and radiosensitivity following oxygen manipulation. Previous work has shown that tumors may contain both diffusion- and perfusion-limited hypoxic cells. Recently, the combination of nicotinamide (NIC) administration plus carbogen breathing has been proposed as a means of targeting both hypoxic cell subpopulations. Intravascular HbO_2 saturations were measured for KHT murine sarcomas following either NIC, carbogen breathing, or the combination, and compared with determinations of tumor cell survival under matched conditions. The percentage of vessels $\geq 25\%$ HbO_2 increased significantly for both the carbogen and NIC-carbogen combination, while remaining unchanged from controls following NIC. These findings contrast with the survival data, where all treatments showed identical cell survival. A possible explanation is that different proportions of clonogenic versus nonclonogenic cells may be oxygenated by the alternative treatments. Thus direct determinations of alterations in tumor oxygenation may not reflect corresponding changes in radiosensitivity.

The oxygenation status of tumors has been the focus of numerous experimental and clinical studies over the past few decades, and several methods have been introduced to directly quantify regional areas of tumor oxygen deprivation or hypoxia. These have included the determination of tumor ^{31}P magnetic resonance spectroscopy energetics (1–3), pO_2 levels (4, 5), intravascular oxyhemoglobin (HbO_2) saturations (6), and the use of radiolabelled sensitizers that preferentially bind to hypoxic cells (4, 7, 8). A key question that remains unanswered is whether a relationship exists between these direct measures of tumor oxygenation and corresponding determinations of tumor radiosensitivity. While clear correlations have been demonstrated within specific tumor lines (6, 9), attempts to define similar relationships across tumor lines have, for the most part, proven unsuccessful (6, 10, 11).

It has been suggested that tumor hypoxia may develop both chronically, as a consequence of diffusion limitations, and acutely, due to perfusion limitations (12). In an effort to target both of these hypoxic cell subpopulations, the combination of nicotinamide (NIC) administration plus carbogen breathing has recently been proposed (13, 14). While NIC has been shown to reduce tumor blood flow intermittencies (15), carbogen breathing is expected to increase the oxygen diffusion distances. In the present study, cryospectrophotometric procedures were used to obtain intravascular HbO_2 saturation profiles for KHT murine sarcomas following either NIC, carbogen breathing, or the combination. These results were compared with determinations of tumor cell survival under matched treatment conditions.

The goal was to determine whether alternative means of manipulating tumor oxygenation produce corresponding changes in both tumor oxygen availability and radiosensi-

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tivity within a given tumor line. If substantially different proportions of clonogenic versus nonclonogenic tumor cells are oxygenated following the two treatments, however, direct measures of tumor oxygenation will not be expected to correlate, since tumor radiosensitivity is based on the ratio of the fractions of anoxic clonogenic tumor cells to total clonogenic cells.

Material and Methods

Mice and tumor model. The KHT sarcoma (16), a tumor maintained in vivo, was used in all experiments. Using 8-week-old female C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME), 2×10^5 KHT cells were inoculated intramuscularly (i.m.) into the hind limbs. Tumors were selected for cryospectrophotometric analysis when they reached a volume of between 320 and 700 mm^3 and selected for radiation survival assays at volumes of ~ 500 mm^3.

Drugs. NIC (Sigma Scientific, St. Louis, MO), was freshly prepared before each experiment in sterile phosphate buffered saline and injected at 1 000 mg/kg intraperitoneally (i.p.) in a volume of 0.01 ml/g, 1 hr before tumor freezing.

Carbogen breathing. Mice were confined to plastic jigs and exposed to carbogen (95% O_2, 5% CO_2) for 7–10 min before tumor freezing or irradiation.

Tumor freezing and cryospectrophotometric determination of HbO_2 saturations. To accelerate the freezing procedure, tumors were first shaved and a depilatory agent was applied. Following treatment, the mice were cervically dislocated and the tumors immediately quick-frozen using a liquid N_2-cooled copper block and stored in cryotanks. Tumor sectioning and sampling procedures were as previously described (17). Four cross-sections of the tumor were exposed using a cooled scalpel blade in a dry ice-ethanol bath at $-73°C$. For HbO_2 determinations, the exposed tumor surface was analyzed on a liquid nitrogen-cooled microscope stage. Approximately 95 blood vessels were systematically selected per tumor, spatial positions of the blood vessels were recorded using stage micrometers, and HbO_2 saturations were determined cryospectrophotometrically (18).

Irradiation and tumor response. Irradiations were performed on non-anesthetized mice using a ^{137}Cs source operating at a dose rate of 3.0 Gy/min. Each mouse was confined to a plastic jig with its tumor-bearing leg extended through an opening in the side to allow local irradiation (19). Clonogenic cell survival was determined immediately after irradiation. Single cell suspensions were prepared, counted, and various dilutions were plated (20). In approximately two weeks, the surviving cells formed colonies which were counted with the aid of a dissecting microscope.

Results

HbO_2 saturations

Fig. 1 presents the intravascular HbO_2 saturations as a function of the distance of the vessels from the tumor surface. Differences between treatment groups are most evident when the data are plotted as the percentage of vessels $\geq 25\%$ HbO_2 saturation, as shown. Untreated controls are contrasted with NIC-treated, carbogen breathing, and the combination of both treatments. Overall, HbO_2 levels decreased rapidly with distance from the tumor surface for each of the four treatment groups. Comparing the NIC and the untreated tumors, significant differences were noted only for the 1–2 mm distance class ($t = 2.4$, $p = 0.03$). Compared to untreated animals, carbogen breathing resulted in significant increases in HbO_2 levels for all but the 3–4 mm distance class ($p < 0.02$), while the combination of NIC plus carbogen produced significant increases at all distances. Differences between carbogen and the combination were not significant for any distance classes ($p > 0.25$).

Fig. 2 illustrates corresponding changes in the percentage of vessels containing no measurable oxygen following treatment. Even though oxygen availability is increased overall following either carbogen or the combination (as shown in Fig. 1), the percentage of vessels containing no oxygen is not significantly different from controls at any of the distance classes. Since the oxygen availability is in-

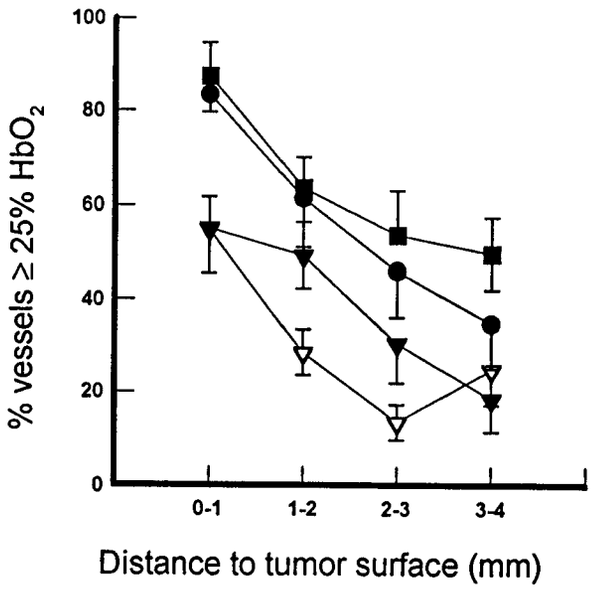

Fig. 1. Percentage vessels $\geq 25\%$ HbO_2 saturation (mean $\pm$ SEM) as a function of distance from the tumor surface. Untreated controls (▽, 529 ± 58 mm^3, $n = 8$) are contrasted with NIC-treated (▼, 530 ± 65 mm^3, $n = 9$), carbogen breathing (●, 483 ± 27 mm^3, $n = 7$), and the combination (■, 430 ± 25 mm^3, $n = 7$).

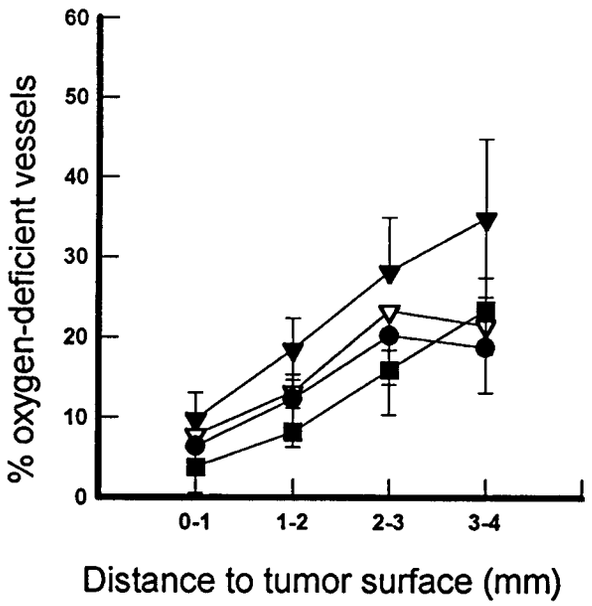

Fig. 2. Percentage of vessels having no measurable HbO_2 content (mean ± SEM) as a function of distance from the tumor surface. ▽ AIR; ▼ NIC; ● CAR; ■ N&C.

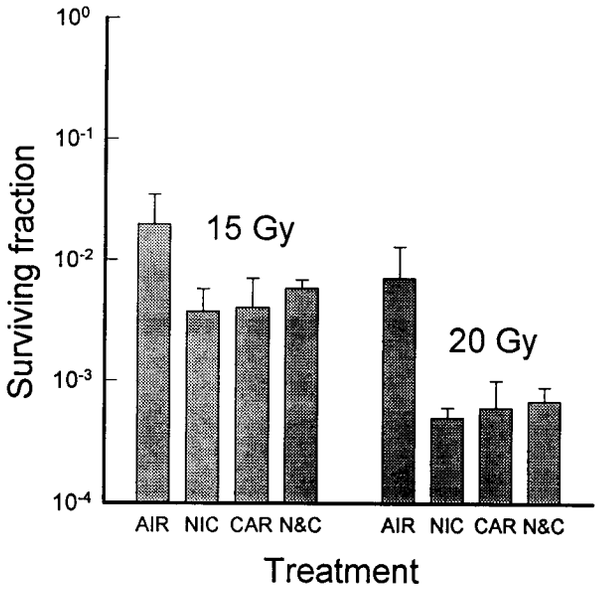

Fig. 3. Surviving fraction as a function of treatment. A single dose of radiation was given and results are the mean (± SEM) of experiments. Air = untreated controls, NIC = 1000 mg/kg nicotinamide , CARB = carbogen breathing, N&C = NIC plus CARB.

creased for the carbogen and the combination without a corresponding decrease in the percentage of vessels containing no oxygen, oxygen availability in some subpopulation of the vessels is most likely not improved following treatment. Furthermore, these 0% HbO_2 vessels are preferentially located towards the tumor interior.

Radiation response

Fig. 3 presents the radiosensitivity results for the untreated controls plus each of the three treatment protocols at radiation doses of both 15 and 20 Gy. Although surviving fraction was significantly reduced by either carbogen breathing or NIC administration, the combination of the two did not result in a further reduction in tumor cell survival for either radiation dose.

Discussion

Finding more effective means for increasing tumor oxygenation, and thereby improving radioresponse, has been a persistent objective over the past few decades, in both experimental and clinical tumors. The current study presents two quite different approaches for evaluating changes in tumor oxygenation following the administration of NIC and carbogen. In the first, oxygen availability is measured directly in individual tumor microvessels. In the second, oxygenation is measured indirectly in terms of tumor radioresponse. Although a quantitative determination of radiosensitivity before and after such manipulations would be ideal, at present no method exists for measuring such changes in patients. Therefore, direct measures of tumor oxygenation have most commonly been used.

A primary concern in the use of such methods is whether these techniques provide a representative appraisal of the ability of different manipulative agents to improve radiosensitivity. The current study clearly demonstrates the opposite, at least for the KHT sarcoma. On the basis of the tumor HbO_2 profiles, both carbogen breathing and the combination of NIC plus carbogen are predicted to provide effective radiosensitization compared to air-breathing controls. Since NIC, on the other hand, produced modest improvements in tumor oxygen availability, minimal effects on radioresponse would be expected following this treatment.

From the radiation survival results, no significant differences in radiosensitivity were observed between any of the three treatments. All three improved radioresponse equally, in spite of corresponding differences in direct measures of tumor oxygenation. Similar findings have been reported by Horsman et al. (10), who compared pO_2 electrode measurements with independent determinations of the radiobiological hypoxic fraction (HF) in a C3H mouse mammary carcinoma. For this tumor line, changes in the fraction of pO_2 readings ≤ 5 mm Hg generally correlated with corresponding changes in the HF following different manipulations of oxygen delivery. Thus, for carbogen breathing and most other manipulations, the HF decreased with increasing tumor oxygenation, as expected. NIC administration was a notable exception. Following NIC, the HF decreased to the same level as with carbogen,

but with no corresponding increase in tumor pO_2 readings. As was found in the current study, tumors were much better oxygenated following carbogen than NIC, in spite of the virtually identical radiosensitivities. In addition, tumor oxygenation following NIC was unchanged from the air-breathing controls.

Martin et al. (11) report somewhat different results. Here, pO_2 profiles and survival were measured following NIC, carbogen, or the combination in three different tumor lines. Among tumor lines, the relationship between pO_2 profiles and surviving fraction varied markedly. In one of the three tumor lines, cell survival again remained essentially constant between NIC and carbogen treatments, in spite of substantial differences in pO_2 profiles. In contrast to the current findings, NIC increased tumor oxygenation more than carbogen for each of the three tumor lines. In still another study, Chaplin et al. (14) found that while both NIC and carbogen improved radioresponse in subcutaneous implanted SCCVII tumors, neither was as effective as the combination. Clearly, response to different manipulative agents varies markedly with tumor line and possibly implantation site.

The radiobiological HF is generally determined by calculating the separation between the air-breathing and anoxic tumor cell survival curves, for radiation doses at which the survival curves under the two treatment conditions are parallel. This means that the separation between the survival curves is mathematically equivalent to the ratio of the radiation survivals of the partially and completely anoxic tumor cell populations (21). In other words, the radiobiological HF depends on the fraction of anoxic clonogenic cells contained in the tumor divided by the total fraction of oxygenated plus anoxic clonogenic cells. Since HF is independent of oxygenation changes in the nonclonogenic cells, the current findings can be explained if it is assumed that the different treatments oxygenate different proportions of clonogenic versus nonclonogenic cells. Thus, if a higher proportion of nonclonogenic versus clonogenic cells is oxygenated in the carbogen-treated tumors compared to the NIC, the carbogen-treated tumors will appear better oxygenated despite similar reductions in cell survival.

If carbogen and NIC increase tumor oxygenation through different physiological mechanisms, these may not be unreasonable assumptions. Carbogen breathing is expected to improve tumor oxygenation primarily by increasing the blood oxygen carrying capacity, and thus the diffusion distance of the oxygen from the blood vessels. This should tend to increase oxygen delivery to both clonogenic and nonclonogenic anoxic tumor cells. For NIC, on the other hand, one suggested mechanism is that the drug acts by reducing intermittent fluctuations in tumor blood flow (15). Since the percentage of oxygen-deficient vessels was not significantly reduced following NIC in the current study, this may not be the primary mechanism in the KHT tumor model. NIC has also been reported to produce an increase in tumor blood flow (22, 23). It is conceivable that different subpopulations of clonogenic and nonclonogenic tumor cells will be oxygenated through these alternative mechanisms, as has been suggested by previous theoretical studies of the relative influences of hemoglobin concentration and blood velocity on tissue oxygenation (24, 25).

In summary, tumor response to carbogen and NIC manipulation varies substantially with tumor line, in terms of both tumor radiosensitivity and direct measures of tumor oxygenation. The difficulty is that no method is presently available for determining whether or not a correlation between the two measures exists for a given tumor. Thus, defining an 'optimal' manipulative agent solely on the basis of its ability to increase tumor oxygenation may lead to erroneous conclusions. Clearly, alterations in tumor oxygenation may not be reflective of corresponding changes in tumor radiosensitivity if significantly different fractions of nonclonogenic tumor 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 variations in tumor radioresponse following oxygen manipulation.

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