Ischemia Reperfusion Injury in Tumors: The Role of Oxygen Radicals and Nitric Oxide

Charles S. Parkins, Madeleine F. Dennis, Michael R. L. Stratford, Sally A. Hill, and David J. Chaplin
CRC Tumor Microcirculation Group, Gray Laboratory Cancer Research Trust, P.O. Box 100, Mount Vernon Hospital, Northwood, Middlesex HA6 2JR, United Kingdom

Abstract

Oxidative stress is a key process involved in the action of several therapeutic modalities used in cancer treatment. Ischemia reperfusion insult provides a model system for investigating the processes involved in determining the sensitivity of tumor tissue to oxidative stress. We have investigated the response of the murine CaNT tumor to ischemia reperfusion injury and the role that oxygen radicals and nitric oxide may play in this phenomenon. Our results show that little or no cell kill is detected in tumors exposed to up to 3 h of ischemia if the tumors are excised immediately before reperfusion. However, if reperfusion is permitted, then extensive cell kill is evident 24 h later. Administration of superoxide dismutase or catalase, at the time when vascular reperfusion occurred, resulted in a significant protection against tumor cell kill, suggesting that the damage was mediated by oxygen radicals. Conversely, administration of an inhibitor of nitric oxide synthase, N\textsubscript{\textgamma}-nitro-L-arginine, resulted in potentiation of tumor cell damage. Administration of a nitric oxide (NO) donor, diethylamine nitric oxide, at the time when vascular reperfusion occurred resulted in significant protection against tumor damage. These results suggest that nitric oxide is a potent mediator in determining tumor damage after ischemia reperfusion injury. The role of intrinsic NO production by murine tumors was investigated by measuring the accumulation of nitrate in the medium of tumor explants cultured in vitro in two tumors with differing sensitivity to ischemia reperfusion damage. The clamp-insensitive tumor SaS showed a greater nitrate accumulation than the clamp-sensitive tumor CaNT, which may confer a greater capacity for preventing tumor and endothelial cell damage after oxidative stress.

Introduction

A sudden and intense exposure of living tissue to reactive oxygen radicals is termed oxidative stress. This phenomenon has been put forward as a key process in the development of many disease pathologies, including ageing, inflammation, diabetes mellitus, arthritis, and cancer. Moreover, oxidative stress is known to be involved in the therapeutic effect of several agents used in cancer treatment, including tumor necrosis factor, interleukin 1, and photodynamic therapy (1, 2). In normal tissues, the role of oxidative stress and how it can be manipulated has been intensively studied in the context of ischemia reperfusion injury. Oxidative stress after reperfusion can affect all the cells in the ischemic tissue. However, evidence indicates that such stress is mainly focussed at the level of the vascular endothelium, primarily because of the oxidative burst after neutrophil activation and adhesion (3).

Little is known about the response of tumors to oxidative stress, although two recent publications have shown that reperfusion injury can be used to advantage in tumors (4, 5). Although these studies indicate the ability of ischemia reperfusion injury to elicit tumor damage and the involvement of oxygen radicals, they do not confirm whether this is vascular mediated, nor do they elucidate the key molecular processes involved in the response.

The response to oxidative stress resulting from ischemia reperfusion is determined by the production of superoxide and hydroxyl radicals, the balance between anti- and pro-oxidant enzymes, neutrophil activation/recruitment, and NO\textsuperscript{3} production. NO is known to play a key role in neutrophil recruitment, and, in addition, it has been shown that NO reacts with superoxide radicals, thereby modulating its activity (6, 7). The aim of the current study was to test the hypothesis that NO is the key molecule determining sensitivity to oxidative stress at the level of the vascular endothelium in tumors, and to determine whether the heterogeneity in the production of NO in different types of tumors influences sensitivity to oxidative stress.

Materials and Methods

Experimental Animals and Tumors

The animals used in these experiments were female CBA/Gy f TO mice. The moderately differentiated adenocarcinoma CaNT and poorly differentiated round cell sarcoma SaS tumors arose spontaneously in this strain of mice and have been serially transplanted using samples from the original frozen stock. Tumors were implanted s.c. on the lower back and treated when the tumor size reached 6–8 mm geometric mean diameter. Tumor growth rate was faster for the CaNT tumor (T\textsubscript{g} = 3.1 ± 0.3 days) compared to the SaS tumor (T\textsubscript{g} = 7.2 ± 0.6 days).

Experimental Model of Vascular Occlusion

Superficial tumors were clamped using a small metal D clamp, hinged along one side to allow placement of the tumor within the central space. The clamp tightens across the skin surrounding the tumor and is held closed with metal spring clips. We assessed damage resulting from changes in e.g., oxygenation, pH, or nutrient supply by excising tumors immediately after clamp release, or damage from oxidative stress associated with reperfusion by excising 24 h after clamp release. Tumors were clamped for 3 or, in the case of growth delay experiments, 6 h at room temperature, followed by removal of the clamp to allow for vascular reperfusion.

Measurement of Relative Tumor Perfusion

Tumor perfusion was measured using an i.v. injection of the radioactive tracer \textsuperscript{86}RbCl, and results were expressed as the percentage of injected activity per gram measured in treated compared to control untreated tumors (8).

Assessment of Antitumor Effect

Clonogenic Assay. An in vivo/in vitro excision assay was performed with tumors treated in vivo, excised, and grown in vitro to determine the cell yield and clonogenicity after treatment. Details of the clonogenic assay have been reported previously but are described briefly as follows. Tumors were enzy-
matically digested, and inocula were plated into culture medium. The product of viable cell yield after digestion and the proportion of colonies grown from each of the innocula resulted in calculation of the relative surviving fraction (clonogens per 100 mg tumor tissue; Ref. 9). A minimum of six tumors was used in each treatment group.

Growth Delay. Tumor size was measured using calipers, and the geometric mean diameter was calculated. The time for tumor size to increase to 3 mm larger than the size at treatment was compared for control and treated tumors (growth delay). Specific growth delay is the growth delay corrected for differences in the absolute growth rate for the two tumors. This method has previously been shown to be a good indicator of the degree of tumor cell survival after treatment.

**Administration of Free Radical and NO Modifiers**

i.v. injections (0.1 ml) of all agents were given via the tail vein 15–30 s before clamp removal to ensure complete vascular mixing by the time of clamp removal. SOD (S-5639; Sigma Chemical Co.) and CAT (C-40; Sigma) were dissolved in sterile saline at a final concentration of 4500 and 3000 units/ml, respectively. The NO synthase inhibitor L-NNA (N-522; Sigma) was prepared in acidified saline at 6.7 mg/ml (0.1 ml equivalent to 20 mg/kg). DEANO (Molecular Probes, Inc.) was initially dissolved in 1 mM sodium hydroxide. Just before administration, DEANO was diluted in an equal volume of saline to a final concentration of 6.7 mg/ml (0.1 ml equivalent to 20 mg/kg).

Tumor Explant and Analysis of Nitrate and Nitrite. Tumors were excised under sterile conditions, finely minced, and suspended in 2 ml of culture medium in small Petri dishes. Explants were incubated at 37°C in a humidified 5% CO2:air incubator for up to 9 h. Aliquots of culture medium from the explant suspension were placed in glass microtubes (Chromacol, United Kingdom) and sealed using teflon-lined caps. All procedures were undertaken in laminar flow hoods, and new glassware was used to ensure minimal risk of contamination from environmental nitrate. Samples were frozen at −20°C until analysis. Nitrate and nitrite were analyzed by high-performance liquid chromatography using a method similar to that reported previously, except that electrochemical detection of nitrite was used (10). Results are expressed as the difference in nitrate concentration in culture medium supernatant from dishes containing tumor explants relative to that from culture medium alone (10–12 μM nitrate concentration, 1 μM nitrite; corrected for equivalent initial weights of the tumor samples as per Thomsen et al. (11]).

**Results**

Our studies have initially focused on the response of the murine CaNT tumor to ischemia reperfusion damage. From the results shown in Fig. 1, it can be seen that if tumors are excised immediately at the end of the clamping period (clamp +0 h), little cell kill is induced by up to 3 h of ischemia; however, if reperfusion is permitted by releasing the clamp and waiting 18 h before excising the tumor, cell kill is dramatically enhanced. Using the 86RbCl extraction method, we found that tumor perfusion is rapidly restored after 3 h of clamping [50.5 ± 6.3% (SEM) of control at 1 h after clamp removal]; therefore, prolonged ischemia due to mechanical damage to the vessels is not involved in the effect.

To determine the role of oxygen radicals in the effects seen, we have evaluated the response of the tumors to ischemia reperfusion insult when either SOD or CAT are injected into the circulation immediately before clamp release. The results are also shown in Fig. 1 and illustrate the dramatic effect these agents have on the response. The administration of SOD or CAT essentially prevents the cell kill induced by the reperfusion, indicating oxygen radicals in the vascular compartment are almost entirely responsible for the effect seen.

The effect of modulating NO levels using either a NO synthase inhibitor or a NO donor during reperfusion is shown in Fig. 2. Of particular interest are the results with the NO synthase inhibitor (L-NNA), which enhances the tumor cell kill induced by reperfusion injury. In contrast, the administration of the NO donor drug DEANO immediately before clamp release prevents a large part of the cell killing.

For comparison with the CaNT tumor, we have initially investigated the response of the SaS tumor, which is grown in the same strain of mice. Because the SaS tumor cannot be cloned in vitro, we undertook the comparison on the basis of growth delay after a 6-h period of ischemia. The results shown in Fig. 3 show that the SaS tumor is resistant to this oxidative insult when compared to the CaNT. The production of NO from freshly explanted CaNT and SaS tumors has been measured as the increase in nitrate in culture medium from freshly explanted tumors, as shown in Fig. 4 (no significant increase in nitrite was measured). The ischemia-resistant SaS tumor produces approximately 4-fold more nitrate compared to the CaNT tumor over a 9-h incubation period. Four tumors contribute to each data point (mean ± SEM).

**Discussion**

The increase in cytotoxicity observed when tumor excision was delayed, allowing resumption of blood flow, is indicative of classical reperfusion injury.

The current study has shown that increasing the plasma levels of either SOD or CAT results in protection against ischemia reperfusion injury. This suggests that these enzymes effectively reduce the number of oxygen radicals formed in the vasculature during the reoxyge

![Fig. 1. The relative surviving fraction of cells in CaNT tumors was assessed using a clonogenic assay after a 3-h vascular occlusion. Tumors were either excised immediately [i.e., no reperfusion (0 h) or 18 h (18 h) after treatment]. Significantly greater cell kill was measured when the excision was delayed (at a time when tumor reperfusion had occurred). Protection against reperfusion injury was found when SOD or CAT were administered i.v. just before clamp removal. *, significant increase in surviving fraction by both SOD and CAT given before clamp release (P < 0.05; 2-sample t test, unpaired). Columns, mean; bars, SEM.](cancerres.aacrjournals.org)
in increased neutrophil adherence (7). The opposing results obtained by the NO donor DEANO strongly support the hypothesis that NO plays a leading role in modulating ischemia reperfusion injury in tumors. The differences observed between the CaNT and SaS tumors in response to ischemia reperfusion insult could be explained by differences in intrinsic NO production. The SaS tumor was more resistant to ischemia reperfusion damage than the CaNT tumor; moreover, explants of the SaS tumor showed a greater accumulation of nitrate, indicative of a greater intrinsic production of NO. These increased NO levels, if they were reproduced in situ, would reduce the half-life and, therefore, toxicity of the oxygen radicals formed during reperfusion and also reduce neutrophil adherence to the tumor endothelium after oxidative stress. The results presented in this paper suggest that ischemia reperfusion injury in tumors involves production of oxygen radicals in the vasculature, which potentially lead to endothelial cell damage. Moreover, the studies indicate that NO plays a key role in modulating tumor cell kill induced by reperfusion insult.

Knowledge of how tumor tissue responds to oxidative stress, particularly at the level of the vascular endothelium, has potentially important applications: (a) it has been shown that temporal heterogeneity in blood flow is a feature of malignant tissue (12, 13). Such heterogeneity results in regions being subjected to ischemia reperfusion. Thus, if it were possible to manipulate the response of tumor tissue to such oxidative stress and make it more sensitive, therapeutic benefit could be achieved by exploiting the phenomenon of intermittent flow; and (b) oxidative stress is known to play a role in the action of other cancer treatments including photodynamic therapy, tumor necrosis factor, and interleukin-1 (1, 2). Thus, identification of the key processes involved in tumor response to oxidative stress should enable the response to these therapies to be improved. Furthermore, the potential importance of endogenous NO production as a prognostic determinant of tumor response to oxidative stress warrants further evaluation.

References


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