Induction of Hypoxia in Experimental Murine Tumors by the Nitric Oxide Synthase Inhibitor, NG-Nitro-L-arginine

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ABSTRACT

The nitric oxide synthase inhibitor NG-nitro-L-arginine (NOARG) was examined for its ability to alter energy metabolism in three mouse tumors using 31P magnetic resonance spectroscopy. NOARG (10 mg/kg, i.v.) increased the inorganic phosphate/total phosphate ratio (Pi:total) 2-3-fold in the KHT, RIF-1, and SCCVII/HA intradermal back tumors from 30 min to 6 h after injection, but the 31P magnetic resonance spectrum from normal tissue on the mouse back was unchanged after this treatment.

The combination of the bioreductive agent RB6145 (300 mg/kg, i.p.) 15 min prior to NOARG (10 mg/kg, i.v.) produced greater than 5 decades of KHT tumor cell killing at 24 h after treatment. This combination also increased Pi: total 4.5-fold over the control value at 24 h in the KHT tumor. Histological examination of tumors at this time indicated extensive necrosis.

INTRODUCTION

NO is a messenger molecule in a range of normal organs and tissues. One site of NO activity is the vascular endothelium, where it is a vasodilator and is responsible in part for maintaining cardiovascular homeostasis (1).

NO is generated in vivo from L-arginine and molecular oxygen by the enzyme NO synthase with L-citrulline as a by-product (2). Some N2 substituted analogues of L-arginine inhibit NO generation and thus increase blood vessel tone, resulting in increased blood pressure and/or reduced local organ perfusion (3).

The role of NO in normal tissue physiology is being characterized, and there is accumulating evidence of NO involvement in a number of human disorders (4, 5), but little is known about its role in tumors.

We have recently shown that the NOS inhibitor NOARG can induce hypoxia in the SCCVII/HA-transplantable murine tumor (11), and in a range of spontaneous murine mammary adenocarcinomas (15), using in vivo 31P MRS. The increase in hypoxia in the SCCVII/HA tumor induced by NOARG was sufficient to increase significantly tumor resistance to X-rays (11).

The aims of this study were 2-fold: (a) to determine whether the effect of NOARG on energy metabolism and X-ray response previously observed in SCCVII/HA tumors also occurred in other transplantable murine tumor systems; and (b) to examine the ability of NOARG to enhance the cytotoxicity of a bioreductive agent.

MATERIALS AND METHODS

Mice and Tumors. The murine-transplantable sarcomas KHT and RIF-1, and the carcinoma SCCVII/HA, implanted in 10-12-week-old male or female C57/HeJ mice were used for this study. KHT was maintained by continuous in vivo passage (16), and RIF-1 and SCCVII/HA were maintained according to the method of Twentyman et al. (17). Tumors were implanted i.d. on the mouse back, 2 cm from the tail base, by injection of 2 × 106 cells in 0.05 ml culture medium. Tumors were used for experiment 12-14 days after implantation when tumor volume was 200-400 mm3.

Mice were anaesthetized for all experiments but gently restrained in specially designed jigs (18), which exposed the tumor on the mouse back. For MRS experiments on normal tissue, the coil was placed on the surface of the mouse back.

Drugs. NOARG (Sigma Chemical Co., Poole, UK) was injected i.v. at 0.01-20 mg/kg in PBS, at a volume of 0.005 ml/g mouse body weight. For MRS experiments, NOARG was administered via a tail vein catheter.

RB6145 (MRC Radiobiology Unit) was injected i.p. at 100-300 mg/kg in acetate buffer (pH 5.4), at a volume of 0.02 ml/g mouse body weight.

MRS Experiments. 31P MRS was carried out using a 4.7 Tesla, 30-cm horizontal bore magnet (Oxford Instruments, Oxford, UK), interfaced with a SISCO 200 spectrometer. A 7-mm diameter double turn surface coil was used for Rf pulsing and signal collection. For experiments with NOARG alone, a tail vein catheter was inserted, and the mouse was placed in the jig and left to settle for 10-15 min. A control spectrum was collected, and NOARG was injected via the tail vein catheter outside the magnet bore. Spectra were collected at intervals of up to 2 h without moving the mouse from its position within the magnet. For later time points (6 and 24 h), the mouse was removed from the jig, returned to its cage, and replaced in the jig at the required time.

This procedure was also used for experiments with RB6145 and NOARG, except that the mouse was removed from the jig after collection of the control spectrum for the i.p. injection of RB6145. The mouse was returned to the jig and replaced in the magnet for collection of a further spectrum prior to the i.v. injection of NOARG.

Each spectrum comprised 256 scans with a 2-s delay, giving a total acquisition time of 8 min. Spectra were analyzed using an in-house baseline and Lorentzian curve fitting program. Data for tumors were routinely expressed as flow could enhance the activity of bioreductive cytotoxic agents (6), hyperthermia (7), and some chemotherapeutic agents (8).

NOS activity has been demonstrated in human tumor cells (9) and biopsies (10). NO donors either alone (11) or in combination with other radiosensitizing treatments (12) can increase tumor sensitivity to X-rays. NOS inhibitors can selectively reduce blood flow in tumor-associated neovascularure induced by sponge implantation (13, 14).

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changes in the Pi:total ratio. In some instances the phosphocreatine:total and βNTP:total ratios were also determined.

Tumor pH was determined from the chemical shift of the inorganic phosphate peak relative to the α-NTP and γ-NTP peaks (19). Since phosphocreatine was not present in all tumors, this peak was not used to determine pH.

Statistical Analysis of MRS Data. MRS data from tumors treated with NOARG were compared to pretreatment controls using a Student’s t test after logarithmic transformation of individual data points. For the NOARG dose-response data, the significance of the differences between sample means was determined using a one-way analysis of variance, following logarithmic transformation of individual data points.

RESULTS

The effect of the NO synthase inhibitor NOARG on the energy metabolism of murine-transplantable tumors and the corresponding normal tissues at the usual site of tumor implant were examined using in vivo 31P MRS. Fig. 1A gives a representative spectrum from a control SCCVII/Ha back tumor; Fig. 1B shows the spectrum from the same tumor 30 min after injection of NOARG at 10 mg/kg, i.v. There was some reduction in high energy NTP and an increase in low energy Pi, consistent with increased tumor hypoxia. Fig. 1C gives a typical 31P MR spectrum from normal tissue on the mouse back, showing high levels of phosphocreatine, indicating a significant contribution from muscle. Fig. 1D gives the spectrum from the same site 30 min after the administration of NOARG (10 mg/kg, i.v.). Table 1 shows the effect of NOARG (10 mg/kg, i.v.) on the metabolite ratios Pi:total and βNTP:total from the SCCVII/Ha tumor, and Pi:total, phosphocreatine:total, and βNTP:total from the normal tissue 31P MR spectra. Since the phosphocreatine signal in the MR spectra from tumors is very variable, this ratio has not been included in the table for SCCVII/Ha. NOARG at 10 mg/kg, i.v., increased Pi:total in the SCCVII/Ha tumor (P < 0.001 at all times), which was accompanied by a small and variable reduction in NTP. However, NOARG at this dose had no effect on phosphorus energy metabolism of the normal tissues at the site of tumor implant (P > 0.05 for all metabolite ratios at all times).

Fig. 2 gives the time course for the increase in Pi:total in the SCCVII/Ha, KHT, and RIF-1 tumors after injection of NOARG (10 mg/kg, i.v.). For SCCVII/Ha (Fig. 2A) the control Pi:total was 0.076 ± 0.006 (mean ± SE), which increased 2.3–3-fold (P < 0.001) from 30 min to 6 h after administration of NOARG, with a return to control levels at 24 h. In the KHT tumor (Fig. 2B), the control Pi:total was 0.103 ± 0.007, which increased 2-fold (P < 0.001) from 30 min to 6 h after NOARG. However, at 24 h Pi:total in this tumor was 0.130 ± 0.005, which was significantly higher than control (P = 0.042). The control Pi:total in the RIF-1 tumor (Fig. 2C), at 0.055 ± 0.007, was considerably lower than that for the other tumors, but NOARG increased this ratio 2-fold (P < 0.01) from 30 min to 6 h after administration, with a return to control levels by 24 h. NOARG had no effect on pH measured by 31P MRS in any of the tumors.

The ability of NOARG to alter the response of the SCCVII/Ha, KHT, and RIF-1 tumors to X-rays was also determined. NOARG at 10 mg/kg, i.v. was given 30 min before irradiation, since this was the earliest time at which the maximal increase in Pi:total from the 31P MRS experiments was observed. Tumor cell survival was
assessed using an in vivo/in vitro clonogenic assay 24 h after treatment. Fig. 3 gives the surviving fraction of clonogenic cells from the three tumors plotted against X-ray dose. The figure also includes the X-ray response of the tumors after physically occluding the blood supply by application of a clamp to the tumor for 10 min prior to and during irradiation. In the SCCVII/Ha tumor (Fig. 3A), 10 mg/kg i.v. NOARG increased tumor survival 3–5-fold, and this survival was equivalent to that attained by clamping the tumor. In contrast, NOARG at 10 mg/kg i.v. produced only a 2-fold increase in survival after X-rays in the KHT tumor (Fig. 3B), which was less than the effect induced by clamping the tumor. The response to NOARG at 10 mg/kg i.v. was more marked in the RIF-1 tumor (Fig. 3C), where a 50–200-fold increase in tumor cell survival was observed, which was equivalent to that seen in clamped tumors.

The large differential in tumor cell survival after X-rays in the RIF-1 between clamped or NOARG-treated tumors and controls led to the use of this tumor for further experiments examining the relationship between effects of NOARG on energy metabolism and X-ray response. Another consequence of the low hypoxic fraction in this tumor may be the variability in the response to X-rays alone; for this reason, each dataset has been presented with the tumor response to X-rays alone determined for that particular experiment.

For the cell survival data given in Fig. 3, the time interval between NOARG and irradiation was 30 min, the earliest time at which the maximal increase in the Pi:total was observed in the 31P MR spectra. In order to determine whether the observed increase in Pi:total over the time range shown in Fig. 2 indicated hypoxia induction sufficient to produce radioresistance over the same time course, NOARG at 10 mg/kg i.v. was administered from 30 min to 24 h prior to 20 Gy X-rays in the RIF-1 tumor. The results are given in Fig. 4. The increase in tumor survival was equivalent to that for clamped tumors for NOARG given up to 6 h prior to X-rays but was not observed at the 24 h time interval. This result is entirely consistent with the time course for the increase in Pi:total induced by NOARG at 10 mg/kg i.v. in this tumor.

The effect of varying the NOARG dose on Pi:total in the RIF-1 tumor at 30 min after i.v. injection is given in Fig. 5A. A significant increase in this ratio over control values was observed at all doses tested from 0.1–20 mg/kg i.v. (P < 0.05). The mean values for Pi:total differed significantly from each other (one-way analysis of variance, following logarithmic transformation; P = 0.011), and the response was dose dependent. Fig. 5B gives the survival of cells from the RIF-1 tumor after various doses of NOARG injected i.v. 30 min prior to 20-Gy X-rays. The increase in cell survival was equivalent to that for clamped tumors for NOARG doses from 5–20 mg/kg i.v. The radioresistance induced by NOARG was reduced with lower doses, with a return to survival levels for radiation alone at 0.2 mg/kg. Again, the NOARG dose-response in terms of RIF-1 tumor cell X-ray survival is consistent with that for the effect of NOARG on tumor energy metabolism.

The 31P MRS results strongly suggest that NOARG induces a prolonged state of hypoxia in three transplantable murine tumors. The degree of hypoxia was sufficient to induce radioresistance in these tumors.

The next experiment carried out was to determine whether NOARG could enhance the effectiveness of bioreductive agents. Fig. 6 gives the survival of the KHT tumor, determined using an in vivo/in vitro clonogenic assay 24 h after treatment with RB6145 given 15 min prior to NOARG. In these experiments, there was a large reduction in tumor cell yield during tumor disaggregation, which was not observed in the X-ray survival experiments. The data have been presented as a relative surviving fraction, as described in "Material and Methods," to take this into account. Fig. 6A gives the relative cell-surviving fraction as a function of NOARG dose from 2–20 mg/kg i.v. for a fixed RB6145

### Table 1: Metabolic ratios from 31P MR spectra of SCCVII/Ha tumors or mouse back without tumor, before and after treatment with 10 mg/kg i.v. NOARG

<table>
<thead>
<tr>
<th>Time after NOARG (min)</th>
<th>SCCVII/Ha Tumor</th>
<th>Mouse Back without Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pi:Σ</td>
<td>βNTP:Σ</td>
</tr>
<tr>
<td>Control</td>
<td>0.076 ± 0.006</td>
<td>0.197 ± 0.009</td>
</tr>
<tr>
<td>10</td>
<td>0.192 ± 0.018</td>
<td>0.157 ± 0.014</td>
</tr>
<tr>
<td>30</td>
<td>0.198 ± 0.019</td>
<td>0.178 ± 0.012</td>
</tr>
<tr>
<td>60</td>
<td>0.199 ± 0.031</td>
<td>0.165 ± 0.018</td>
</tr>
<tr>
<td>120</td>
<td>0.190 ± 0.040</td>
<td>0.181 ± 0.009</td>
</tr>
</tbody>
</table>

Values are mean ± SE for 4–6 mice.
HYPOXIA INDUCTION BY NO-NITRO-L-ARGININE

Fig. 3. X-ray dose response curves for (A) SC-CVII/Ha, (B) KHT, and (C) RIF-1 tumors determined using an in vivo/in vitro clonogenic assay 24 hr after treatment. △, X-rays alone; ○, 10 mg/kg i.v. NOARG 30 min prior to X-rays; •, clamp 10 min prior to and during X-rays. Points, geometric mean for 3–6 mice; bars, SE.

The observation that the tumor cell yield was greatly reduced when tumors were disaggregated 24 hr after treatment led to examination of the effect of the combination of RB6145 and NOARG on the KHT tumor metabolism using 31P MRS. The results are given in Fig. 7.

RB6145 at 300 mg/kg i.p. injected 15 min before 10 mg/kg i.v. NOARG produced no increase in Pi:total over that for NOARG alone for early times after administration. However, at 24 h after NOARG, the combination with RB6145 increased Pi:total 4.5-fold over control. Histological examination of the tumors at this time indicated extensive necrosis.

DISCUSSION

In vivo 31P MRS provides a useful method for examining the relative changes in energy metabolism of solid tumors following physiological manipulation with vasoactive agents, where reducing tumor blood flow and hence oxygenation result in reduced NTP signal from the MR spectrum, together with increased inorganic phosphate (23, 24). The results of the present study strongly suggest that NOARG reduces tumor blood flow and that the resulting increase in tumor hypoxia is responsible for the observed changes in the 31P MR spectrum.

NOARG increased Pi:total to a maximal value of about 0.3 in the SC-CVII/Ha tumor and to 0.2 in the KHT tumor, but to only 0.12 in RIF-1. These ratios are all increases over the pretreatment values for the tumors but are not as large as that observed following clamping of the tumor to occlude the blood supply, when the ratio is 0.4–0.5 (23). On the basis that the size of the Pi:total ratio is related to the severity of tumor hypoxia, then NOARG does not appear to be inducing hypoxia to the same degree as clamping the tumor. The results would also suggest that hypoxia induction by NOARG is even less in the RIF-1 tumor than in the SC-CVII/Ha and KHT tumors. However, the pretreatment value of Pi:total for RIF-1 is considerably less than that for the other tumors. If the increase in Pi:total by NOARG is expressed relative to the control value, a 2–3-fold increase in Pi:total is obtained for all 3 tumors, irrespective of the pretreatment ratio.

Increases in Pi:total of this magnitude were also observed in a range of spontaneous murine mammary adenocarcinomas for up to 2 h after treatment with NOARG (15).

The duration of the response to NOARG is similar in the 3 transplantable murine tumors and is prolonged for at least 6 h, which may be related to the pharmacokinetics of NOARG. This agent exerts
HYPOXIA INDUCTION BY \text{N}^{0}\text{-NITRO-L-ARGININE}

Fig. 5. (A) Pi:total in the RIF-1 tumor 30 min after NOARG administration, measured using in vivo \text{\textsuperscript{31}P} MRS as a function of NOARG dose. Points, mean for 4–6 mice; bars, SE. ■, control Pi:total prior to treatment. (B) Surviving fraction of RIF-1 tumors after NOARG given 30 min prior to 20-Gy X-rays, determined by an in vivo/in vitro clonogenic assay 24 hr after treatment as a function of NOARG dose. Points, geometric mean for 3–6 mice; bars, SE. □, surviving fraction after 20-Gy X-rays alone.

The increase in tumor hypoxia induced by NOARG was sufficient to increase resistance to X-rays in all three tumors, but the size of the effect varied. In SCCVII/Ha and RIF-1, NOARG induced full radiobiological hypoxia, since the cell survival in these tumors was equivalent to that achieved by clamping, whereas in the KHT, the increase in cell survival was significantly less. This suggests that the 30-min interval between the injection of NOARG and irradiation in the KHT

prolonged pressor effects in the rat because of a lack of metabolism to L-arginine found with other, shorter acting NOS inhibitors (25).

One problem often associated with the systemic administration of vasoactive agents to alter tumor physiology is the effects on the host cardiovascular system, and NOARG at 10 mg/kg i.v. increases blood pressure in the anesthetized rat by 20% (26). Interestingly, the response of the SCCVII/Ha tumor to NOARG was the same in anaesthetized and unanaesthetized mice (15). As part of a study to assess the normal tissue response to NOARG, \text{\textsuperscript{31}P} MRS was carried out on the mouse back at the site where tumors would normally be implanted. The results indicate that at the same dose of NOARG which increases Pi:total 2–3-fold in the tumors, no significant changes in normal tissue phosphorus metabolism are observed. Thus, NOARG is producing a differential response in terms of energy metabolism in the tumors compared with that in the corresponding normal tissue. Data presented here also demonstrate that the dose of NOARG may be reduced 2–5-fold and still induce tumor hypoxia. These small doses of NOARG may be expected to result in only minor increases in systemic vascular resistance. However, the systemic versus tumor effects must be carefully investigated if agents of this type are to be considered for clinical use.

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Fig. 6. Relative surviving fraction for the KHT tumor, determined using an in vivo/in vitro clonogenic assay 24 hr after treatment with i.p. RB6145, 15 min prior to i.v. NOARG (A) as a function of NOARG dose for a fixed RB6145 dose of 300 mg/kg, and (B) as a function of RB6145 dose for a fixed NOARG dose of 10 mg/kg. Arrows, survival below the level of detection. Points, individual tumors. Curve was fitted by eye.

Fig. 7. Pi:total in the KHT tumor, measured using in vivo \text{\textsuperscript{31}P} MRS at 30 min, 60 min, and 24 hr after 10 mg/kg i.v. NOARG alone (C) or with 300 mg/kg i.p. RB6145 15 min prior to NOARG (B). ■ (C), control Pi:total prior to treatment. Columns, mean for 4–7 mice; bars, SE.

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tumor may not be optimal for NOARG induction of full radiobiological hypoxia, despite the finding that the increase in Pi:total induced by NOARG in this tumor was similar to that observed for the other tumors examined. Furthermore, the time course for the induction of radioresistance given in Fig. 4 for the RIF-1 tumor may not be the same for the other two tumors.

The ability of a NO synthase inhibitor to increase hypoxia in tumors to a level sufficient to enhance the efficacy of a bioreductive agent is a significant finding. This is apparent in the KHT tumor (Fig. 6), where the effect of NOARG alone was not sufficient to induce full radiobiological hypoxia. However, the combination of RB6145 and NOARG may be sufficient to do so, although it is not apparent from the MRS measurements at early times. This implies that even larger increases in cell killing may be attainable for the combination of RB6145 and NOARG in SCCVII/Ha and RIF-1 tumors. This possibility is under investigation.

The combination of RB6145 and NOARG induced severe hypoxia in the KHT tumor at 24 h after treatment, as demonstrated by the large increase in Pi:total. This was emphasized by the reduced tumor cell yield during disaggregation and the presence of necrosis in histological sections. This phenomenon is only seen after some types of antitumor treatments, such as hyperthermia (27), and may reflect interactions between RB6145 or its metabolites and NOARG other than enhancement of bioreductive drug cytotoxicity by increased tumor hypoxia. The interaction of NOARG with other bioreductive and chemotherapeutic agents is also under investigation.

NOARG is one of several NO synthase inhibitors available for study, and is relatively nonselective for NO synthase isoforms (28, 29). Although the induction of hypoxia by this agent is substantial, it may not be optimal. Therefore, further studies are necessary to determine the relative contributions of the various NO synthase isoforms in tumors to the induction of hypoxia by NOS inhibitors. This may be approached by both directly measuring the activity of the NO synthase isoforms in tumor extracts, and examining other NOS inhibitors with known selectivity toward the different isoforms of NO synthase.

If the administration of inhibitors of NO synthase can differentially induce sufficient hypoxia in tumors to substantially increase the effectiveness of bioreductive cytotoxic agents, then this may be a useful addition to the regimens presently available for anticancer therapy.

REFERENCES

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