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BIOLOGY CONTRIBUTION

CHANGES IN TUMOR OXYGENATION/PERFUSION INDUCED BY THE NO DONOR, ISOSORBIDE DINITRATE, IN COMPARISON WITH CARBOGEN: MONITORING BY EPR AND MRI

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Purpose: In an effort to improve radiotherapy treatments, methods aimed at increasing the quantity of oxygen delivered to tumors were investigated. The aim of this study was to evaluate the effect of one nitric oxide (NO) donor (isosorbide dinitrate) on pO_2 and blood flow in a murine tumor model. The effect was compared to carbogen, used as a reference treatment.

Methods and Materials: Thirty-six liver tumors implanted in mouse thighs were imaged using magnetic resonance imaging (MRI) at 4.7 Tesla with dynamic Gd-DTPA and blood oxygen level-dependent (BOLD) contrast-enhanced imaging after administration of isosorbide dinitrate or carbogen. The effect on the pO_2 was also tested by EPR oximetry (1.1 GHz) on 52 mice.

Results: A significant increase in MRI intensity was observed for both treatments in comparison with the control group. EPR oximetry showed a dose-dependant increase in tumor pO_2 for isosorbide dinitrate (by 5.9 mmHg at 0.2 mg/kg) and a substantially greater change for carbogen breathing (by 23 mmHg).

Conclusion: Both tumor blood flow and pO_2 were increased by isosorbide dinitrate and carbogen. Carbogen is more efficient than isosorbide dinitrate in increasing the BOLD image intensity, as well as the tumor pO_2, but as efficient as isosorbide dinitrate in the Gd-DTPA contrast-enhanced imaging. We conclude that the effects of carbogen on improving tumor pO_2 involve both improved blood flow and improved hemoglobin oxygenation, whereas the effects of isosorbide dinitrate are predominantly mediated by improved blood flow alone.   © 2000 Elsevier Science Inc.

Nitrovasodilators, Oxygenation, Blood flow, Tumor, Magnetic resonance.

INTRODUCTION

The most limiting factor for complete control of tumors by radiotherapy is the presence of hypoxic cells in tumors, which are about three times more resistant to radiotherapy than normoxic cells (1). In consequence, radiation therapy of tumors is largely dependent on oxygen concentration, which is mainly governed by local blood flow. The area of interest is radioresistance caused by hypoxia, which is the result of two mechanisms: diffusion-limited or chronic hypoxia (2), due to reduced oxygen diffusion to regions distant from the tumor blood vessels, and acute hypoxia due to transient occlusion of vessels (3). Therefore, methods aimed at increasing the amount of oxygen delivered to tumors are under investigation. Studies assessing tumor blood flow and oxygenation during the administration of pharmacological vasoactive agents or carbogen breathing could be of great clinical value.

It has been shown that carbogen breathing enhances the radiosensitivity of rodent and human tumors (4, 5). Carbogen is thought to reduce chronic hypoxia by increasing the amount of dissolved oxygen in blood plasma and by saturating hemoglobin with oxygen. In addition, the acidification of blood and tissue due to the CO_2 component of carbogen may cause increased systemic blood pressure and vasodilation of microvessels possessing responsive smooth musculature, which can change tumor blood perfusion (6). Our group recently investigated different classes of vasoactive agents able to modulate the pO_2 in tumors by electron paramagnetic resonance (EPR) oximetry (7). In this preliminary study, the class of nitric oxide (NO) donors was one of the most effective, with a significant increase of the tumor oxygenation 30 min post-treatment in a majority of tumors.

NO is an endogenous vasodilator and is believed to be the main EDRF (endothelium-derived relaxing factor), contrib-

uting to cardiovascular homeostasis. Nitrovasodilators are NO donors that also cause vasodilation through release of NO *in vivo*. It has been reported that systemic administration of an NO donor (SIN-1:3-morpholinosydnonimine-N-ethylcarbamide) to mice bearing the SCCVII/Ha tumor improved the energy level in tumors and produced an 3–4-fold increase in the response of tumors to X-rays (8). Conversely, the NO synthase inhibitor nitro-L-arginine induced a 3–5-fold increase in tumor resistance (9).

The aim of this study was to characterize the effect of isosorbide dinitrate administration on the tumor pO_2 and the tumor blood flow in a murine model. The effect was compared with carbogen breathing, used as a reference treatment. This effect was evaluated using EPR oximetry, Gd-DTPA contrast-enhanced dynamic MRI, and the MR blood oxygen level-dependent (BOLD) contrast method. These methods were previously used to assess modifications of local pO_2 (partial oxygen pressure) as well as perfusion of the tumors (10, 11). A preliminary dose-effect curve was carried out for isosorbide dinitrate with EPR oximetry to determine the most efficient intraperitoneal (i.p.) dose.

METHODS AND MATERIALS

A transplantable mouse liver tumor model (TLT) with low pO_2 was implanted in the thighs of mice, as described elsewhere (7, 12, 13). To restrain the mice during the experiments, anesthesia was first induced by an i.p. injection of ketamine (80 mg/kg)/xylazine (8 mg/kg), and maintained with ketamine alone (30 mg/kg). The mice were placed in a bird cage to position the tumor in the center of the coil.

Treatments

Isosorbide dinitrate (Cedocard®, Byk Belga, 1 mg/mL for i.v. injection), was diluted in saline (to 1 mg/40 mL) for further use.

Carbogen was flushed (4 L/min) through the bird cage where the mouse was positioned.

EPR oximetry

EPR spectra were recorded using an EPR spectrometer (Magnettech, Germany) with a low-frequency microwave bridge operating at 1.1 GHz and extended loop resonator (14). Charcoal (Charcoal wood powder, CX0670-1, EM Science, Gibbstown, NJ) was used as the oxygen-sensitive probe in all experiments. Calibration curves were made by measuring the EPR line width as a function of the pO_2 (15). TLT mice were injected in the center of the tumor (8-mm diameter) using the suspension of charcoal (100 mg/mL, 50 μl injected, 1–25-micron particle size). The EPR measurements were started 2 days after the injection. The tumor under study was placed in the center of the extended loop resonator in which sensitive volume extends 1 cm into the tumor mass. EPR recordings were acquired before and 30 min after the administration of the vasodilator. A set of 14 experiments for each of the three doses of isosorbide dinitrate tested was carried out for the dose-effect study. A

standard experiment showing the effect of carbogen (4 L/min) was achieved ($n = 10$) and a control experiment was carried out using 0.9% NaCl ($n = 10$).

MRI experiments

MRI acquisition was performed with a 4.7 Tesla Bruker Biospec experimental imager.

Gd-DTPA contrast-enhanced MRI was carried out using a spin-echo T1-weighted pulse sequence. The data acquisition parameters, repetition time (TR), echo time (TE), flip angle (α), slice thickness (ST), field of view (FOV), number of averages (NA), and matrix size were as follows: TR = 380 ms, TE = 9 ms, ST = 2 mm, FOV = 6 cm, NA = 6, and matrix size = 128 × 128. The total data acquisition time was 4 min, 55 s for each image series. Three precontrast images were acquired before injection of the contrast agent. The i.v. injection of Gd-DTPA (0.1 mmol/kg) and either i.p. injection of isosorbide dinitrate (0.2 mg/kg, 240 μl of diluted solution injected for a 30-g mouse, $n = 9$) or carbogen breathing (4 L/min, $n = 9$) were simultaneously carried out. A total of 9 contrast-enhanced images for each mouse were obtained. A control experiment was performed with the contrast agent alone (Gd-DTPA 0.1 mmol/kg, $n = 9$).

BOLD images were acquired using a regular gradient-echo (GE) pulse sequence. The raw data acquisition parameters were: TR = 120 ms, TE = 20 ms, $\alpha = 60°$, FOV = 6 cm, matrix size = 128 × 128, NA = 8, and ST = 3 mm through the tumor center. The total data acquisition time was 2 min, 2 s per slice. As in the Gd-DTPA experiment, 9 mice were used for each group (control, carbogen, and isosorbide dinitrate). Five BOLD images were first acquired to establish the baseline; isosorbide dinitrate or carbogen was then administrated and the image acquisition continued for 30 min.

For both MRI techniques, a preliminary anatomical rapid T_2 image (RARE sequence: TR = 1500 ms, TE = 11.5 ms, NA = 4, echo train length = 8, and acquisition time = 1 min, 41 s) was acquired to define a region of interest encompassing the whole tumor. This ROI was next further copied to the corresponding T1-weighted and BOLD images. To eliminate external sources of variation, the signal intensity was reported to a reference that was placed near the mouse in the coil.

RESULTS

EPR oximetry

The tumor pO_2 was modified by injection of isosorbide dinitrate. The effect is dose-dependant (Fig. 1). Three doses were tested by EPR oximetry. In Table 1, the results are presented as the proportion of responsive tumors. Mean pO_2 before and 30 min after treatment are also presented. A tumor was considered responsive when pO_2 was elevated by an arbitrary additional 3 mmHg after a 30-min treatment, as
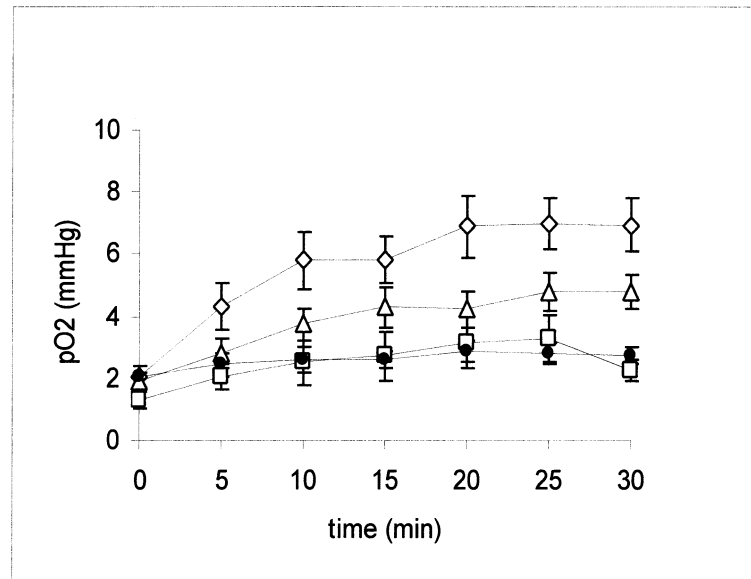
Fig. 1. EPR oximetry: effect of isosorbide dinitrate on TLT mean tumor pO_2 (mean $\pm$ SD) is a function of the dose. Solid circles = control (0.9% NaCl). Open squares = 0.04 mg/kg isosorbide dinitrate. Open triangles = 0.1 mg/kg isosorbide dinitrate. Open diamonds = 0.2 mg/kg isosorbide dinitrate.

it is well documented that the radiosensitivity of cells dramatically increases when the pO_2 is higher than 3–5 mmHg (which corresponds to half of the maximal effect) (16). The initial pO_2 observed in the center of TLT tumors was very low (0–4 mmHg) (7). The absence of response was considered as an increase below 1 mmHg. The most efficient dose tested (0.2 mg/kg with 11 of 14 responding tumors) was selected for all MRI experiments. It was shown by EPR measurements that tumor pO_2 dramatically increased after the administration of isosorbide dinitrate. Both the pO_2 reached after injection and the time course of increasing pO_2 were quite variable from one tumor to another. The effect of higher doses of isosorbide dinitrate was not tested in this study. The aim was to show the possible effect of the NO donor on tumor oxygenation and perfusion at a minimal relevant dose for radiosensitization, *i.e.*, increasing the tumor pO_2 above 5 mmHg. Using a minimal dose, we as-

sumed that it would be possible to avoid secondary effects of the drug at the same time. Carbogen breathing also induced a significant increase of the tumor oxygenation in all tumors tested. The mean pO_2 reached after carbogen breathing was higher than after isosorbide dinitrate administration. No significant change of pO_2 was observed in the control experiment.

Gd-DTPA contrast-enhanced MRI

The results of T1-weighted dynamic Gd-DTPA contrast-enhanced images are shown in Fig. 2. The post-contrast images presented a higher contrast enhancement in isosorbide dinitrate- or carbogen-treated groups than in the control group (ANOVA, $p < 0.01$). As in the EPR experiment, the rate and the degree of increase in signal enhancement were quite variable from one tumor to another, but higher in treated groups than in the control group. There was no

Table 1. Dose-dependent effect of isosorbide dinitrate in comparison with carbogen on pO_2 in TLT tumors

Treatment	pO_2 ($\pm$ standard error) before treatment (mmHg)	pO_2 ($\pm$ standard error) 30 min after treatment (mmHg)*	Reponsive tumors[§] (*n*)
Isosorbide dinitrate			
0.04 mg/kg	1.29 ± 0.25	2.26 ± 0.35	0% (14)
0.1 mg/kg	1.91 ± 0.28	4.78 ± 0.56[†]	54% (13)
0.2 mg/kg	2.05 ± 0.37	6.95 ± 0.83[‡]	79% (14)
Carbogen			
4 L/min	1.01 ± 0.32	24.17 ± 5.06[‡]	100% (10)
Control			
0.9% NaCl	2.05 ± 0.56	2.82 ± 0.29	0% (10)

*Significant difference before and after treatment (*t*-test): [†]$p < 0.05$; [‡]$p < 0.01$; [§]A tumor is considered responsive when pO_2 is elevated by an arbitrary additional 3 mmHg after a 30-min treatment.
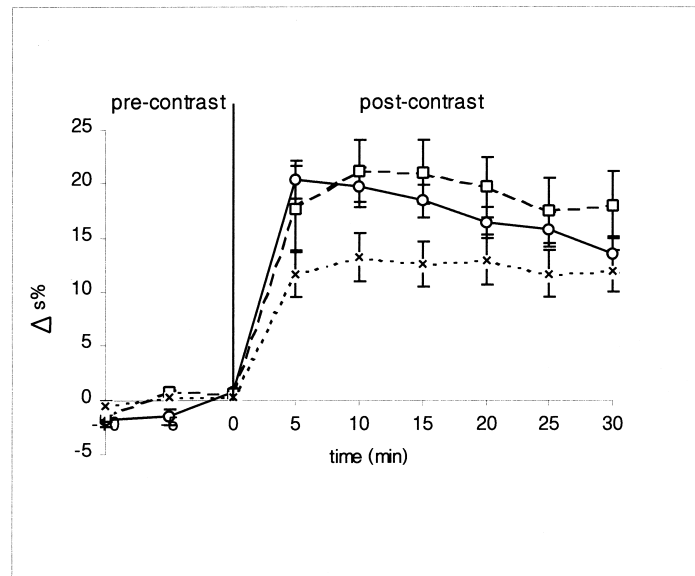
Fig. 2. Gd-DTPA contrast-enhanced MRI. Signal enhancement (mean ± SD, $n = 9$) as represented as a function of the time for each treatment. Open triangle s% (y-axis) corresponds to the percent variation in signal intensity at each time, normalized to the mean intensity of the three pretreatment images (considered as 0% of increase). Open circle = isosorbide dinitrate (0.2 mg/kg). Open square = carbogen (4 L/min). × = control.

significant difference between the effect of isosorbide dinitrate and that of carbogen.

Bold MRI

Both treatments also induced increases in the BOLD image intensity of tumors. The change in average image intensity is shown in Fig. 3. Each point corresponds to the increase in signal intensity at each time normalized to the mean intensity of the three pretreatment images (mean is considered 0% of increase). These high-resolution images demonstrate the frequent heterogeneous distribution of vascular response throughout the tumor. Signal intensity increased during treatments, while it remained at the pretreatment level in the control group. Although we observed a significant difference between the effect of isosorbide dinitrate and carbogen concerning the degree
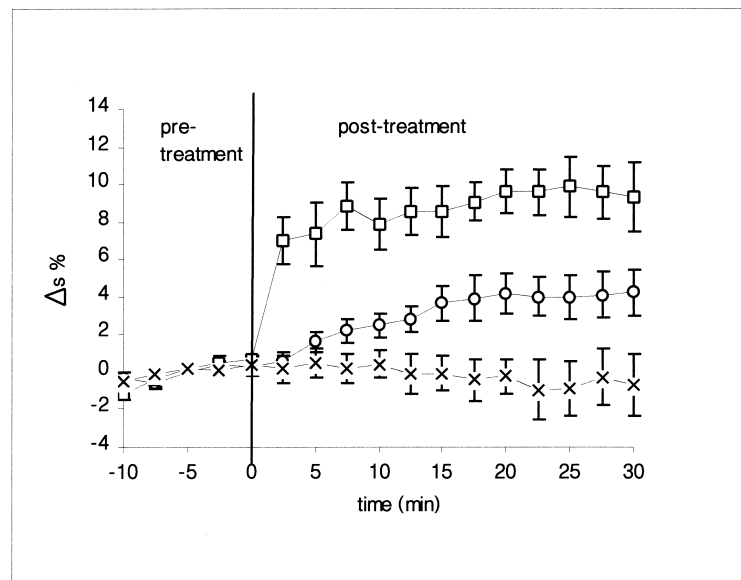

Fig. 3. BOLD imaging: evolution of the image intensity changes (mean ± SD, $n = 9$) as a function of time. Open triangle s% (y-axis) corresponds to the percent variation in signal intensity at each time normalized to the mean intensity of the five pretreatment images (considered as 0% of increase). Open circle = isosorbide dinitrate (0.2 mg/kg). Open square = carbogen (4 L/min), × = control (0.9% NaCl).

of increase in BOLD signal intensity (ANOVA, $p < 0.01$), both treatments were effective. Resumption of air breathing after 30 min of carbogen breathing caused a fall-off in signal intensity to the level of the baseline in maximum 10 min (data not shown).

DISCUSSION

As the initial pO_2 observed in the center of the TLT tumors is very low (but not necrotic, as observed in histological studies), this model of murine tumor is very sensitive to subtle increases of local pO_2, and is convenient for evaluating the efficacy of treatments aimed at increasing the tumor oxygenation (7). EPR oximetry is helpful in measuring treatment-induced modifications of pO_2 in tumors because changes of tissue oxygenation can be monitored with a paramagnetic material that is sensitive to variations of 1 mmHg in tissues. Using this technique, it was possible to demonstrate a significant effect of isosorbide dinitrate and carbogen treatments on tumor pO_2. Changes in blood flow induced by carbogen or isosorbide dinitrate are believed to be the main factor increasing tumor pO_2, as the T1-weighted contrast images, which are sensitive to increase in blood flow, indicate a higher contrast enhancement after treatments. However, Bussink *et al.* observed a significant decrease in tumor blood flow during carbogen breathing in one of the two tumor lines studied (SCCNij3 but not for SCC-Nij19), suggesting a difference in vascular responsiveness between various tumor lines (17). In the RIF-1 tumor, Honess and Bleehen observed an increase in blood perfusion during carbogen breathing (18). In addition, changes in tumor blood flow caused by external factors are greatly influenced by the structural relationship between vascular beds of tumor and surrounding normal tissues. Because the majority of tumor vessels are unable to autoregulate (except those incorporated from normal tissues), modifications of tumor blood flow are mainly due to changes in blood flow in adjacent normal tissues and arterial blood pressure. When the vascular beds in tumors are in parallel with those in normal tissues, changes in perfusion would be opposite; an increase in normal tissue blood flow due to vasodilation would shunt away the blood flow from the tumor to the normal tissue, resulting in a decrease in tumor blood flow (steal effect). Conversely, when the tumor blood flow is in series with the normal tissue blood flow, changes are similar because the blood that leaves the normal tissue vascular bed directly flows into the tumors (19). This is the main reason why the potential effect of vasodilators on tumor perfusion is a controversial topic; in many tumors, series and parallel types may be mixed or combined. Modifications of tumor perfusion vary depending on the relative contribution of these two types.

Using these three complementary methods, we were able to show the potential effect of an NO donor compound on tumor oxygenation and perfusion. This effect was compared with carbogen. As observed in the Gd-DTPA study, the two treatments seem to induce the same effect on tumor blood flow. Conversely, the effect on the tumor pO_2 is significantly higher for carbogen than for isosorbide dinitrate, as shown by EPR oximetry. BOLD imaging is predominantly used for detecting changes in T_2, which is sensitive to the blood deoxyhemoglobin concentration, but is also sensitive to flow (20). The higher rate and degree of increase in BOLD image intensity for carbogen than for isosorbide dinitrate can be explained by an important effect of the hemoglobin saturation caused by the O_2 content of carbogen. Isosorbide dinitrate can be identified as an interesting modifier of the pO_2, and also of the tumor perfusion.

A probable additional effect of isosorbide dinitrate on blood rheology has to be considered. Indeed, the effect of nitrovasodilators on blood viscosity has already been studied *in vitro* and *in vivo*. Both the blood cell shape and the blood viscosity are influenced *in vitro* by some nitro-vasodilators (21). *In vivo*, it has been observed that isosorbide dinitrate improved hemodynamic parameters and wall viscoelastic properties in case of atherosclerosis (22). This effect could be important by decreasing the transient occlusion of vessels (acute hypoxia).

NO has recently been evaluated as a potent radiosensitizer of tumor cells (23). The ability of the gas NO to radiosensitize hypoxic tumor cells appeared to be equal to that of oxygen, whereas chemical NO donors possess variable activity, depending on the mechanism and rate of NO generation (24). The activation of inducible NO synthase by IFN-γ resulted in radiosensitization of hypoxic EMT-6 tumor cells. The evidence was provided that NO was the molecule responsible for the increased radiosensitivity (25). The NO donor S-nitroso-N-acetylpenicillamine (SNAP) has been demonstrated to generate NO by bioreduction and at radiobiologically active concentrations (26). To date, no clear rationale exists on how to extend the promising radiosensitizing properties of NO in cell culture to *in vivo* applications. It would be interesting to test the radiosensitive effect of isosorbide dinitrate. *In vivo*, the radiosensitivity effect of NO could be enhanced by the flow effect produced by these compounds and the consequent increase of pO_2.

CONCLUSION

In conclusion, it is suggested that isosorbide dinitrate could be of great interest in the modification of the tumor response to irradiation, as we observed significant effects on tumor blood flow and pO_2 in experimental tumor models. A combination of EPR and MRI techniques is a convenient tool to study the modifications of tumor parameters and to differentiate the effects on pO_2 and blood flow.

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