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Advances in Brief

In Vivo Radiation Protection by Nitric Oxide Modulation

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Abstract

Drugs that affect blood flow have been shown to be whole body radiation protectors. Using N^G-nitro-L-arginine, a specific inhibitor of nitric oxide synthase, and the NO-releasing agent $(C_2H_5)_2N[N(O)NO^-]Na^+$ (DEA/NO), we have studied the ability of NO to modulate whole body radiation toxicity in C3H mice. N^G-Nitro-L-arginine given to mice between 15 and 60 min prior to radiation afforded significant protection from whole body irradiation, *e.g.*, the estimated whole body irradiation dose required to kill 50% of mice by 30 days after radiation ($LD_{50/30}$) in mice treated with N^G-nitro-L-arginine 60 min before irradiation was 1051 cGy compared with a whole body radiation $LD_{50/30}$ of 822 cGy in control mice ($P < 0.00001$). Treatment of mice with DEA/NO prior to whole body irradiation also significantly reduced toxicity; the estimated whole body radiation $LD_{50/30}$ was 1063 and 945 cGy in mice treated with DEA/NO 10 or 30 min before irradiation, respectively ($P < 0.00001$ for radiation $LD_{50/30}$ of either DEA/NO-treated group compared with control). Measurement of [^{14}C]etanidazole binding to bone marrow demonstrated that DEA/NO and N^G-nitro-L-arginine exacerbated bone marrow hypoxia. Perturbations of NO levels have profound effects on *in vivo* radiosensitivity of normal tissues. We hypothesize that alterations in regional blood flow may underlie the changes in radiosensitivity that we have observed.

Introduction

Over 40 years ago cysteamine was discovered to be an *in vivo* radiation protector (1). Since then a number of other compounds have been identified which also are able to protect mammals from otherwise lethal irradiation (2). The mechanism(s) by which these agents provide *in vivo* radiation protection is (are) not known with certainty, although a number of hypotheses exist. For example, aminothiols, such as WR-2721, may protect normal tissue from radiation by their ability to scavenge ·OH, metal chelation, or other mechanisms (3). Nitroxides, stable free radicals represented by tempol, are thought to function as radiation protectors in part through their superoxide dismutase-like activity, reaction with radiation-induced carbon-centered free radicals or peroxy radicals, and prevention of hydrogen peroxide reduction to hydroxyl radical (4). In addition to these diverse potential mechanisms of radiation protection, another property which many radiation protectors share is their ability to induce hypoxia in normal tissues, particularly in bone marrow. Oxygen is a very effective radiation sensitizer (5); conversely, both normal and malignant hypoxic cells are resistant to radiation compared with their aerated counterparts. Bone marrow cells may reside in a hypoxic environment *in vivo* (6). It appears that many radiation protectors, including cysteamine and WR-2721, exacerbate bone marrow hypoxia (7).

Agents which have a direct effect on blood flow might also be expected to affect the radiation sensitivity of bone marrow or other tissues. Both serotonin and 16,16-dimethyl prostaglandin E_2 have significant effects on blood flow in mammals, are *in vivo* radiation protectors, and induce bone marrow hypoxia (7–9). NO is a potent vasodilator which has been shown to be endothelium-derived relaxing factor (10). Like oxygen, NO might be able to react with carbon-centered free radicals generated by radiation and "fix" the damage on important biological macromolecules. Evidence that NO can be a radiation sensitizer independent of effects on blood flow or oxygen delivery was first provided by Howard-Flanders in 1957 (11). He showed that NO gas could efficiently sensitize hypoxic bacterial cells to radiation and that NO by itself was not toxic. Recently, we have found that NO, produced from the NO-releasing agent DEA/NO[2] (12), is a very effective hypoxic cell radiation sensitizer in mammalian cells *in vitro* (13). The addition of DEA/NO to a final concentration of 1 mM in the medium of hypoxic Chinese hamster V79 cells resulted in a sensitizer enhancement ratio of 2.5. No radiosensitization by DEA/NO was seen in aerobic V79 cells. Further, treatment of hypoxic cells with a solution of DEA/NO which had been allowed to completely release NO resulted in no radiosensitization, indicating that NO, not the by-products of DEA/NO decomposition, nitrite and diethylamine, was responsible for radiosensitization. The results of our *in vitro* work with DEA/NO, together with the well-known effects of NO on blood flow, have led us to examine the effect of perturbing NO levels on the whole body radiation sensitivity of C3H mice.

Materials and Methods

Materials. N^G-Nitro-L-arginine was purchased from Sigma Chemical Co. (St. Louis, MO). It was dissolved in PBS adjusted to pH 10 at a concentration of 10 mg/ml just prior to use. DEA/NO (Chemical Abstracts Service Registry No. 92382-74-6) was synthesized as previously described (12). Because DEA/NO is stable at alkaline pH but releases NO at physiological pH, it was dissolved initially in 1 mM NaOH at a concentration of 16 mg/ml. Just before administration to mice, an aliquot of DEA/NO was added to an equal volume of PBS (pH 7.4), and the resulting solution was immediately injected into mice. To assess the effect of the diethylamine and nitrite decomposition products of DEA/NO on mice, DEA/NO was dissolved in 0.9% saline at a concentration of 8 mg/ml. The pH of the solution was adjusted to 7.4 and the solution was allowed to stand at 37°C for 24 h before use. Previous work has shown that the half-life for NO release from a solution of DEA/NO at physiological pH is approximately 2.1 min at 37°C and that no detectable NO remains after 24 h (12). [^{14}C]Etanidazole was the generous gift of Stanford Research Institute (Palo Alto, CA).

Mice. Female C3H mice were supplied through the Frederick Cancer Research and Development Center Animal Production Area (Frederick, MD). The animals were received at 6 weeks of age, housed 5/cage in climate-controlled, circadian rhythm-adjusted rooms, and allowed food and water *ad libitum*. The animals were 60–80 days old at the time of irradiation and weighed between 22 and 27 g. Experiments were conducted according to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, National Research Council.

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[2] The abbreviations used are: DEA/NO, $(C_2H_5)_2N[N(O)NO^-]Na^+$; PBS, phosphate-buffered saline; $LD_{50/30}$, dose of radiation required to kill 50% of mice by 30 days after irradiation; DMF, dose modifier factor.

Determination of DEA/NO Dose. To determine the maximally tolerated dose of DEA/NO in C3H mice, animals were given injections i.p. of DEA/NO in doses ranging from 60 to 120 mg/kg. Survival was assessed up to 14 days after injection, although all deaths after DEA/NO administration occurred within 1 h of drug administration. No mice died after injections of less than 90 mg/kg. A dose of 80 mg/kg of DEA/NO was used throughout all subsequent experiments.

Whole Body Irradiation. A total of 10–15 mice were used per radiation dose. Mice were weighed in groups of five. The weights of each group ranged from 110 to 135 g or an average weight of between 22 and 27 g/mouse. In all experiments, drugs and diluent in controls were injected i.p. into mice in volumes equivalent to 1% of the weight of each animal (0.22 to 0.27 ml). For irradiation, groups of 5 to 10 mice were placed in round (30.5-cm diameter and 10.5-cm height) Plexiglas containers with holes for ventilation. Two separate containers were placed in the sample tray of the irradiator. A ^{137}Cs gamma cell 40 (Norion International, Inc., Kanata, Ontario, Canada) was used as the ionizing radiation source. The irradiator was calibrated with thermoluminescent dosimetry chips planted in phantom mice, and the radiation dose was determined according to previously described methodology (14). The dose rate used was 1 Gy/min. Total time of irradiation varied as a function of the dose delivered. Immediately after irradiation, the mice were separated into groups of five and returned to climate-controlled cages for observation. Mice were assessed daily for survival. A dose modifer factor was calculated by dividing the dose of radiation necessary to kill 50% of treated mice by the dose of radiation required to kill 50% of control mice.

[^{14}C]Etanidazole Binding to Bone Marrow. A modification of a previously described technique was used to assess marrow hypoxia (7). C3H mice were given injections i.p. of DEA/NO (80 mg/kg) or N^G-nitro-L-arginine (100 mg/kg). Ten min later, DEA/NO animals were given injections of [^{14}C]-etanidazole (300 μg/mouse i.p.). Thirty min after injection of N^G-nitro-L-arginine, those animals were given injections of [^{14}C]etanidazole. Control animals were given injections i.p. of 0.25 ml PBS and then given injections of [^{14}C]etanidazole 10 min later. Sixty min after injection of [^{14}C]etanidazole, all animals were killed by cervical dislocation. Femurs were removed from each animal and the marrow was flushed out with cold PBS. Bone marrow cells were washed 4 times with PBS and then lysed in 1.6 ml of 0.1 M NaOH overnight at 37°C. An 0.8-ml aliquot of the bone marrow cell lysate was counted in a liquid scintillation counter. The remainder of the lysates were used for protein determination (15). Data are expressed as cpm/μg bone marrow cell lysate protein.

Statistical Analysis. To assess survival of mice after irradiation, logistic regression analysis was used to fit smooth curves to the survival proportions as a function of radiation dose for each treatment. The doses were logarithmically transformed for analysis in order to obtain an improved overall fit, although the transformation had little effect upon the significance of the results. Differences between pairs of curves were assessed by using the likelihood ratio test of the logistic model, which is a 2-tailed test. The maximum likelihood estimates of $LD_{50/30}$ were derived from the parameters of the logistic fit, and confidence intervals based on the profile likelihoods were calculated.

Differences in binding of [^{14}C]etanidazole to bone marrow cells of animals treated with DEA/NO or N^G-nitro-L-arginine were compared with those of control animals, using the 2-tailed Student's t test.

Results

Whole Body Radiation Protection. Inhibition of endogenous NO synthesis by N^G-nitro-L-arginine afforded significant protection from the lethal effect of whole body irradiation. Animals given injections of N^G-nitro-L-arginine (100 mg/kg) 15 or 60 min before irradiation were protected from radiation doses of up to 11.5 Gy. Fig. 1 is an example of the time course of animal survival after 10 Gy whole body irradiation from one experiment. Of note is that the radioprotective effect of N^G-nitro-L-arginine disappeared if 2 h were allowed to elapse between injection of the drug and irradiation. We found little difference in radiation protection from N^G-nitro-L-arginine in animals treated between 15 and 60 min before irradiation. Most of our studies with N^G-nitro-L-arginine were conducted with administration of the drug 60 min before irradiation. Table 1 includes the survival of mice 30 days after whole body irradiation that were treated with N^G-nitro-L-arginine 60 min before irradiation.

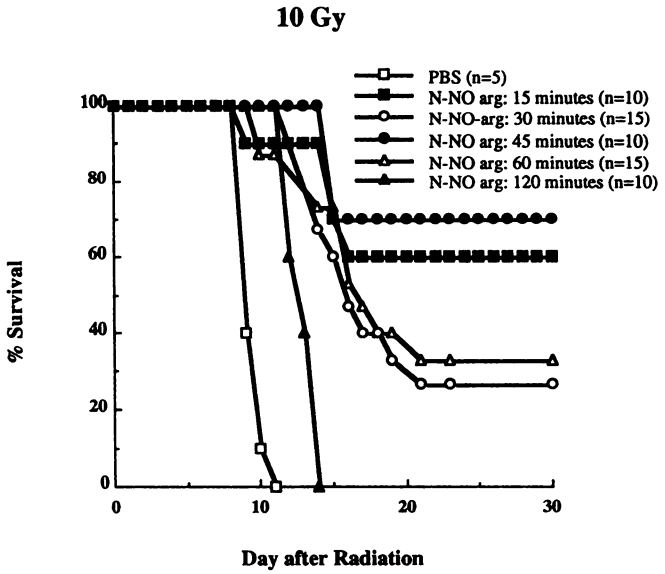

Fig. 1. Survival of C3H mice after irradiation with 10 Gy. Mice were given injections i.p. of either 0.25 ml of PBS 15 min before irradiation or with N^G-nitro-L-arginine, 100 mg/kg, at 15, 30, 45, 60, or 120 min before irradiation. Also shown are the number of mice in each group in this experiment.

Table 1 *C3H mice surviving 30 days after irradiation*

Mice were treated with i.p. injections of PBS, 0.25 ml, 15 min before irradiation, or with DEA/NO, 80 mg/kg, 10 or 30 min before irradiation, or with N^G-nitro-L-arginine, 100 mg/kg, 60 min before irradiation. The numerator for each group is the number of animals surviving; the denominator is the total number of animals treated in each group. Numbers in parentheses, percentages of animals surviving in each group.

cGy	PBS	DEA/NO-10 min	DEA/NO-30 min	N-NO-Arg[a] 60 min
700	10/10 (100)	10/10 (100)	10/10 (100)	10/10 (100)
750	18/20 (90)	10/10 (100)	10/10 (100)	10/10 (100)
775	10/10 (100)			
800	23/30 (77)	10/10 (100)	19/20 (95)	10/10 (100)
825	2/10 (20)			
850	4/30 (13)	29/29 (100)	24/29 (83)	19/19 (100)
900	1/15 (7)	16/18 (89)	22/29 (76)	15/19 (79)
950	3/20 (15)	23/29 (79)	12/27 (44)	10/19 (53)
1000	1/15 (7)	14/19 (74)	3/29 (10)	9/19 (47)
1050	0/10 (0)	17/28 (61)	7/29 (24)	8/19 (42)
1100	0/15 (0)	14/21 (67)	6/30 (20)	13/20 (65)
1150	0/10 (0)	0/20 (0)	0/19 (0)	3/10 (30)
1250	0/5 (0)	0/10 (0)	0/10 (0)	

[a] N-NO-arg, N^G-nitro-L-arginine.

Treatment with DEA/NO also protected mice from whole body irradiation. Fig. 2 shows the time course of animal survival after whole body irradiation with 11 Gy from one experiment. Animals that were treated with DEA/NO 60 min or longer before irradiation were not protected from radiation toxicity (data not shown). Also, solutions of DEA/NO that were depleted of NO by prolonged storage at pH 7.4 did not protect animals from whole body irradiation (data not shown). Table 1 includes the survival of animals 30 days after whole body irradiation that were treated with DEA/NO 10 or 30 min before irradiation. The data in Table 1 were accumulated from several separate experiments. The results shown in Figs. 1 and 2, while consistent with Table 1, do not exactly match those of Table 1 because of the inclusion of additional data in the Table.

Fig. 3 shows the survival of mice treated with DEA/NO 10 or 30 min before irradiation or with N^G-nitro-L-arginine 60 min before irradiation. The estimated $LD_{50/30}$ ranged from 822 cGy for control animals to 1063 cGy for mice treated with DEA/NO 10 min before

irradiation (DMF = 1.29; $P < 0.00001$). Mice treated with DEA/NO 30 min before irradiation had an estimated $LD_{50/30}$ of 945 cGy (DMF = 1.15; $P < 0.00001$ compared with control mice), and animals treated with N^G-nitro-L-arginine had an estimated $LD_{50/30}$ of 1051 cGy (DMF = 1.28; $P < 0.00001$ compared with control mice).

Bone Marrow Hypoxia. Both DEA/NO and N^G-nitro-L-arginine induced bone marrow hypoxia in C3H mice. Fig. 4 shows that binding of ^{14}C-etanidazole to bone marrow cells was significantly increased in animals given injections of either DEA/NO ($P < 0.05$) or N^G-nitro-L-arginine ($P < 0.01$) compared to control animals.

Discussion

We have shown that modulation of NO levels in mice, either by the inhibition of NO synthase or by the administration of the NO-releasing agent DEA/NO reduces the lethal toxicity of whole body irradiation. It is curious that two agents that would be expected to change NO levels in opposite directions would both yield radiation protection. Although we have not yet defined the mechanism by which these drugs could affect radiation response, possible explanations which are amenable to further study do exist.

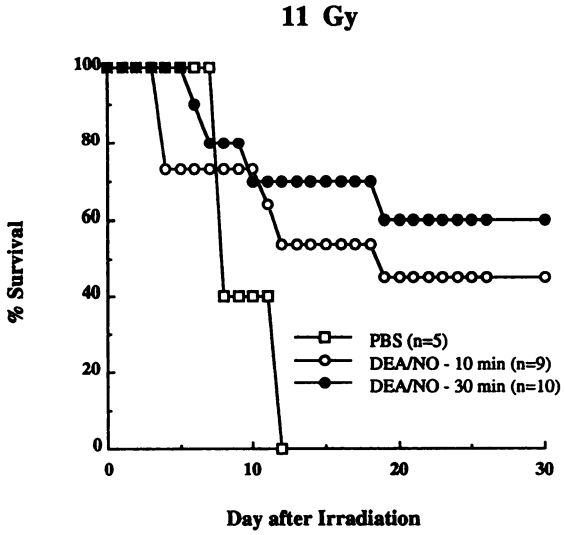

Fig. 2. Survival of C3H mice after irradiation with 11 Gy. Mice were given injections i.p. of 0.25 ml of PBS 15 min before irradiation or of DEA/NO, 80 mg/kg, 10 or 30 min before irradiation. Also shown are the number of mice in each group in this experiment.

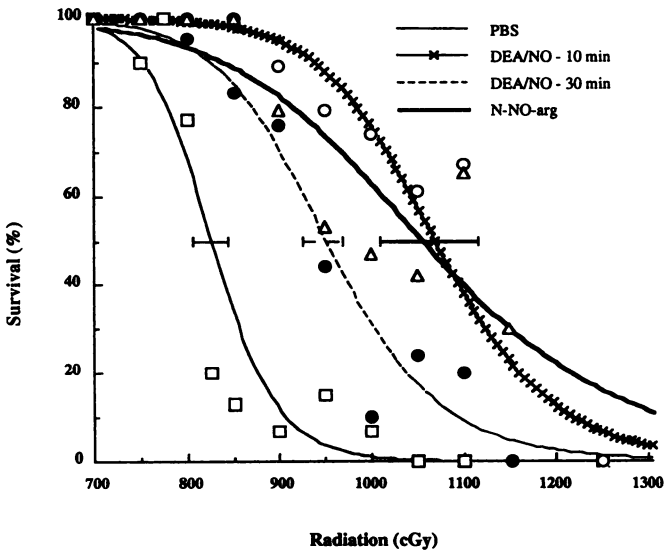

Fig. 3. Survival of C3H mice at 30 days after irradiation. Mice were treated with PBS 15 min before, or DEA/NO (80 mg/kg) 10 min or 30 min before, or N^G-nitro-L-arginine (*N-NO-arg*) (100 mg/kg) 60 min before irradiation. *Data points* shown are taken from Table 1. □, PBS animals; ○, animals treated with DEA/NO 10 min before irradiation; ●, animals treated with DEA/NO 30 min before irradiation; and △, N^G-nitro-L-arginine-treated animals. Lines drawn are smoothed curves fit to the survival data as described in "Materials and Methods." *Bars*, 95% confidence intervals about the $LD_{50/30}$ for each treatment.

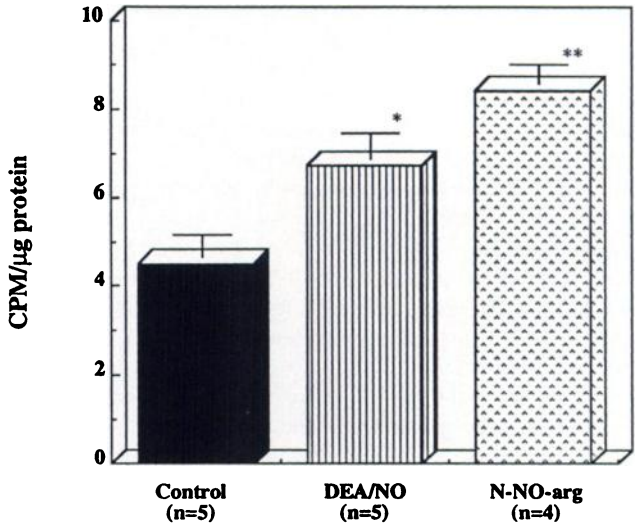

Fig. 4. Binding of [^{14}C]etanidazole to bone marrow cells of mice treated with PBS (*Control*), DEA/NO (80 mg/kg), or N^G-nitro-L-arginine (*N-NO-arg*, 100 mg/kg). *Bars*, SEM. *, $P < 0.05$ for DEA/NO mice compared with control mice. **, $P < 0.01$ for N^G-nitro-L-arginine mice compared with control mice.

The profound effects of NO on blood flow have been well documented (16). Bone marrow contains populations of stem cells which exist in a relatively hypoxic and radiation-resistant environment (6). A number of other agents which protect animals from whole body irradiation decrease blood flow and oxygen content within bone marrow (7). The bone marrow hypoxia which is caused by cysteamine, WR-2721, 5-hydroxytryptamine, or 16,16-dimethyl prostaglandin E_2 has been shown to be sufficient to account for a significant degree of the radioprotection afforded by those agents (7). It is likely that *in vivo* manipulations of NO levels will also affect bone marrow oxygen levels. One would expect that DEA/NO would have cardiovascular effects similar to those of nitroglycerine or sodium nitroprusside (17), and we have found[3] that DEA/NO is profoundly hypotensive in swine. If mice also became hypotensive after DEA/NO administration, it is likely that blood flow to bone marrow would be reduced below normal levels and that worsening hypoxia would result. In contrast to the effects of NO administration, treatment of lambs with N^G-nitro-L-arginine results in an increase in systemic vascular resistance and blood pressure and does not affect cardiac output (18). It is possible that vasoconstriction resulting from NO synthesis inhibition, in the presence of an unchanged cardiac output, would result in decreased perfusion of bone marrow. This would also result in enhanced hypoxia of the marrow and an increase in radiation resistance.

Regardless of the mechanism(s) of the protective effect of manipulation of NO levels from the lethality of whole body irradiation, it is likely that bone marrow protection accounts for the enhanced survival. Bone marrow damage from whole body irradiation becomes apparent at radiation doses of less than 10 Gy. Above 10 Gy, however, gastrointestinal injury also appears and can be fatal. In the present study, control mice irradiated with 11 Gy or less died during the second week after irradiation (Figs. 1 and 2). We have observed no enhancement of survival with either DEA/NO or N^G-nitro-L-arginine

[3] J. E. Liebmann, A. M. DeLuca, and F. Sullivan, unpublished observation.

with radiation doses above 12 Gy. All animals irradiated with doses of 12 Gy or higher died regardless of any treatment, with a median time to death of 6 days (data not shown), a timing of death that is consistent with gastrointestinal effects of whole body irradiation (19). These findings suggest that these agents protect the bone marrow but fail to protect the gut at higher doses of radiation.

The radiation protector that would be ideal for use in radiation therapy should protect normal tissue but not tumors from radiation. Recently Wood *et al.* (20) demonstrated that the NO donor, SIN-1, enhanced the *in vivo* radiation sensitivity of the implantable murine tumor SCCVII/Ha. Using ^{31}P magnetic resonance spectroscopy, they found that SIN-1 increased blood flow and oxygen delivery to the tumor. If perturbations in NO levels *in vivo* resulted in decreased blood flow to normal tissues (*e.g.,* bone marrow) but enhanced blood flow to tumors, then agents which alter NO levels should be excellent radiation protectors.

We have found that DEA/NO is an effective *in vitro* hypoxic cell radiation sensitizer (13). Since *in vitro* conditions do not permit modulation of blood flow or oxygen levels, some other mechanism(s) of NO radiosensitization must be invoked. NO, like oxygen, can bind to carbon-centered radicals. The formation of stable covalent bonds on important macromolecules (*e.g.,* DNA) can prevent repair of radiation-induced damage and increase the cytotoxicity of radiation. It is possible that NO released from DEA/NO could sensitize tumors to radiation *in vivo,* while at the same time protecting bone marrow from radiation.

In summary, we have shown that the NO synthase inhibitor, N^G-nitro-L-arginine, and the NO-releasing agent, DEA/NO, both protect mice from whole body irradiation. We suggest that the whole body radiation protection afforded by manipulations of NO levels was due to bone marrow protection mediated at least in part by changes in blood flow and oxygen delivery.

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