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Hypoxic Mammalian Cell Radiosensitization by Nitric Oxide

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Abstract

The bioregulatory molecule, nitric oxide (NO), was evaluated as a hypoxic cell radiosensitizer. Authentic NO gas was nearly as effective as oxygen in radiosensitizing hypoxic Chinese hamster V79 lung cells as evaluated using clonogenic assays. When NO was delivered to hypoxic Chinese hamster V79 cells using the NO-releasing agent (C$_2$H$_5$)$_2$N[NO(O)-NO]+Na"*, radiosensitization was also observed with a sensitizer enhancement ratio of 2.4 (1 mM (C$_2$H$_5$)$_2$N[NO(O)-NO]+Na"*). Aerobic radiosensitivity was not affected at this concentration. The hypoxic cell radiosensitization properties of (C$_2$H$_5$)$_2$N[NO(O)-NO]+Na"*, coupled with the vasodilatory effects of NO on tumor vasculature, suggest that such agents open a new avenue of research in radiation oncology.

Introduction

The successful local treatment of some tumors with ionizing radiation is believed to be limited, in part, by the presence of hypoxic cell subpopulations within the tumor (1-3). Since hypoxic cells are approximately 3-fold more resistant to radiation than aerobic cells, a primary objective of clinical radiation oncology and basic radiobiology is identifying new approaches to sensitize and eliminate hypoxic cells from tumors (4). To address the problem of hypoxic cell radioresistance in tumors, radiobiologists and radiotherapists have evaluated high linear energy transfer radiation (i.e., neutrons), hyperbaric oxygen, and chemical hypoxic cell radiosensitizers both experimentally and clinically (4). Thus far, results from clinical trials using these approaches have not been overwhelmingly successful. Therefore, the search continues for safe and effective means to sensitize hypoxic cells to radiation.

In the course of mechanistic studies, Howard-Flanders (5) showed in 1957 that a gas, NO, could efficiently radiosensitize hypoxic bacterial cells to ionizing radiation. More recently, NO has been shown to be a key bioregulatory molecule generated endogenously in mammalian systems, suggesting that sensitization of hypoxic mammalian cells by NO may provide a new approach to this problem. The use of NO gas in animals (or humans) to radiosensitize hypoxic cells is problematic since breathing high concentrations of NO gas can damage lung tissue, and pharmacological delivery of adequate concentrations of short-lived gaseous NO to distant solid tumor sites is not possible. It would be useful to have an agent (in solution) that would be more amenable to pharmacological delivery of NO to solid tumor sites. Recently, a series of compounds called NONOates (complexes of NO with various nucleophiles) have been shown to release NO in a predictable manner under physiological conditions (6). To test whether NONOates act as hypoxic cell radiosensitizers, we have used an in vitro clonogenic assay to evaluate radiation sensitivity of cells exposed to radiation under both aerobic and hypoxic conditions. The present study shows that the NO-releasing agent DEA/NO is a potent and controllable hypoxic mammalian cell radiosensitizer.

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2 The abbreviations used are: DEA/NO, (C$_2$H$_5$)$_2$N[NO(O)-NO]+Na"*; SER, sensitizer enhancement ratios.

Materials and Methods

Chemicals. Sodium nitrite and diethylamine were purchased from Aldrich Chemical Co. (Milwaukee, WI). DEA/NO (Chemical Abstracts Service Registry No. 92382-74-6) was synthesized and assayed for NO production via chemiluminescence techniques as described previously (6). DEA/NO stock solutions were made up in 0.02 N NaOH, and the concentration was confirmed spectrophotometrically using an extinction coefficient of 8000 M$^{-1}$ cm$^{-1}$ at the characteristic absorbance band (247 nm). The rate of decomposition of DEA/NO as well as the amount of NO released from DEA/NO was found to be independent of oxygen tension. Exposure of cells to DEA/NO was done in F-12 medium with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.0. Addition of DEA/NO (or 0.02 N NaOH for control cells) to this medium gave a final pH of 7.1-7.3.

Cell Culture and Experimental Protocols. Chinese hamster V79 lung fibroblasts were cultured in F-12 medium supplemented with 10% fetal calf serum and antibiotics. Cell survival under aerobic and hypoxic conditions was assessed by clonogenic assay with the plating efficiency ranging between 70-90%. Two methods of hypoxia induction were used. For experiments involving NO-releasing agents and respective controls, hypoxia induction was achieved by metabolism-mediated oxygen depletion in dense cell suspensions as described previously (7, 8). Briefly, stock cultures of exponentially growing cells in 850-cm$^2$ roller bottles were trypsinized, rinsed with medium, and counted. Samples of 10$^6$ cells were then placed in 15-ml centrifuge tubes and rinsed once with medium or medium plus various concentrations (final concentrations ranging from 0.1-2.0 mM) of DEA/NO, nitrite, diethylamine, or DEA/NO that had been allowed to decompose in full medium overnight at 37°C prior to cell exposure. The cell pellet (0.15-0.25 ml packed cell volume) was then resuspended at 10$^6$ cells/ml in complete medium (plus 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with or without respective concentrations of the drugs and controls cited above. The cell suspension was then taken up into a 3-ml glass syringe with an 18-gauge spinal needle. Air bubbles were removed, and a 25-gauge needle was fitted on the syringe. The syringe was then rotated by hand at room temperature for a total of 20 min to allow for cellular metabolic oxygen consumption. The syringe was then placed in a lucite buildup block and irradiated. After a given dose of radiation, 1-3 drops of cell suspension were discarded, and 1-2 drops were collected in tubes containing drug-free medium. Thus, an entire hypoxic survival curve for a given condition could be obtained from a single syringe. Following irradiation, the various cell suspensions were pipeted, counted, diluted, and plated for macroscopic colony formation. Each dose determination was plated in triplicate, and experiments were repeated a minimum of 2 times. Plates were incubated 7 days. After which colonies were fixed with methanol:acetic acid (3:1), stained with crystal violet, and counted. Samples of 10$^6$ cells were then placed in 15-ml centrifuge tubes and rinsed once with medium or medium plus various concentrations (final concentrations ranging from 0.1-2.0 mM) of DEA/NO, nitrite, diethylamine, or DEA/NO that had been allowed to decompose in full medium overnight at 37°C prior to cell exposure. The cell pellet (0.15-0.25 ml packed cell volume) was then resuspended at 10$^6$ cells/ml in complete medium (plus 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with or without respective concentrations of the drugs and controls cited above. The cell suspension was then taken up into a 3-ml glass syringe with an 18-gauge spinal needle. Air bubbles were removed, and a 25-gauge needle was fitted on the syringe. The syringe was then rotated by hand at room temperature for a total of 20 min to allow for cellular metabolic oxygen consumption. The syringe was then placed in a lucite buildup block and irradiated. After a given dose of radiation, 1-3 drops of cell suspension were discarded, and 1-2 drops were collected in tubes containing drug-free medium. Thus, an entire hypoxic survival curve for a given condition could be obtained from a single syringe. Following irradiation, the various cell suspensions were pipeted, counted, diluted, and plated for macroscopic colony formation. Each dose determination was plated in triplicate, and experiments were repeated a minimum of 2 times. Plates were incubated 7 days, after which colonies were fixed with methanol:acetic acid (3:1), stained with crystal violet, and counted. Colonies containing >50 cells were scored. Error bars represent SD of the mean and are shown when larger than the symbol. Survival curve data were fitted using a linear quadratic model of Albright (9), and the survival curves shown in the figures represent the best fit curves for the experimental points. SERs were calculated by dividing the radiation dose for control hypoxic conditions by the radiation dose for various agents under hypoxic conditions at the 1% surviving fraction level.

Aerobic irradiations were carried out at a lower cell density (3 × 10$^5$/ml) in 5-ml plastic dilution tubes. To minimize air space above the cell suspension which might allow NO released from DEA/NO to escape from the solution, the dilution tubes were filled to the top with cell suspensions and capped with or without drug. Various concentrations (final concentrations ranging from 0.1-2.0 mM) of DEA/NO, nitrite, diethylamine, or DEA/NO allowed to decompose in full medium overnight at 37°C were added to the cell suspensions 20 min prior to radiation exposure. The tubes were placed in the lucite block and irradiated; following irradiation, the cell suspensions were rinsed and processed for survival as described above.

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To study the effects of NO and radiation in hypoxic cells, a second method of hypoxia induction was achieved by plating cells into glass flasks and gassing the medium over the cell monolayer with nitrogen. Cells from exponentially growing stock cultures were plated into specially designed 25-cm² glass flasks (2.5 × 10⁶ cells in 2 ml of medium/flask) (10) and incubated at 37°C overnight. Two needles (19-gauge) were pushed through a rubber stopper inserted into each flask to provide entrance and exit ports for a humidified gas mixture of 95% nitrogen/5% CO₂ (Matheson Gas Products). Stoppered flasks were connected in series, mounted on a reciprocating platform, and gassed at 37°C for 45 min. The gassing procedure resulted in an equilibrium between the gas and liquid phase and yielded oxygen concentrations in the effluent gas phase of <10 ppm as measured by a Thermox probe (10). After 45 min of deoxygenating, flasks were disconnected from the gassing system, hypoxic radiobiological conditions can be maintained for several hours (10). The various partial pressures of NO in the head space above the cell monolayer were achieved by the following method. The head space volume in the flask above the cell monolayer was determined by weighing the amount of water subsequently required to fill the flask and subtracting the volume of medium present during gassing (2.0 ml). Specific volumes of NO were delivered by an airtight syringe containing a teflon-tipped plunger into the head space to achieve the desired partial pressures of NO ranging from 10³ to 10⁵ ppm. After NO gas injection, the flasks were gently rocked for 10 min to facilitate equilibration of NO with the medium and then irradiated. After irradiation, the stoppers were quickly removed, and the medium over the cell monolayer was immediately gassed with 95% N₂ for 2 min. This precaution was taken to prevent the reaction of NO in the gas phase with oxygen which can form potentially cytotoxic oxides of nitrogen as well as acidifying the medium. After gassing, the cell monolayer was rinsed, trypsinized, counted, and plated for macroscopic colony formation as described above.

Irradiation Conditions. Cells were irradiated at room temperature with 4 MeV photons from a linear accelerator at a dose rate of 1.5 Gy/min. Full electron equilibrium was ensured for all irradiations.

Results and Discussion

To determine the extent of hypoxic radiosensitization by NO for mammalian cells, various partial pressures of NO gas were administered to cell cultures in order to compare its radiosensitizing activity with that of oxygen. For these studies, cells growing as monolayers in glass flasks were gassed with nitrogen, then known amounts of NO gas were injected into the sealed flask to yield final head space concentrations ranging from 10³ to 10⁵ ppm NO at the time of irradiation. NO gas was found to be a potent hypoxic mammalian cell radiosensitizer as shown in Fig. 1A. NO gas treatment resulted in a concentration-dependent enhancement of hypoxic radiosensitivity with the highest concentration (10⁵ ppm) yielding a SER of 3.2. The radiosensitization of hypoxic V79 cells by NO is compared to that for oxygen in Fig. 1B. NO gas was nearly as efficient as oxygen in hypoxic cell radiosensitization, the k (concentration required to yield one half the maximum effect) of 10⁴ ppm for NO being greater than the 2.5 × 10³ ppm value for oxygen. The results from this experimental system suggest that hypoxic cells could be treated clinically by irradiation if means could be found for doing so in the presence of locally high NO concentrations.

Unfortunately, exposure to gaseous NO as in the protocol described above will not be generally useful because NO is extremely reactive toward many cellular constituents; therefore, it is impractical to use the gas per se for achieving adequate exposures of any organel except lung and skin. On the other hand, NO-donor drugs should be more useful for this purpose. To test this hypothesis, we exposed cells to DEA/NO instead of NO gas before irradiating them. DEA/NO releases NO without electron transfer activation, allowing controllable dosing of biological systems with NO (6). Survival radiation curves for V79 cells exposed to varying concentrations of DEA/NO and various DEA/NO decomposition products under hypoxic conditions (metabolic hypoxia induction) are shown in Fig. 2. DEA/NO treatment alone was not cytotoxic for concentrations ranging from 0.1 to 1.0 mM and resulted in minimal cytotoxicity at 2 mM (30%). Survival curves were corrected for drug cytotoxicity when appropriate. Fig. 2A and Table 1 show that DEA/NO treatment resulted in concentration-dependent radiosensitization of hypoxic V79 cells. While the extent of radiosensitization was modest for 0.1 mM DEA/NO, significant enhancement was observed for 0.5 and 1.0 mM treatments. Increasing the DEA/NO concentration to 2.0 mM resulted in a SER of 2.9 ± 0.4, indicating that at this concentration DEA/NO was approximately as effective as oxygen in eliminating radiation resistance due to hypoxia.

To determine if the radiation sensitization resulted solely from NO originating from DEA/NO, several control experiments were conducted evaluating two decomposition products of DEA/NO, nitrite, and diethylamine. As an additional control, DEA/NO (2.0 mM) was allowed to decompose completely (and hence liberate NO) in medium maintained at 37°C overnight. This medium (containing DEA/NO decomposition products) was then used to evaluate possible effects on radiosensitization. Neither nitrite, diethylamine, or decomposed DEA/NO resulted in any cytotoxicity, nor did any of these agents significantly modify hypoxic radiosensitivity (Fig. 2B). Hence from the results shown in Fig. 2A and Table 1, it can be concluded that the
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Fig. 2. Radiation survival curves for V79 cells exposed to varying concentrations of DEA/NO or DEA/NO decomposition products under hypoxic conditions. (A) Control (○); DEA/NO, 0.1 mM (△); DEA/NO, 0.5 mM (○); DEA/NO, 1.0 mM (●); DEA/NO, 2.0 mM (★). (B) Control (○); nitrite, 2.0 mM (△); diethylamine, 2.0 mM (●); decomposed DEA/NO, initial concentration 2.0 mM (★).

Table 1 SERs for DEA/NO and related decomposition products

<table>
<thead>
<tr>
<th>Agent</th>
<th>Concentration (mM)</th>
<th>SER*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEA/NO</td>
<td>0.05</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>1.1 ± 0.06b</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>2.1 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>2.9 ± 0.40</td>
</tr>
<tr>
<td>Nitrite</td>
<td>2.00</td>
<td>1.0</td>
</tr>
<tr>
<td>Diethylamine</td>
<td>2.00</td>
<td>1.0</td>
</tr>
<tr>
<td>DEA/NO (decomposed)</td>
<td>2.00</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* SER = Survival Fraction

The oxygen enhancement ratio using this hypoxic system was 2.8 ± 0.40.

Under hypoxic conditions very few, if any, oxygen-related adducts are formed because of "repair" of the primary adduct (—C—) by endogenous reducing species; hence, less cell killing is observed.

We speculate that, being itself a free radical, NO can substitute for oxygen in reacting with carbon-centered radicals of equation A and

THREE Squares for V79 cells exposed to varying concentrations of DEA/NO or DEA/NO decomposition products under aerobic conditions. (A) Control (○); DEA/NO, 0.5 mM (△); DEA/NO, 1.0 mM (●); DEA/NO, 2.0 mM (★). (B) Control (○); nitrite, 2.0 mM (△); diethylamine, 2.0 mM (●); decomposed DEA/NO, initial concentration 2.0 mM (★).

The effects of DEA/NO on aerobic radiosensitivity are shown in Fig. 3A. DEA/NO concentrations of 0.5 and 1.0 mM had no effect on aerobic radiosensitivity; however, 2.0 mM DEA/NO enhanced aerobic radiosensitivity (enhancement at the 1% survival level was 1.3). Controls similar to those conducted above were also performed under aerobic conditions. Nitrite, diethylamine, and decomposed DEA/NO did not appreciably alter the aerobic radiosensitivity of V79 cells (Fig. 3B).

Collectively, these data clearly establish that NO is a potent hypoxic mammalian cell radiosensitizer and that NO can be delivered to cells by a chemical delivery system (DEA/NO) in sufficient concentrations to result in hypoxic cell radiosensitization. While the mechanism of NO radiosensitization is not clearly understood, Howard-Flanders (5) suggested that NO-mediated hypoxic radiosensitization is similar to that of oxygen. Radiation can produce carbon-centered radicals (in cellular DNA, the presumed target for radiation-induced cell killing) that can react with molecular oxygen (a diradical molecule) to yield an adduct that is toxic and that if not repaired will result in death of the cell (Ref. 11; see Equations A and B below).

\[
\begin{align*}
-\text{CH} + \text{X-rays} & \rightarrow -\text{C}\cdot \quad (A) \\
-\text{C}• + -\text{O}—\text{O}— & \rightarrow -\text{C}—\text{O}—\text{O}• \quad \text{(damage fixation)} \quad (B)
\end{align*}
\]

Under hypoxic conditions very few, if any, oxygen-related adducts are formed because of "repair" of the primary adduct (—C•) by endogenous reducing species; hence, less cell killing is observed.

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\[
\begin{align*}
-\text{C}• + \text{RH} & \rightarrow -\text{CH} \quad \text("repair") \quad (C)
\end{align*}
\]

We speculate that, being itself a free radical, NO can substitute for oxygen in reacting with carbon-centered radicals of equation A and

The oxygen enhancement ratio using this hypoxic system was 2.8 ± 0.40.

* Mean ± SD.
hence “fix” damage to a critical cellular/molecular species similar to that seen for oxygen.

\[ \text{C} + \text{NO} \rightarrow \text{C} \equiv \text{N}=\text{O} \text{ (damage fixation)} \]  \hspace{1cm} (D)

This scheme is consistent with the data presented regarding hypoxic radiosensitization by NO; however, for high concentrations of DEA/NO (2 mM), sensitization of aerobic cells was observed (Fig. 3B). This sensitization could result from several possibilities. NO reacts with oxygen in the gas phase and hydrophobic media (12) such as cellular lipid layers to form NO$_2$. NO can also react with oxygen in aqueous media to yield a yet uncharacterized oxide of nitrogen (NO$_3$) which can nitrosate or oxidize substrate (13).

\[
\begin{align*}
2 \text{NO}^\cdot + \text{O}_2 & \rightarrow 2 \text{NO}_2^\cdot \text{ (nonaqueous)} \hspace{1cm} (E) \\
\text{C} + \text{NO}_2^\cdot & \rightarrow \text{C} \equiv \text{N}=\text{O} \hspace{1cm} (F) \\
2 \text{NO}^\cdot + \text{O}_2 & \rightarrow \text{NO}_3^- \text{ (aqueous)} \hspace{1cm} (G) \\
\text{C} + \text{NO}_3^- & \rightarrow \text{C}^+ + \text{H}_2\text{O} \rightarrow \text{C}-\text{OH} \hspace{1cm} (H)
\end{align*}
\]

Thus, various combinations of reactions in Equations D–H may account for the enhanced aerobic radiosensitization (30% enhancement; Fig. 3B). However, it should be noted that, at a DEA/NO concentration of 1 mM, no aerobic radiosensitization was observed, yet significant hypoxic cell radiosensitization was observed (SER, 2.4) suggesting that optimal DEA/NO concentrations can yield selective hypoxic radiosensitization. Furthermore, the decomposition products of DEA/NO were shown not to influence either aerobic or hypoxic radiation sensitivity. NO was found to be somewhat less efficient in hypoxic cell radiosensitization than oxygen (Fig. 1B). This may be possibly due to the less efficient scavenging of carbon-centered radicals by NO as compared to that by oxygen.

It has recently been shown that tumor blood flow can be selectively reduced in tumor versus normal tissue by administering inhibitors of NO synthase (14), consistent with a general role for NO in regulation of vascular tone (15). Furthermore, Wood et al. (16) recently showed that SIN-1, an NO-donor molecule, when administered to tumor-bearing mice decreased inorganic phosphate in the tumor, suggesting increased tumor oxygenation. Wood et al. (16) also noted that SIN-1 administration increased X-ray sensitivity of tumor cells with a SER of ~1.2 (1% survival level). A potential problem with SIN-1 as an NO-releasing agent is that equimolar oxygen is required for NO release (17) which may be a serious limitation to its use in a hypoxic environment. In contrast, NO release from DEA/NO does not require metabolism, and the rate of release of NO is independent of oxygen concentration.

In summary, we have shown that NO gas and NO released from DEA/NO result in hypoxic mammalian cell radiosensitization. This finding, coupled with the vasodilatory effects of NO on tumor vasculature, warrants further study of such agents in cancer therapy.

References

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