Activation of Inducible Nitric Oxide Synthase Results in Nitric Oxide-mediated Radiosensitization of Hypoxic EMT-6 Tumor Cells

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Abstract

EMT-6 cells treated for 16 h with 1-10 units/ml IFN-γ showed a gradual activation of inducible nitric oxide synthase (iNOS) in Western and Northern blots, a simultaneous raise in NO output, and an increase in hypoxic cell radiosensitivity almost to the level of aerobic cells. Both the NO signal and radiosensitization were counteracted by the NO scavenger oxyhemoglobin, by the specific iNOS inhibitor aminoguanidine, and by the L-arginine analogue N^2-monomethyl-L-arginine. Collectively, these data demonstrate that IFN-γ can radiosensitize EMT-6 cells through iNOS induction and that NO is the effector molecule responsible for radiosensitization. Compared with the spontaneous NO releaser (Z)-1-{N-(3-ammoniopropyl)-N-[L-1,2-diolate]| the iNOS-generated NO signal appeared to be 10 times lower yet resulting in the same enhancement ratio of 2.4. Direct stimulation of NO synthesis in tumor cells through the L-arginine/iNOS pathway represents a novel approach to exploit the radiosensitizing properties of NO.

Introduction

The NO radical, a natural mediator involved in vasorelaxation, neurotransmission, and immunological reactivity (1), has been evaluated recently as a potent radiosensitizer of tumor cells (2). The ability of the authentic 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 (2-5). To date, no clear rationale exists on how to extend the promising radiosensitizing properties of NO in cell cultures to in vivo applications. Mitchell et al. (2, 3) suggested spontaneous NO releasers of the class of NONOates [2-(N,N-diethylamino)-diazenolate-2-oxide-Na^+, (Z)-1-{N-[3-aminopropyl]N-[4-[3-aminopropylammonium]butyl-2-amino]-diazen-1-ium-1,2-dioliate] and PAPA/NO1 as future sensitizers, considering that their constant and predictable decomposition rate may be advantageous to control the NO level. The NO donors sodium nitroprusside and SNAP cause radiosensitization through a bioreductive mechanism of NO generation (5, 6), that fits better to the concept of hypoxia-selective cytotoxicity in the bioreductive microenvironment of tumor tissue (7). Both spontaneous and bioreductive NO donors, however, would provoke systemic vasodilatation in vivo through cGMP cyclase, a physiological target for NO in vascular smooth muscle cells. One possibility to reduce these systemic effects may be to activate the NO synthesis directly in tumor tissue using the natural L-arginine/iNOS pathway. Indeed, endotoxin/cytokine-inducible iNOS is expressed in many tumors (8-11) and has already been evaluated in tumor biotherapy (12), whereas its significance to modify tumor cell radiosensitivity remains to be explored. As a first step to verify such a possibility, we have investigated whether activation of iNOS by IFN-γ in mammalian carcinoma EMT-6 cells can enhance NO production and radiosensitivity. These parameters were validated by comparison with the NO donors PAPA/NO and SNAP and various inhibitors of the L-arginine metabolism; iNOS and NO itself were used to implicate NO in radiosensitization.

Materials and Methods

Chemicals. SNAP and PAPA/NO were purchased from Alexis Corp. (Laufelfingen, Switzerland). Other chemicals were obtained from Sigma Chemical Co (St. Louis, MO).

Cell Culture. Murine mammary adenocarcinoma EMT-6 cells were kindly provided by Dr. Edith Lord (University of Rochester, Cancer Center, New York, NY). The cells were propagated in our laboratory in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% bovine calf serum (HyClone Laboratories, Inc, Logan, UT) at 37°C in 5% CO2/95% air. The cultures were split twice per week, and passages 100-140 were used to conduct our experiments.

Radiation. Cultures grown to early confluence were exposed to IFN-γ at the specified concentrations for 16 h, washed, and incubated for another hour without IFN-γ before trypsinization. Metabolic hypoxia in cell pellets was achieved as described earlier (5, 6). Briefly, 0.5 × 10^5 cells in 100 μl of medium were placed in conical plastic tubes, and pellets were produced by centrifugation at 300 × g for 5 min. Metabolic oxygen depletion in pellets was induced by a 10-min incubation at 37°C prior to radiation. In separate experiments, the radiosensitizing activity of the NO donors SNAP and PAPA/NO was estimated. For this, the NO donors were added to the cell suspension to a final concentration of 0.3 mm before pellet formation. When used, the inhibitors AG, L-NMMA, D-NMMA, and HbO were added to the cells to final concentrations of 1, 3, and 0.03 mm, respectively, and cell suspensions were incubated for 30 min before forming the pellets. In the inhibitory assays, the concentration of L-arginine in the medium was reduced to 0.1 mm. Cell pellets were irradiated at 37°C at doses 0-16 Gy, and cell survival was measured by an 8-day colony formation assay using a serial dilution approach (5, 6). The enhancement ratios were calculated at the level of 0.1 surviving fraction by dividing the radiation dose of hypoxic cells by the radiation dose of hypoxic cells treated with IFN-γ or NO donors.

Amperometric Measurement of NO. Cultures were exposed to IFN-γ as described above, and amperometric measurements were performed in cell suspensions (30 × 10^6/ml) at 37°C. The NO signal was registered by an ISO-NOP200 microsensor connected to an ISO-NO Mark II meter (both from World Precision Instruments, Hertfordshire, United Kingdom), and data were expressed in nA (6). To estimate the NO release from SNAP or PAPA/NO, these agents were used at a concentration of 0.3 mm. The conditions for inhibitory assays were specified above.

Western Blot Analysis. The cell lysates (1 × 10^5 cells) were resolved in a 7.5% polyacrylamide-SDS gel and transferred onto Bio-Rad nitrocellulose membrane (Amersham International, Buckinghamshire, United Kingdom). The blots were stained for 1 h at 20°C with the primary monoclonal antibody to iNOS (Affiniti Research Products, Exeter, United Kingdom) and analyzed by an immunoperoxidase-based ECL technique (Amersham) according to the manufacturer’s protocol.

Northern Blot Analysis. Total RNAs were prepared from EMT-6 cells treated with the TRIZol reagent (Life Technologies, Inc.). RNAs (10 μg) were electrophoresed on formaldehyde-agarose gels, transferred to HyBond-N membranes (Amersham), and probed with 32P-labeled 1.8-kb mouse iNOS cDNA.
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cDNA fragment (Alexis Corp.). To verify equal loading of RNA, a probe specific for 18S rRNA was used in the second hybridization.

Statistics. All assays were repeated at least three times. Data are expressed as means with corresponding SDs.

Results and Discussion

The synthesis of NO from L-arginine by a NOS family represents a fundamental biochemical pathway involved in the regulation of vascular tone, neurotransmission, and immunoreactivity and underlies some pathological disorders as well (1). The overexpressed iNOS is thought to be an essential component of tumor development (8–11) and therefore may be an appropriate target for therapeutic strategies based on sensitizing properties of the NO radical. Despite a well-documented radiosensitizing activity of NO released from NO donors (2–6), no data are available about whether iNOS-mediated NO output is high enough to cause radiosensitization.

To address this issue, we have investigated iNOS expression, radiosensitivity, and NO production in EMT-6 tumor cells that were exposed to IFN-γ in the range of 0.3–30 units/ml during 16 h. These conditions have been selected with the Griess assay that indicated the accumulation of nitrite, an oxidation product of NO, up to 25–50 μM at 3–10 units/ml IFN-γ. Western blot analysis showed a steep activation of the iNOS expression at 3–10 units/ml, leveling out at 30 units/ml (Fig. 1A). Northern blots revealed a similar pattern of mRNA expression, suggesting a transcriptional level of iNOS up-regulation by IFN-γ (Fig. 1B). The iNOS expression could be stimulated further when IFN-γ was combined with other activators, similar to interleukin 1β and endotoxin (data not shown), but the resulting cytotoxicity of the treatment was too high to perform radiosensitizing experiments.

The radiosensitivity of control and IFN-γ-treated EMT-6 cells was assessed under hypoxic conditions induced by metabolic oxygen depletion, a model generally applied to estimate the radiosensitizing potency of NO donors (2–6). Fig. 2A shows the radiation survival curves for EMT-6 cells treated with 1, 3, and 10 units/ml IFN-γ, which were corrected for the direct cytotoxicity of IFN-γ (surviving fraction, 0.6 or higher). The ERs for these concentrations of IFN-γ were 1.0, 1.8, and 2.4, which is in agreement with the profile of iNOS expression described above. The radiosensitizing effect observed at 10 units/ml IFN-γ was close to that of oxygen (ER, 2.5), although a trend to decreased activity was found at high radiation doses. We draw attention to the incubation interval between IFN-γ addition and irradiation of the cells, allowing transcriptional up-regulation of iNOS to develop (Ref. 1 and this study). Indeed, a 16-h incubation interval was appropriate to reveal the radiosensitizing activity of IFN-γ, whereas its addition to cells immediately before hypoxia induction and radiation did not alter radiosensitivity (data not shown). It is worth noting that the NO donors PAPA/NO and SNAP, used here at the maximal noncytotoxic concentration of 0.3 mM, demonstrated comparable or lower radiosensitizing activity (ER, 2.4 and 1.7), as shown in...
exposed to different concentrations of IFN-γ (A) or to 10 units/ml IFN-γ and treated with various inhibitors (B). To estimate the level of NO generation, the NO signal was allowed to stabilize during 4–5 min at 37°C and was corrected with the background signal in medium. In A, the bioreductive NO release from 0.3 mM SNAP in the presence of 30 × 106 cells/ml or the spontaneous NO release from 0.3 mM PAPA/NO in the absence of cells is indicated for comparison. In B, cells exposed to 10 units/ml IFN-γ were further treated for 30 min with 0.03 mM HbO, 1 mM AG, 3 mM L-NMMA, or 3 mM d-NMMA before NO measurement. Bars, SD.

Fig. 4. Amperometric measurement of NO generated by EMT-6 cells (30 × 10⁶/ml) exposed to different concentrations of IFN-γ (A) or to 10 units/ml IFN-γ and treated with various inhibitors (B). To estimate the level of NO generation, the NO signal was allowed to stabilize during 4–5 min at 37°C and was corrected with the background signal in medium. In A, the bioreductive NO release from 0.3 mM SNAP in the presence of 30 × 10⁶ cells/ml or the spontaneous NO release from 0.3 mM PAPA/NO in the absence of cells is indicated for comparison. In B, cells exposed to 10 units/ml IFN-γ were further treated for 30 min with 0.03 mM HbO, 1 mM AG, 3 mM L-NMMA, or 3 mM d-NMMA before NO measurement. Bars, SD.

Fig. 2B. Several inhibitors of the L-arginine/iNOS pathway were tested to clarify the role of NO in radiosensitization. To achieve competitive inhibition, the concentration of L-arginine in assays was reduced from 1 mm (standard RPMI 1640) to 0.1 mm (physiological level). Under these conditions, a slight decrease in radiosensitization from 2.4 to 2.3 was observed for 10 units/ml IFN-γ (Fig. 3). HbO, a potent scavenger of NO, markedly inhibited radiosensitization (ER, 1.3). AG, a specific inhibitor of iNOS, and L-NMMA, a stereoreactive analogue of L-arginine, abolished radiosensitization (ER, 1.1 or less), whereas the inactive enantiomer d-NMMA showed low inhibitory activity (ER, 1.9).

Collectively, these data provide evidence that NO is the effector molecule responsible for the increased radiosensitivity of EMT-6 cells treated with IFN-γ, and that the synthesis of NO proceeds through the L-arginine/iNOS pathway. Whether iNOS-generated NO can itself fix radiation-induced DNA damage as suggested by Howard-Flanders (13) or whether other NO-reactive species contribute to radiosensitization is still to be elucidated. Because activation of iNOS is associated with the formation of peroxynitrite from NO and the superoxide anion (14), this highly reactive NO adduct might also affect radiosensitivity. Another contributing mechanism might be NO-induced oxygen sparing through the inhibition of respiration (3), although our recent study with SNAP did not indicate such a possibility (6). To further strengthen the central role of NO in IFN-γ-induced radiosensitization, direct NO measurements in suspensions of EMT-6 cells were performed using a NO-specific microsensor. The NO signal in IFN-γ-activated EMT-6 cells increased substantially in the range of 1–10 units/ml (Fig. A4), in line with the concentration-dependent profiles of the iNOS expression and radiosensitization (see Figs. 1 and 2A). All inhibitors but d-NMMA efficiently counteracted the NO output (Fig. 4B), consistent with the survival curves in Fig. 2B. An interesting finding is that the NO signal generated by iNOS was 3- and 10-fold lower compared with that of SNAP and PAPAO/NO while resulting in a comparable or higher radiosensitizing activity.

We already introduced the hypothesis that the extracellular NO signal registered by a NO sensor represents only a part of the NO pool responsible for radiosensitization (discussed in Refs. 5 and 6). Intracellular NO adducts in the form of nitrosothiols may contribute to radiosensitization as well, yet are not detectable amperometrically. Indeed, the appearance of intracellular S-nitroso-L-glutathione upon exposure of cells to NO has been demonstrated clearly (15, 16), and this agent was shown to be a radiosensitizer (3, 5). Previous reports (3, 6) and present data indicate that SNAP-induced radiosensitization operates at lower levels of extracellular NO than those observed for NONOates, consistent with the model of membrane-catalyzed activation of bioreductive NO donors (17, 18). The present study now suggests that: (a) iNOS-mediated NO production is a further step to decrease the extracellular NO background that might be advantageous in vivo regarding vascular effects of NO; (b) direct stimulation of the NO synthesis in tumor cells represents a novel and efficient approach to radiosensitization that favors the production of the radiosensitizing molecule in the proximity of radiation targets; and (c) IFN-γ may be considered as a radiation response modifier that acts through activation of the natural L-arginine/iNOS pathway.

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References

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