Nitric Oxide Radicals Choreograph a Radioadaptive Response

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Abstract
The reduced biological effects of radiation exposure seen in cells after conditioning exposures to a low dose or at a low-dose rate (i.e., the acquisition of resistance against high-dose irradiation) is called the "radioadaptive response" and many studies concerning this phenomenon have been reported since the 1980s. Radioadaptive responses have been observed using various end points, such as chromosomal aberrations, mutations, and clonogenic survival. However, the mechanisms of the radioadaptive response are not fully known. Here, we show that radiation-induced nitric oxide (NO) radicals contribute to the induction of radioresistance as determined by cell survival after a subsequent high-dose exposure. An accumulation of inducible NO synthase was produced, and the concentration of nitrite in the culture medium increased when cells were exposed to γ-rays at a low-dose rate or to X-rays for a low dose followed by an acute high-dose X-irradiation. In addition, the induction of radioresistance was not observed in the presence of an inhibitor of inducible NO synthase or a scavenger of NO radicals. Moreover, radioresistance was observed when cultures were treated with a NO radical–generating agent. These findings suggest that NO radicals are an initiator of the radioadaptive response. [Cancer Res 2007;67(18):8574–9]

Introduction
In 1984, the term, "radioadaptive response" was coined by Olivieri and colleagues (1) who studied chromosomal aberrations in human lymphocytes following irradiation. They showed that the frequency of chromosomal aberrations after irradiation with X-rays was less than expected when the cells were preexposed to [3H]thymidine (1). Subsequently, several other reports, using a range of human and mammalian cells, have confirmed these findings (2–7). Although the mechanisms responsible for the radioadaptive response are still not fully understood, several key findings have been reported. It was shown, for example, that the transcription and translation of genes that participate in antioxidant machinery (8), DNA repair, and cell cycle regulation are required for the adaptive response in human lymphocytes and fibroblasts (9, 10). Thus, the radioadaptive response depends on the de novo synthesis of proteins, most of which are involved in DNA damage responses, such as antioxidant enzymes (11) and/or DNA repair enzymes (12).

Sasaki reported that the activation of protein kinase C was required for radioadaptive responses in murine m5S cells (13). Furthermore, he and his colleagues showed that in radioadapted murine cells, p53 plays a key role in regulating and guiding radiation-induced DNA double-strand breaks into the appropriate legitimate repair pathway (14, 15). It has also been reported that the accumulation of p53 observed after high-dose irradiation (acute irradiation) was strongly suppressed by a prior low-dose rate irradiation (chronic irradiation; ref. 16). Our group has reported that p53-dependent apoptosis after exposure to high-dose radiation was also found to be suppressed by chronic irradiation in cultured cells in vitro and in the spleens of mice in vivo (17). In addition, it seems that p53 can control the secretion of certain growth-inhibitory factors in response to stress in vitro and in vivo (18). On the other hand, p53 which accumulated after irradiation can attenuate the induction of inducible nitric oxide (NO) synthase (iNOS, also known as NOS2), which catalyzes the conversion reaction of L-arginine into L-citrulline, through the interaction between p53 and TATA binding protein and/or nuclear factor κB, which are essential for iNOS expression (19–22). Moreover, NO radicals secreted from irradiated cells with mutant p53 induced radioresistance in unirradiated wild-type p53 cells through intercellular signaling (21, 22). Other published works have shown that down-regulation of endogenous murine double minute 2 (Mdm2) following NO radical treatment was likely to contribute to the activation of p53 (23), and that NO radicals induced a specific feature of p53 phosphorylation for activation, distinct from the pattern evoked by other inducers, such as ionizing radiation, UV light, and Adriamycin (24). These findings led to the proposal that the repressed p53-dependent response is one of the mechanisms involved in the radioadaptive response. In the work reported here, additional observations of the role of p53 and NO radicals in the radioadaptive response are described.

Materials and Methods

Reagents. Aminoguanidine was purchased from Research Biochemicals International. Isosorbide dinitrate (ISDN) was purchased from Shionogi & Co., Ltd. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was purchased from DuPont/NEN Research Products. Giemsa solution was purchased from Shionogi & Co., Ltd. Aminoguanidine was purchased from Research Biochemicals International. Isosorbide dinitrate (ISDN) was purchased from Shionogi & Co., Ltd. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was purchased from DuPont/NEN Research Products. Aminoguanidine was purchased from Research Biochemicals International. Isosorbide dinitrate (ISDN) was purchased from Shionogi & Co., Ltd. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was purchased from DuPont/NEN Research Products. Aminoguanidine was purchased from Research Biochemicals International. Isosorbide dinitrate (ISDN) was purchased from Shionogi & Co., Ltd. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was purchased from DuPont/NEN Research Products. Aminoguanidine was purchased from Research Biochemicals International. Isosorbide dinitrate (ISDN) was purchased from Shionogi & Co., Ltd. 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was purchased from DuPont/NEN Research Products.

Cell culture. Human glioblastoma cell line A-172 was purchased from the JCRB Cell Bank. This cell line bears the wild-type p53 gene (25). Cells were cultured at 37°C in a conventional humidified CO2 incubator using DMEM containing 10% fetal bovine serum, penicillin (50 units/mL), streptomycin (50 μg/mL), and kanamycin (50 μg/mL); DMEM-10).

Irradiation of cells with γ-rays or X-rays. Chronic irradiation with γ-rays (1 mGy/min) was delivered with a 137Cs γ-source (Sangyo Kagaku...
Treatment with aminoguanidine or c-PTIO. Just before irradiation at a low-dose rate or at a low dose, cells were washed twice with DMEM-10 and then exposed to DMEM-10 containing 50 μmol/L of aminoguanidine or c-PTIO. After the irradiation, cells were subsequently irradiated at a high-dose rate, and then incubated at 37°C in a conventional humidified CO₂ incubator, without changing the medium, to allow the formation of colonies. When cells were treated with aminoguanidine, only during the irradiation at a low-dose rate, after the irradiation was done, cells were washed twice with DMEM-10, exposed to DMEM-10, exposed to a subsequent irradiation at a high-dose rate, and then allowed to form colonies. When cells were treated with aminoguanidine during and after an irradiation at a high-dose rate, just after an irradiation at low-dose rate in the absence of aminoguanidine, cells were exposed to DMEM-10 containing aminoguanidine, exposed to an irradiation at high-dose rate, and then allowed to form colonies.

Treatment with ISDN and/or c-PTIO. One, 6, or 12 h before X-irradiation at a high-dose rate, cells were washed twice with DMEM-10 and then exposed to DMEM-10 containing 5 μmol/L of ISDN with or without 10 μmol/L of c-PTIO. After irradiation, cells were incubated at 37°C in a conventional humidified CO₂ incubator without changing the medium, and allowed to form colonies.

Western blotting. Aliquots of proteins (20 μg) were used for Western blotting for p53, Hdm2, and iNOS. Following electrophoresis on 10% polyacrylamide gels containing 0.1% SDS and transfer by electrophoresis to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation), proteins on the membrane were incubated with primary antibodies. For visualization of protein bands, horseradish peroxidase–conjugated anti-mouse or antirabbit IgG antibodies and BLAST was used.

Measurement of nitrite concentration in medium. Nitrite concentrations in the medium were measured using the method of Saltzman (26) with some modifications (27). One hundred microliters of medium was mixed with 150 μL of a reagent containing 0.5% sulfanilic acid, 0.002% N-1-naphthylethylenediamine dihydrochloride, and 14% acetic acid. After incubation at room temperature for 15 min, the absorbance of the samples at 550 nm was measured. A solution of sodium nitrite dissolved in medium was used as a standard.

Survival curves. The surviving cell fraction after irradiation was determined using colony-forming assays, and adjusted by the plating efficiency of untreated cells as a control. Two replicate flasks were used per experiment, and two or more independent experiments were done for each survival point. Colonies were fixed with methanol and stained with a 2% Giemsa solution. The D₀ value represents the dose required to reduce the survival to 1/e on the exponential portion of the survival curves. The D₁₀ dose is defined as the dose required reducing the survival to 10% of the control.
Results

Induction of radioresistance. We first analyzed the changes in cellular radiosensitivity after acute X-irradiation in cells pretreated with chronic \( \gamma \)-irradiation to confirm the induction of the radioadaptive response and to examine whether NO radicals could mediate such a response. After chronic irradiation for 25 h (dose rate, 1.0 mGy/min; total dose, 1.5 Gy), human glioblastoma A-172 cells showed a significant reduction in radiosensitivity following a subsequent acute irradiation to graded doses of X-rays (Fig. 1B). The \( P \) value was 0.01 for the reduction in radiosensitivity after a chronic irradiation followed by an acute irradiation at 1 Gy. The \( D_{0} \) value in chronically irradiated cells was 4.8 \( \pm \) 0.2 Gy (\( P = 0.02 \)), but was 2.7 \( \pm \) 0.3 Gy after a single acute irradiation. The \( D_{10} \) value was 12.4 \( \pm \) 0.5 Gy (\( P = 0.09 \)) after chronic irradiation versus 7.4 \( \pm \) 0.5 Gy following an acute irradiation alone. In addition, the reduction of radiosensitivity in the cells was almost completely suppressed by the addition of aminoguanidine (a specific inhibitor of iNOS, 50 \( \mu \)mol/L) to the culture medium at any time during pretreatment with a low-dose rate irradiation (Fig. 1B). Similar observations were also made with aminoguanidine treatment during the colony-formation period following an acute irradiation (Fig. 1C), or when the drug was present throughout the experiment (Fig. 1D). Aminoguanidine did not affect the intrinsic radiosensitivity of the cells (Fig. 1A).

The findings in Fig. 1 confirm that a radioadaptive response in cell survival can be induced in cells pretreated with a chronic, low-dose rate irradiation and subsequently challenged by a single, high-dose exposure. These results suggest that the accumulation or activation of iNOS and the secretion of NO radicals may contribute to the radioadaptive response.

Lack of p53 accumulation coupled with activation of Hdm2.

We further analyzed the accumulation and activation of Hdm2 during chronic irradiation. Although an accumulation of p53 was initially induced by chronic irradiation for 1 and 3 h (at doses of 0.06 and 0.18 Gy, respectively), the level of p53 gradually declined from its peak levels for the remainder of the chronic irradiation period of 25 h (Fig. 2). In contrast, the accumulation of Hdm2 was induced by the chronic, 3-h irradiation, and the higher level of Hdm2 was maintained throughout the irradiation period. At the same time, Hdm2 phosphorylation at serine 166 was observed.

These results strongly suggest that the lack of p53 accumulation in cells pretreated with a low-dose chronic exposure followed by an acute irradiation may be due to the accumulation and activation of Hdm2 during chronic irradiation.

Accumulation of iNOS. To verify the hypothesis described above, we analyzed the changes in iNOS levels and the concentrations of nitrite in the medium after acute X-irradiation alone, chronic \( \gamma \)-irradiation alone, and acute X-irradiation following pretreatment with chronic \( \gamma \)-irradiation. Figure 3 shows the accumulation of iNOS in the cells after pretreatment with chronic irradiation followed by an acute irradiation, but not after either irradiation alone, resulting in an increase in nitrite concentrations in the medium.

Accumulation of iNOS and induction of radioadaptive response. We confirmed the induction of the radioadaptive response following the classic mode of irradiation in which cells were preirradiated with priming X-rays at a low dose (specifically, 20 mGy at 240 mGy/min), incubated at 37°C for an appropriate interval (12 h) and then irradiated with acute X-rays (challenging irradiation of 1–5 Gy at 1 Gy/min). The accumulation of iNOS in the cells and an increase in nitrite concentration in the medium were observed after an acute irradiation following preirradiation, but not after either the priming or challenging irradiation alone (Fig. 4A–C). In addition, an increased radioresistance in the cells was detected after the classic mode of irradiation. The \( P \) value was 0.05 for the increase in radioresistance after a low-dose irradiation followed by an acute irradiation at 1 Gy. The \( D_{0} \) values after an acute irradiation with or without preirradiation were 4.9 \( \pm \) 0.5 Gy (\( P = 0.05 \)) and 3.3 \( \pm \) 0.2 Gy, respectively. The \( D_{10} \) values were 12.1 \( \pm \) 0.5 Gy (\( P = 0.09 \)) and 8.1 \( \pm \) 0.5 Gy (Fig. 4D). In addition, the induction of radioresistance was almost completely suppressed by the addition of c-PTIO, a NO radical-specific scavenger, to the culture medium. The results shown in Fig. 4 were consistent with the results represented in Figs. 1 and 3.

Induction of radioresistance by exogenous NO. Changes in cellular radiosensitivity were analyzed after acute X-irradiation in...
the presence of ISDN with or without c-PTIO to examine whether the NO radical–generating agent induced radioresistance. Figure 5 shows the survival curves of the cells in the presence of ISDN with or without c-PTIO. A significant radioresistance in the cells was observed after pretreatment with ISDN for 1, 6, or 12 h prior to irradiation.

The \( D_0 \) value for the cells pretreated with ISDN for 6 and 12 h before exposure to an acute radiation dose was 3.4 \( \pm \) 0.3 Gy (\( P = 0.04 \)) and 3.2 \( \pm \) 0.3 Gy (\( P = 0.05 \)), respectively, compared with a \( D_0 \) of 2.7 \( \pm \) 0.3 Gy after acute irradiation alone. The \( D_0 \) for the cells pretreated with ISDN for 6 and 12 h before an acute irradiation was 9.6 \( \pm \) 0.5 Gy (\( P = 0.01 \)) and 9.2 \( \pm \) 0.5 Gy (\( P = 0.02 \)), respectively, compared with a value of 7.4 \( \pm \) 0.5 Gy after a single acute irradiation. The acquisition of radioresistance in the cells following treatment with ISDN was almost completely suppressed by the addition of c-PTIO.

**Discussion**

The radioadaptive response is a phenomenon in which the biological effects of a challenging radiation exposure to high doses were reduced by a priming exposure to low doses or at a low-dose rate (1). However, a variety of environmental agents, stress-inducing agents, and chemotherapeutic agents are able to induce an increased resistance to the subsequent mutagenic effects induced by ionizing radiation (28, 29). In addition, in experiments using human lymphocytes with introduced restriction enzymes Alu, Dra, or NotI, all three of the restriction enzymes induced radioadaptive responses because they reduced the number of chromosome breaks produced by a subsequent exposure to X-rays (30). These findings imply the possibility that a priming exposure to low doses or exposure at a low-dose rate is not essential for the induction of the radioadaptive response.

In the work reported here, the role of p53 and NO radicals in the radioadaptive response was examined. The findings in Fig. 1 indicated that a radioadaptive response, as measured by cell survival, could be induced after a chronic irradiation followed by an acute irradiation. In addition, the radioadaptive response was almost completely suppressed by the addition of aminoguanidine to the medium at any time during the chronic irradiation period (Fig. 1B), during the colony formation period following an acute irradiation (Fig. 1C), or when the agent was present throughout the experiment (Fig. 1D). Especially, the results in Fig. 1C indicate that post-challenge modification of cellular responses could modify the radioadaptive response and that the induction of antioxidant and/or DNA repair enzymes by the priming irradiation is not required. Wiencke et al. (31) observed similar results using the poly(ADP-ribose) polymerase inhibitor, 3-aminobenzamide. We reported that poly(ADP-ribose) polymerase was deeply involved in p53-dependent intracellular signaling pathways from the experiment using 3-aminobenzamide (32). In addition, Ibuki et al. (33) found a correlation between poly(ADP-ribose) polymerase and NO in the responses induced by radiation. Therefore, the correlation between poly(ADP-ribose) polymerase and NO may play an important role in the induction of radioadaptive response. The results in Fig. 1 suggest that NO radicals generated by the accumulated and activated iNOS might be responsible for the induction of radioresistance in the cells. These observations also suggest that the response of wild-type p53 could be blunted by chronic irradiation followed by an acute irradiation. Indeed, it has been previously reported that an accumulation of p53 was not induced after a chronic irradiation (dose rate, 1.0 mGy; total dose, 10 for the cells treated with ISDN for 6 and 12 h after low-dose and/or high-dose irradiation, and nitrite concentrations were measured. The irradiation conditions are indicated by the rows in A and the line graphs in B and C. a, after a low-dose X-irradiation (0.02 Gy); b, after a high-dose X-irradiation (2.0 Gy); c, after a low-dose X-irradiation (0.02 Gy) followed by a high-dose X-irradiation (2.0 Gy). C, no irradiation. *, \( P < 0.05 \), for the comparison between control and irradiations. D, radiosensitivity after a high-dose X-irradiation following a low-dose X-irradiation. ○, high-dose X-irradiation alone; ●, high-dose X-irradiation given after a low-dose X-irradiation; △, irradiation in the presence of c-PTIO throughout the entire procedure. 

**Figure 4.** Accumulation of iNOS and induction of radioadaptive response after challenging irradiation following priming irradiation. A, cells were harvested at various time points (0–12 h) after a low-dose and/or high-dose irradiation, and NO radicals were detected by Western blotting. B, relative amounts of iNOS were calculated from the scanning profiles of Western blots (three independent experiments). C, medium over the cells was sampled at various time points (0–12 h) after low-dose and/or high-dose irradiation, and nitrite concentrations were measured. The irradiation conditions are indicated by the rows in A and the line graphs in B and C. a, after a low-dose X-irradiation (0.02 Gy); b, after a high-dose X-irradiation (2.0 Gy); c, after a low-dose X-irradiation (0.02 Gy) followed by a high-dose X-irradiation (2.0 Gy). C, no irradiation. *, \( P < 0.05 \), for the comparison between control and irradiations. D, radiosensitivity after a high-dose X-irradiation following a low-dose X-irradiation. ○, high-dose X-irradiation alone; ●, high-dose X-irradiation given after a low-dose X-irradiation; △, irradiation in the presence of c-PTIO throughout the entire procedure.

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chronic irradiation followed by an acute irradiation, but not by irradiation followed by an acute irradiation. The secretion of NO radicals could be induced after a chronic irradiation. In addition, these findings suggest that iNOS may be down-regulated by NO if iNOS was accumulated and NO cannot be accumulated during a chronic irradiation because Hdm2 was activated (37). Briefly, the repressed p53-dependent response, and the acquisition of radioresistance was almost completely suppressed by the addition of c-PTIO (Fig. 5). It was observed after treatment with ISDN at extremely low concentrations because NO radicals are relatively stable and easily excreted from irradiated cells through the induction of cytoprotective actions because NO radicals secreted endogenously from cells after such irradiations could protect the irradiated cells through the induction of cytotoxic actions. Radioresistance seems to be dependent on p53, which suppresses the accumulation of iNOS and nitrite in the medium of the cells were induced after a low-dose irradiation followed by an acute irradiation with an intervening period as is the case in Fig. 3. Moreover, the radioresistance following the classic mode of irradiation was induced when compared with the results of a single acute irradiation in Fig. 1. These results strongly suggest that NO radicals generated by the accumulated and activated iNOS might actually contribute to the induction of radioresistance of the cells.

Finally, we analyzed the changes in cellular radiosensitivity after acute X-irradiation in the presence of ISDN with or without c-PTIO to examine whether ISDN can induce radioresistance in cells. In accordance with expectations, the acquisition of radioresistance was observed after treatment with ISDN at extremely low concentrations in a manner similar to the so-called radioadaptive response, and the acquisition of radioresistance was almost completely suppressed by the addition of c-PTIO (Fig. 5). It was previously reported that NO radicals secreted from irradiated p53 cells induced radiosensitivity in the wild-type p53 cells. Tokumizu et al. (42) reported that the treatment of cultured macrophages, RAW264.7 cells, with several NO radical donors reduced the micronuclei frequency induced by γ-irradiation, suggesting that NO radicals may act as a signal for repair systems. In addition, soluble factors released from irradiated cells, such as reactive oxygen species and NO radicals, seem to be important for the induction of radioresistance (43). These results

Figure 5. Effect of ISDN on radiosensitivity after single acute X-irradiation. (A) in the absence of drugs; (B) in the presence of ISDN; (C) in the presence of both ISDN and c-PTIO. These drugs were added at 1 (A), 6 (B), and 12 h (C) before irradiation.

Figure 6. Possible model of the signaling pathways involved in the radioadaptive response. Radioresistance seems to be induced by the radiation-induced NO radicals at extremely low concentrations because irradiation at a low dose or at a low-dose rate followed by an irradiation at a high-dose rate induced iNOS through the activation of Hdm2 during the low-dose or low-dose rate irradiation, which suppressed the accumulation and activation of p53.
strongly resemble those obtained when cells were irradiated with low-dose/low-dose rate radiation. From the abovementioned reports, it could be concluded that NO radicals can initiate the radioadaptive response.

Many reports concerning the radioadaptive response have been published since the 1980s. Most mechanisms associated with the radioadaptive response have been understood to involve the induction of antioxidant and/or DNA repair enzymes. Here, it is proposed that radioreistance observed in the radioadaptive response can be induced by NO radicals at extremely low concentrations, which are endogenously generated after exposure to radiation. This could occur because irradiation at a low-dose rate followed by an irradiation at a high-dose rate induces NO radical generation through the activation of Hdm2, which is induced by the initial low-dose rate irradiation, and depresses p53 accumulation (Fig. 6). Additional studies of this proposed mechanism are still required to resolve the mechanisms involved in cellular responses to low-dose/low-dose rate radiation.

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