Nitric Oxide Radical-induced Radioadaptation and Radiosensitization 
Are G2/M Phase-dependent

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Nitric oxide/Radiosensitivity/Cell cycle/Clonogenic cell survival/Chromosome aberrations.

The aim of this study was to examine biological effects of nitric oxide (NO) on radiosensitivity and chromosome aberrations in different phases of the cell cycle in human cancer cells with a wild-type p53 (wt-p53) genotype. H1299/wt-p53 cells were pre-treated with isosorbide dinitrate (ISDN) at different concentrations or pre-irradiated with a low dose of X-rays, and then exposed to a high dose of X-rays. Cell synchronization was achieved with serum starvation. Cellular radiosensitivity, cell cycle distributions, and chromosome aberrations were assayed with colony-forming assays, flow cytometry and chromosome banding techniques, respectively. After treatment with ISDN at a low concentration or after an exposure to 0.02 Gy of X-rays, radioresistance and a reduction in the number of chromosome aberrations were observed mainly 17.5 h after plating mitotic cells. This radioadaptation effect was observed during a clearly shortened G2/M phase and a slightly prolonged S phase. In contrast, in the presence of a high concentration of ISDN, radiosensitization and the enhancement of chromosome aberrations were detected principally 17.5 h after plating mitotic cells, and this radiosensitization was observed during a significantly prolonged G2/M phase and a slightly shortened S phase. A range of concentrations of NO induced opposing effects on radiosensitivity and chromosome aberrations in human non-small cell lung cancer cells bearing wt-p53 gene status, and these different effects produced by NO depended on the cell cycle phase.

INTRODUCTION

Nitric oxide (NO) is a highly reactive free radical which participates in numerous reactions.1–3 NO is a ubiquitous molecule which is capable of inducing a multitude of biological effects, and which also plays an extremely critical role in the regulation of tumor evolution and progression.4–7 In recent years, the influence of NO on radiosensitivity in cancer cells has become a very active research area.8–11 In addition, there have been reports on investigations of the mode of action of NO and its products.12,13 Radiosensitization and radioresistance have been examined in different types of tumor cells exposed to a range of doses of NO, delivered through different routes, and with different time courses.14–21 However, the precise mechanisms through which NO is able to mediate the induction of radiosensitivity are still not completely known.

Evidences suggest that the cell cycle and NO radicals may interact in determining cell survival or cell death. NO can induce a cell cycle arrest leading to the induction of irreversible growth arrest and cell death.22–26 In particular, the position in the cell cycle in which cells are exposed to NO radicals may determine cell survival or cell death.27,28

Previous researches have led to a signaling pathway model which attempts to describe the induction of radioresistance and the depression of chromosome aberrations via the action of p53 and NO radicals.19,21 This model includes the following steps: (a) a pre-irradiation exposure activates the human homolog of murine double minute 2 (Hdm2) during the interval between radiation exposures; (b) Hdm2 leads to the degradation of p53 through ubiquitination; (c) the tran-
scriptional suppression of inducible NO synthase (iNOS, or alternatively, NOS2) is released by the loss of p53; (d) the second irradiation induces an accumulation of iNOS; (e) iNOS generates NO radicals; and (f) NO radicals induce radioresistance and the depression of chromosome aberrations. Further research has indicated that low and high concentrations of isosorbide dinitrate (ISDN), which can act as an NO donor,\textsuperscript{30} can result in opposing effects on radiosensitivity, apoptosis and chromosome aberrations in human lung cancer cells, and that NO radicals can affect the fate of wild-type p53 (wt\textsubscript{p53}) cells.\textsuperscript{29} However, it is unclear if the radiosensitivity of tumor cells exposed to NO depends on the cell cycle phases in which the exposure occurs.

The purpose of the study described here was to examine the dual effects of different doses of NO radicals on radiation-induced cell killing and chromosome aberrations as a function of the cell cycle phase in human cells bearing a wt\textsubscript{p53} genotype. A novel finding described here indicates that the biphasic effects of NO radicals on radiosensitivity and chromosome aberrations are tightly related to the cell cycle position in human lung cancer cells with a wt\textsubscript{p53} gene status.

**MATERIALS AND METHODS**

**Reagents**

ISDN was provided by the Takata Seiyaku Co. Ltd. (Tokyo, Japan). Giemsa solutions, dimethyl sulfoxide (DMSO), and colcemid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). RNase and propidium iodide (PI) were provided by Sigma Chemical Co. (St. Louis, MO, USA).

**Cells**

Human H1299 non-small cell lung cancer cells with a deleted p53 gene (provided by Dr. Moshe Oren, Weizmann Institute of Science, Rehovot, Israel) were stably transfected with a wt\textsubscript{p53} gene. The resulting cell line with a wt\textsubscript{p53} gene is designated H1299/wt\textsubscript{p53}.\textsuperscript{31} This H1299/wt\textsubscript{p53} cell line was kindly provided by Dr. Hideki Matsumoto (University of Fukui, Fukui, Japan). All cells were cultured in Dulbecco’s modified Eagle’s medium (MP Biomedicals Inc., Illkirch, France) containing 10% (volume/volume) fetal bovine serum (MP Biomedicals Inc.), 20 μmol/ml 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Nacalai Tesque, Inc.), 50 units/ml penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), 50 μg/ml streptomycin (Meiji Seika Kaisha Ltd.), and 50 μg/ml kanamycin (Nacalai Tesque, Inc.) (DMEM-10). The doubling time of this cell line is about 24 h. Exponentially growing cells which were grown to a density of about 80% of confluence were used for each experiment, and were cultured at 37°C in a conventional humidified CO\textsubscript{2} incubator.

**X-irradiation**

Acute X-irradiation (1.0 Gy/min, 20 mA) and pre-irradiation X-ray exposures (250 mGy/min, 5 mA) were delivered with a 150-kVp X-ray generator (Model MBR-1520R, Hitachi, Tokyo, Japan). Under the present experimental conditions, no marked change of pH was detected in the medium during the treatment.

**Treatment with ISDN**

ISDN was used as an NO donor as previously described.\textsuperscript{17} Six hours prior to acute X-irradiation, cells were exposed to DMEM-10 containing ISDN at different concentrations (5 or 500 μM). After treatments, cells were incubated at 37°C in a conventional humidified CO\textsubscript{2} incubator, with no subsequent medium changes.

**Cell synchronization**

Cell synchronization was accomplished as previously described.\textsuperscript{32} Cells with a 95% mitotic index were obtained 28 h after the initiation of serum starvation (Fig. 1A). Mitotic cells floating in the medium were collected by centrifugation and re-plated in DMEM-10. Cells synchronized at various parts of the cell cycle were treated with ISDN or exposed to X-rays. After a 6 h incubation, the cell cycle profile was analyzed (Fig. 2).

**Survival analysis**

After synchronization, 400 or 1,000 of the floating mitotic cells were re-plated in 25 cm\textsuperscript{2} flasks and used as controls or were exposed to 6 Gy of X-rays, respectively, for survival analysis. The surviving cell fraction was determined using colony forming assays. Three replicate flasks were used per experiment, and two independent experiments were performed. Eight days after treatments, colonies were fixed.

**Fig. 1.** Cell cycle distribution. Panel A shows a typical photograph (×400) of cells synchronized at mitotic phase. Panel B shows the percentage of cells in the various phases of the cell cycle after plating mitotic cells.
with methanol and stained with a 2% Giemsa solution. Microscopic colonies containing more than approximately 50 cells were counted as having arisen from single surviving cells. The error bars in the figures indicate standard deviations (n = 6).

Cell cycle analysis
Cells were fixed with cold 67% methanol in phosphate-buffered saline (PBS) and kept in a freezer at −20°C for 3 h to 2 weeks before analysis. Cells were centrifuged and rinsed with PBS containing 0.05% Tween 20 (TPBS). The cells were blocked with 4% BSA in TPBS for 15 min at room temperature and rinsed with TPBS. Cells were then incubated for 30 min at room temperature with 1 mg/ml RNase and 50 μg/ml PI. Cell cycle distributions were assayed by determining cellular DNA content. Cell cycle profiles were determined using no fewer than 10,000 cells. Profiles were generated using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Before flow cytometric analysis, samples were filtered through a 35-μm nylon mesh. Three independent experiments were performed for each data point (n = 6).

Analysis of chromosome aberrations
The induction of chromosome aberrations was scored by the observation of dicentrics as previously described.29) Cells were irradiated with 3 Gy of X-rays 6 h after exposures to 5 or 500 μM ISDN, or after a 0.02 Gy irradiation. The cells were then exposed to DMEM-10 containing 0.2 μg/ml colcemid 40 h after the treatments, and the cells were fixed 42 h after the treatments. Cells were allowed to swell for 20 min in 75 mmol/l KCl, fixed with three changes of methanol/acetic acid (3:1), and dropped onto a clean micro-slide glass (Matsunami Glass Industrial, Ltd., Osaka, Japan) with a pipette. Slides were stained with 2% Giemsa for 30 min, and metaphase spreads were scored for dicentrics per cell. In all cases, dicentrics were counted in a minimum of 1,500 chromosomes (36 cells in every group). The error bars in the figures indicate standard deviations (n = 6).

Statistical analysis
Significance levels were calculated using the Student’s t-test. Asterisks (*, ** and ***) indicate statistically significant differences (P < 0.05, 0.01 and 0.001, respectively).

RESULTS

Cell cycle distribution
The cell cycle distribution was analyzed in the cells with flow cytometry. Figure 1B shows the population of cells in each cell cycle phase after plating mitotic cells. The population of cells in G2/M phase was decreasing 0.5 h after plating mitotic cells, whereas the population of cells in the G1 phase was increasing. The population of cells in the G1 phase had increased to 81.2 ± 1.4% 2.5 h after plating mitotic cells, and the percent of the cells in the S and G2/M phases was less than 20%. The population of cells in the G1 phase decreased slowly, and the increase in the population of cells in S phase was not more rapid than that of population of cells in the G2/M phase, but was observed earlier than that of population of cells in the G2/M phase. The population of cells in the S phase was 57.5 ± 0.7% 12.5 h after plating mitotic cells, which was 2.6 times the population of cells in the G2/M phase. Thereafter, the population of cells in the S phase began to decrease, and the population of cells in the G2/M phase continued to increase. The increase in the number of G2/M phase cells reached a peak 17.5 h after plating the mitotic cells, and the population of cells in the G2/M phase was 69.0%; concurrently, the population of cells in both, the S and G1 phases was about 15%.

Effect of different concentrations of ISDN on the cell cycle distribution
A pre-irradiation (0.02 Gy) or pre-treatment with ISDN (5 or 500 μM) was delivered 0.5, 2.5, 7.5, 12.5 and 17.5 h after plating wt p53 mitotic cells. Following these treatments, changes in the cell cycle distribution were observed with flow cytometry (Fig. 2). Noticeably, 17.5 h after plating mitotic cells, the cells pre-irradiated with a low dose of X-rays or exposed to a low dose pre-treatment with ISDN were able to contribute to the introduction of a clearly shortened G2/M phase (P < 0.01 when compared to the control group)
and to a slightly prolonged S phase ($P < 0.05$ when compared to the control group). In addition, pre-treatment with ISDN at a high dose resulted in a significant increase of cell numbers in the G$_2$/M phase ($P < 0.001$ when compared to the control group) and to a reduction of cell numbers in S phase ($P < 0.05$ when compared to the control group).

**Effect of ISDN on cell survival depends on the cell cycle position**

Cell survival was examined by counting the number of colonies per dish 8 days after treatments (Fig. 3). Noticeably, 17.5 h after plating mitotic cells, a significant radioadaptation effect was observed in the presence of a low concentration of ISDN or after a pre-irradiation with a low dose of X-rays, if these treatments were administrated before an acute irradiation. When cells were pre-treated with 5 μM ISDN or pre-irradiated with 0.02 Gy of X-rays, the number of dicentrics per cell was reduced by 45.9 ± 1.4% or 44.2 ± 1.7% when compared with the irradiated control group ($P < 0.01$). On the other hand, the number of dicentrics per cell increased to 48.8 ± 0.8% in the presence of 500 μM ISDN in the irradiated cells ($P < 0.01$ vs the irradiation control group). Cells at the G$_2$/M phase were clearly the most sensitive to high ISDN dose induced chromosome aberrations, indicating that the effect of ISDN on chromosome aberrations is dependent on the cell cycle phase.

**DISCUSSION**

Exponentially growing cells are distributed asynchronously throughout the four phases of the cell cycle: G$_1$, S, G$_2$, and M. Because metabolic processes are different in the various phases of the cell cycle, the cell cycle phase can determine a cell’s relative sensitivity to genotoxic stresses, and synchronized populations of cells are not only useful, but almost necessary in studies of the effects of NO radicals on cellular radiosensitivity and chromosome aberrations in each phase of the cell cycle in cancer cells. The research described here utilized synchronized cells collected at various cell cycle phases after serum starvation (Fig. 1). The cell cycle phase
distributions after plating synchronized mitotic cells are shown in Figure 1B. Most of the cells were in G1 phase 2.5 h after plating mitotic cells. Majority of the cells in the G1 phase had entered S phase 12.5 h after plating. Most of the cells in S phase had progressed into the G2/M phase 17.5 h after plating. These data were obtained with flow cytometry and are consistent with previous reports. This implies that the present experimental protocol and model of cell synchronization is valid.

In the work reported here, the effect of the cell cycle on radioadaptation or the radioenhancement of cell survival levels induced by different concentrations of ISDN (an NO radical generating agent) was determined with the use of colony forming assays (Fig. 3). It was found that a high concentration of ISDN resulted in radiosensitization in the G2/M phase (Fig. 3E). Cell survival and cell death associated with exposure in the G2/M phase have been observed in several experimental systems involving damage responses. Previously, it has also been reported that PAPA NONOate (pNO) induced cell death in the G2/M phase in colon cancer cells, whereas confluent HT29-C1.16E cells, predominantly in the G0/G1 phase, were insensitive to NO treatment. The entry of pNO-treated cells into the G2/M phase was associated with actin depolymerization and actin S-glutathionylation. The dynamic balance of glutathione associated with progression into the G2/M phase was shifted toward an oxidized state following NO treatment. E6 cells were arrested by oxidants such as H2O2 and died at the prophase or prometaphase stages of mitosis, which can be inhibited by B-cell lymphoma gene 2 (bcl-2) over-expression or by caspase inhibition. Arsenite induced cell death is associated with mitosis in HeLa-S3 cells. The diverse effects of NO radicals on radiosensitivity in cancer cells were primarily dependent on its concentration. When a low dose pre-irradiation stimulated tumor cells to produce low levels of NO radicals after an acute irradiation in wt p53 cells, or when an NO donor such as ISDN released extremely low levels of NO radicals in the cells, radioresistance was observed instead of radiosensitization, and this was easily observed from the cellular survival rate. The work described here appears to be a novel report showing that the radioadaptation induced by low doses of NO radicals is also G2/M phase-dependent (Fig. 3E). Cells in the G2/M phase were also the most sensitive to ISDN-induced cell lethality. Due to these observations, it appears desirable to elucidate the precise mechanisms responsible for this G2/M phase role in NO radical mediated radioadaptation and radioenhancement.

An important finding in this study is the coupling of G2/M arrest and cell death at the G2/M phase, because a cell cycle arrest is a mechanism commonly used to explain alterations in sensitivity to radiation.
of radiosensitivity in tumor cells. Also, cell cycle regulation is perhaps the most important determinant of ionizing radiation sensitivity. A common cellular response to DNA-damaging agents is the activation of cell cycle checkpoints. DNA damage initiates signals which can ultimately activate either temporary checkpoints to permit genetic repair or an irreversible growth arrest which results in cell death.90 High doses of NO radicals produce genetic stresses and can induce DNA damage leading to alterations in cell cycle regulation.91–93 It has been reported that two NO donors (exogenous nitric oxide), S-nitroso-N-acetylpenicillamine (SNAP, 0.4 mM, 24 h) and sodium nitroprusside (SNP, 1 mM, 24 h) significantly inhibit cell growth and increase the fraction of cells in the G2/M phase, and induce cytotoxicity and cell death in lung carcinoma cells.94 Other reports show that endogenous nitric oxide can affect the microfilament system in interleukin-6 (IL-6) treated Mm1 cells and block the cell cycle in the early G2/M phase,95 and that NO-induced DNA damage can lead to a prolonged G2 arrest.96 These previous reports are consistent with the work reported here (Fig. 3). A high concentration of ISDN can lead to significant increases in cell numbers in the G2/M stage, and to a slight cell number reduction in S phase (Fig. 2). The cell cycle phase determines a cell’s relative radiosensitivity, with cells being most radiosensitive in the G2/M phase, and least sensitive during the latter part of the S phase.90 A larger number of cancer cells were arrested in the G2/M phase, which increased cellular sensitivity and promoted cell death. It appears clear that fewer cancer cells were blocked in the G2/M phase, which reduced cellular sensitivity and decreased levels of cell death. This implies that the dual effects of NO on the cell cycle are concentration-dependent and the impact of ISDN on cell survival depends on the cell cycle phase.

In a manner similar to cell cycle arrest, chromosome aberrations also play an important role in NO radical induction of cancer cell radiosensitivity. Previous studies have shown that NO induced mutagenesis both in vitro as well as in vivo.97–99 In the work reported here, the number of dicentrics per cell was greatly increased in the presence of 500 μM ISDN in irradiated cells (Fig. 4J). Possibly NO and its oxidative products were able to induce deamination, nitration, and oxidation of guanine, producing xanthine, 8-nitro-guanine, and 8-oxo-guanine, respectively. In particular, xanthine and 8-nitro-guanine are unstable in DNA and can be quickly converted to apurinic/apyrimidinic sites through depurination.100 This reaction can then conceivably lead to DNA damage, chromosome aberrations and cell death. This report demonstrates that a treatment with ISDN at a low concentration, or an irradiation with 0.02 Gy of X-rays, before a 3 Gy X-irradiation reduced the number of radiation induced dicentrics per cell (Fig. 4J). This is also one of reasons for using H1299/wtp53 cells in the present study: a previous report indicated that during the radioadaptive response, chromosome aberrations were not found in p53 knockout cells.101 Other work has shown that the treatment of cultured macrophages (RAW264.7 cells) with a variety of NO radical donors resulted in reduced micronuclei frequencies induced by γ-irradiation, suggesting that NO radicals may act as a signal leading to a repair system activation which reduces micronuclei frequency.102 Mutations are usually formed in damaged chromosomes during cell division. In fact, in a manner similar to that seen in cell survival, alterations in the frequencies of chromosome aberrations mediated by different doses of NO radicals was also G2/M phase-dependent (Figs. 4E and J). Thus it can be seen that the different effects of ISDN on X-ray induced chromosome aberrations depend on the cell cycle phase.

In conclusion, this report documents novel opposing effects of NO radicals and a conditioning irradiation on radiosensitivity and chromosome aberrations: these are dependent on the position of the cells in the cell cycle in human lung cancer cells bearing a wt p53 gene status. The radioadaptation and radioenhancement phenomena generated by these conditioning agents are G2/M phase-dependent. However, additional studies of this proposed mechanism are still required to define precise details involved, such as the up-regulation of DNA repair during these cellular responses to low and high doses of NO radicals.

ACKNOWLEDGMENTS

This work was sponsored by Grants-in Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 21310040). This work was funded in part by a grant from the Central Research Institute of the Electric Power Industry of Japan. This work was also partially supported by a grant from the China Scholarship Council.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Received on February 22, 2011
Revision received on May 12, 2011
Accepted on May 12, 2011
J-STAGE Advance Publication Date: July 15, 2011