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BIOLOGY CONTRIBUTION

BIPHASIC EFFECTS OF NITRIC OXIDE RADICALS ON RADIATION-INDUCED LETHALITY AND CHROMOSOME ABERRATIONS IN HUMAN LUNG CANCER CELLS CARRYING DIFFERENT *p53* GENE STATUS

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Purpose: **The aim of this study was to clarify the effects of nitric oxide (NO) on radiation-induced cell killing and chromosome aberrations in two human lung cancer cell lines with a different *p53* gene status.**

Methods and Materials: **We used wild-type (wt) *p53* and mutated (m) *p53* cell lines that were derived from the human lung cancer H1299 cell line, which is *p53* null. The w*tp53* and m*p53* cell lines were generated by transfection of the appropriate *p53* constructs into the parental cells. Cells were pretreated with different concentrations of isosorbide dinitrate (ISDN) (an NO donor) and/or 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) (an NO scavenger) and then exposed to X-rays. Cell survival, apoptosis, and chromosome aberrations were scored by use of a colony-forming assay, Hoechst 33342 staining assay and TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP [deoxyuridine triphosphate] nick end labeling) assay, and chromosomal banding techniques, respectively.**

Results: **In w*tp53* cells the induction of radioresistance and the inhibition of apoptosis and chromosome aberrations were observed in the presence of ISDN at low 2- to 10-μmol/L concentrations before X-irradiation. The addition of c-PTIO and ISDN into the culture medium 6 h before irradiation almost completely suppressed these effects. However, at high concentrations of ISDN (100–500 μmol/L), clear evidence of radiosensitization, enhancement of apoptosis, and chromosome aberrations was detected. However, these phenomena were not observed in m*p53* cells at either concentration range with ISDN.**

Conclusions: **These results indicate that low and high concentrations of NO radicals can choreograph inverse radiosensitivity, apoptosis, and chromosome aberrations in human lung cancer cells and that NO radicals can affect the fate of w*tp53* cells.** © **2010 Elsevier Inc.**

NO, *p53*, Radiosensitivity, Apoptosis, Chromosome aberration.

INTRODUCTION

Nitric oxide (NO) is involved with multiple signaling mechanisms that lead to the modifications of proteins. These protein changes are concentration dependent and include immediate binding to heme centers, nitrosylation of thiol and amine groups, nitration of tyrosine, oxidation of thiols, and conjugation of soluble guanylyl cyclase and transcription factors. Sustained production of NO acts as a proapoptotic modulator by activating caspases, whereas low or physiologic concentrations of NO prevent cells from entering apoptosis (1). Nitric oxide can directly motivate cytochrome *c* release through losses of potential in mitochondrial membranes where cytochrome *c* can induce caspase-dependent apoptotic signaling pathway (2). Nitric oxide has also been found to upregulate the *p53* tumor suppressor gene in the nucleus. P53 is phosphorylated, can not bind to Mdm2 and Mdm2 can not lead to the degradation of P53 through ubiquitination (3). Activation of downstream genes then occurs and results in increasing the ratio of Bax to Bcl-XL, which are apoptotic regulatory molecules involved in the apoptotic pathway (4).

Ionizing radiation (IR) can lead to deoxyribonucleic acid (DNA) double-strand breaks that activate DNA damage

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checkpoints, leading to the initiation of signals, which ultimately result in a binary decision between cell death and cell survival. P53 has been recognized as an important checkpoint protein involved in the cell's complex response to IR. The resulting sequence of events will determine the cell's fate: induction of growth arrest coupled with DNA damage repair, leading to the cell's survival, or induction of irreversible growth arrest and apoptosis, leading to the cell's death (5).

Current questions about these systems are as follows: How do NO and IR synergistically affect survival and growth in cancer cells? What role does *p53* play in this process? It has been reported that conditioning exposures of X-irradiation at low doses and at low dose rates can reduce radiation-induced (*p53*)–dependent apoptosis in cultured cells (6). A conditioning radiation exposure has also been reported to suppress P53 function (7). These findings have led to a proposal suggesting that this suppressed *p53*-dependent response is one of the mechanisms likely to be involved in the radioadaptive response (8). A signaling pathway model has been proposed to describe the induction of radioresistance and the depression of chromosome aberrations via the action of *p53* and NO radicals (9, 10). This model includes the following steps: (*1*) a priming irradiation exposure activates Hdm2 during the interval between radiation exposures; (*2*) Hdm2 leads to the degradation of P53 through ubiquitination; (*3*) the decrease of P53 relaxes the depression of inducible NO synthase (iNOS) induction; (*4*) the second challenging irradiation induces an accumulation of iNOS; (*5*) iNOS generates NO radicals; and (*6*) NO radicals induce radioresistance and the depression of chromosome aberrations. In addition, it has been reported that acquisition of radioresistance in wild-type (wt) *p53* cells was observed after treatment with an NO-generating agent at extremely low concentrations in a manner similar to that seen with the radioadaptive response. This acquisition of radioresistance was almost completely suppressed by the addition of an NO radical scavenger (9, 10). However, these initial observations are very recent, and the detailed mechanisms that are responsible for the induction of radiosensitivity are still not fully understood.

The aim of this study is to clarify the effect of different doses of NO radicals on radiation-induced cell killing and chromosome aberrations in cancer cells, as well as possible molecular mechanisms related to the action or involvement of *p53*. We report, for the first time to our knowledge, that the biphasic effects of NO radicals on radiosensitivity and chromosome aberrations are *p53* dependent in human lung cancer cells.

METHODS AND MATERIALS

Reagents

Isosorbide dinitrate (ISDN), an NO radical–generating agent (11), was provided by Takata Seiyaku Co. Ltd. (Tokyo, Japan). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO), an NO radical scavenger (12), Giemsa solutions, glutaraldehyde, Hoechst 33342, dimethyl sulfoxide, and colcemid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). ApopTag *in situ* Detection Kit was provided by Millipore Co. (Billerica, MA). Hematoxylin was provided by Muto Pure Chemicals Co., Ltd. (Tokyo, Japan).

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 either a wild-type (wt) *p53* gene or a mutated (m) *p53* gene (in which codon 248 is altered to code for Trp [TGG] rather than Arg [CGG]). The resulting cell lines with a wt*p53* or an m*p53* gene are designated H1299/wt*p53* or H1299/m*p53* cells (13). These H1299/wt*p53* and H1299/m*p53* cell lines were kindly provided by Dr. Hideki Matsumoto (Fukui University, Fukui, Japan). H1299/m*p53* cells have lost *p53* functions such as the induction of apoptosis and *p53*-regulated gene products after exposure to X-rays (14). The doubling time of these cell lines was about 24 h. Exponentially growing cells that 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 carbon dioxide incubator.

X-irradiation

X-ray (1.0 Gy/min, 20 mA) exposures were delivered with a 150-kVp (peak kilovoltage) X-ray generator (Model MBR-1520R; Hitachi, Ltd., Tokyo, Japan). H1299/wt*p53* and H1299/m*p53* cells were irradiated with 3 or 6 Gy.

Treatment with ISDN and/or c-PTIO

Isosorbide dinitrate and/or c-PTIO was used as previously described (10, 15). Six hours before X-irradiation, cells were washed twice with DMEM-10 (Dulbecco's modified Eagle's medium [MP Biomedicals Inc., Illkirch, France] containing 10% [v/v] fetal bovine serum [MP Biomedicals Inc.]) and then exposed to DMEM-10 containing different concentrations of ISDN (1, 2, 5, 10, 20, 50, 100, 200, and 500 μmol/L) with or without 10-μmol/L c-PTIO. After treatments, cells were incubated at 37°C in a conventional humidified carbon dioxide incubator, with no subsequent medium changes.

Survival analysis

Exponentially growing cells were treated at 6 h after plating in 25-cm^2 culture flasks. The surviving cell fraction was determined by use of colony-forming assays. Three replicate flasks were used per experiment, and two or more independent experiments were performed for each survival point. Eight days after a 6-Gy X-irradiation, colonies were fixed 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 SDs.

Analysis of apoptosis

Two days after a 6-Gy X-irradiation, attached and detached cells were collected with trypsin. Induction of apoptosis was analyzed by the detection of apoptotic bodies and DNA fragment with Hoechst 33342 staining assay and TUNEL (terminal deoxynucleotidyl transferase–mediated dUTP [deoxyuridine triphosphate] nick end labeling) assay, respectively.

In Hoechst 33342 staining assay, cells were fixed with 1% glutaraldehyde in phosphate-buffered saline solution (PBS) at 4°C, washed with PBS, stained with 0.2-mmol/L Hoechst 33342, and then observed under a fluorescence microscope. Apoptosis was characterized by nuclear and cytoplasmic condensation leading to the formation and release of apoptotic bodies.

In TUNEL assay cells were fixed with 66.7% methanol in PBS at 4°C, and apoptotic cells were examined by staining with an