

Mechanism of genotoxicity induced by targeted cytoplasmic irradiation

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BACKGROUND: Direct damage to DNA is generally accepted as the main initiator of mutation and cancer induced by environmental carcinogens or ionising radiation. However, there is accumulating evidence suggesting that extracellular/extranuclear targets may also have a key role in mediating the genotoxic effects of ionising radiation. As the possibility of a particle traversal through the cytoplasm is much higher than through the nuclei in environmental radiation exposure, the contribution to genotoxic damage from cytoplasmic irradiation should not be ignored in radiation risk estimation. Although targeted cytoplasmic irradiation has been shown to induce mutations in mammalian cells, the precise mechanism(s) underlying the mutagenic process is largely unknown.

METHODS: A microbeam that can target the cytoplasm of cells with high precision was used to study mechanisms involved in mediating the genotoxic effects in irradiated human–hamster hybrid (A_L) cells.

RESULTS: Targeted cytoplasmic irradiation induces oxidative DNA damages and reactive nitrogen species (RNS) in A_L cells. Lipid peroxidation, as determined by the induction of 4-hydroxynonenal was enhanced in irradiated cells, which could be suppressed by butylated hydroxyl toluene treatment. Moreover, cytoplasmic irradiation of A_L cells increased expression of cyclooxygenase-2 (COX-2) and activation of extracellular signal-related kinase (ERK) pathway.

CONCLUSION: We herein proposed a possible signalling pathway involving reactive oxygen/nitrogen species and COX-2 in the cytoplasmic irradiation-induced genotoxicity effect.

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Direct damage to DNA caused by environmental carcinogens and ionising radiation has been considered as a significant initiator of deleterious effects, such as mutation and cancer. However, recent reports suggest that extracellular/extranuclear targets may have a role in mediating the genotoxic effects of radiation (Little, 2000; Morgan, 2003). In our previous studies, we have shown that irradiation of the cytoplasm produces gene mutations in the nucleus of the hit cells and that this process is mediated by free radicals (Wu *et al*, 1999).

One of the most abundant oxidative lesions caused by reactive oxygen species (ROS) in DNA is 8-hydroxydeoxyguanosine (8-OHdG), a potential mutagenic lesion (Gajewski *et al*, 1990). Although evidence has indicated that X-ray, γ -ray, and ultraviolet radiation are able to induce the formation of 8-OHdG in purified DNA and various cultured cells (Kamiya *et al*, 1992; Takeuchi and Morimoto, 1994), direct proof of oxidative DNA damages induced by extranuclear events including targeted cytoplasmic irradiation is lacking. Another type of bioactive molecules, the reactive nitrogen species (RNS) such as peroxy nitrite anions (ONOO⁻), are formed through interaction of

nitric oxide (NO) with superoxide anions. Reactive nitrogen species have been shown to be potent inducers of apoptosis and necrosis. They may also inhibit DNA repair mechanisms leading to mutation and carcinogenesis (Burney *et al*, 1997). Although there is evidence suggesting a close association between RNS and cellular response induced by irradiation (Hirst and Robson, 2007), the role of RNS in the mutagenic effect of α -particle irradiation is not clear.

The presence of oxidants as well as free radicals in cellular systems can also adversely alter lipids, proteins, and DNA (Devasagayam *et al*, 2004). Decomposition of the lipid peroxyl radicals or the primary free radical intermediate of lipid peroxidation lead to the generation of reactive aldehydes such as 4-hydroxynonenal (4-HNE). 4-Hydroxynonenal has been implicated in a number of oxidative stress-related diseases, including atherosclerosis (Requena *et al*, 1996), neurodegenerative diseases (Floyd and Hensley, 2002), and fibrosis (Leonarduzzi *et al*, 1997). Moreover, there have been numerous studies indicating that 4-HNE can stimulate cellular proliferation (Dianzani *et al*, 1999), differentiation (Yang *et al*, 2003), and cytoprotective response (Dickinson *et al*, 2002; Zhang *et al*, 2005) through effects on multiple signalling pathways.

To investigate the underlying mechanisms involved in the mutagenicity of cytoplasmic irradiation, we used a charged particle microbeam developed at the Radiological Research Accelerator

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Facility of Columbia University, in which the cytoplasm of individual cells could be targeted and irradiated with defined numbers of α -particle traversals at $90 \text{ keV } \mu\text{m}^{-1}$ (Hei *et al*, 1997). A sensitive mutagenic assay system using the human–hamster hybrid A_L cells was used for the study. We show here that cytoplasmic irradiation induced the formation of 8-OHdG, and that inhibition of nitric oxide synthase with N^G -methyl-L-arginine (L-NMMA) could suppress the mutant fraction. As downstream products generated from free radicals and oxidants, we also detected significantly increased levels of 4-HNE, cyclooxygenase-2 (COX-2), and the activation of extracellular signal-related kinase (ERK).

MATERIALS AND METHODS

Cell culture

The human–hamster hybrid A_L cells that contain a standard set of Chinese hamster ovary-K1 chromosomes and a single copy of human chromosome 11 were used (Waldren *et al*, 1979). These cells were cultured in Ham's F12 medium supplemented with 8% heat inactivated fetal bovine serum (Atlanta Biological, Norcross, GA, USA), $2 \times 10^{-4} \text{ M}$ glycine, and $25 \mu\text{g ml}^{-1}$ gentamycin at 37°C in a humidified 5% $\text{CO}_2/95\%$ air incubator and passaged as described (Wu *et al*, 1999). All the chemicals are from Sigma (St Louis, MO, USA) except where otherwise indicated.

Exposure to α -particles

The layout and methods for the cytoplasmic irradiation using the microbeam have been described previously (Hei *et al*, 1997; Wu *et al*, 1999). For precision targeting of cellular cytoplasm, exponentially growing A_L cells were plated at a density of 500–600 cells per microbeam dish 24 h before irradiation. An image analysis system was used to locate each cell and a precision beam was delivered to the cytoplasmic sites with an accuracy of $\pm 1 \mu\text{m}$ as described. The cytoplasm of each cell was irradiated at each of two sites chosen to be $8 \mu\text{m}$ from the ends of the major axis of each nucleus as described (Wu *et al*, 1999). Dose measurement and accuracy were determined using the detector mounted on the microscope lens.

Treatment with dimethyl sulfoxide (DMSO)

A_L cells were treated with 8% DMSO 10 min before and 3 min after irradiation with eight α -particles targeted through the cytoplasm. The dose of DMSO used was nontoxic and nonmutagenic under the conditions used in the study and as shown by others (Wu *et al*, 1999). After treatment, cells were fixed with 5% acetic acid–ethanol at -20°C for immunoperoxidase staining.

Treatment with inhibitors

A_L cells were treated with the lipid peroxidation inhibitor, butylated hydroxyl toluene (BHT, $100 \mu\text{M}$), the mitogen-activated protein (MAP) kinase cascade inhibitor PD98059 ($50 \mu\text{M}$), L-NMMA or its inactive D-enantiomer, D-NMMA (both at 1 mM from Molecular Probes, Eugene, OR, USA), or the COX-2 inhibitor NS-398 ($50 \mu\text{M}$) before irradiation with eight α -particles targeted through the cytoplasm. Medium containing the inhibitor was added back to the microbeam dish after irradiation. Cells were incubated for 4 h and fixed with 4% paraformaldehyde for immunocytochemistry analysis. For peroxynitrite detection, cells were incubated for 16 h before fixation and immunostaining. For the mutagenesis assay, cells were incubated overnight in medium containing inhibitors. The medium was removed just before the irradiation and then fresh medium with inhibitors was added back

to the cells and they were grown for 16–18 h and then returned back to grow in medium without inhibitors.

Immunoperoxidase staining for 8-OHdG

Induction of 8-OHdG in the nucleus of A_L cells irradiated with α -particles through the cytoplasm was quantified using the monoclonal antibody 1F7 specific for 8-OHdG coupled with immunoperoxidase staining and image analysis software as described (Hei *et al*, 1998; Xu *et al*, 1999). A Cell Analysis System CAS 200 microscope and a Cell Measurement Software package (Becton Dickinson, San Jose, CA, USA) were used to quantify the relative staining intensity from 50 randomly selected cells per dish. A total of 150–200 cells were measured from either the control or the irradiated groups. Data presented are the average absorbance of three experiments multiplied by 1000.

Immunostaining

At 4 h after irradiation, A_L cells were washed three times with PBS, fixed for 15 min at room temperature in 4% (w/v) paraformaldehyde in PBS and then washed in PBS. The fixed cells were permeabilised with 0.1% Triton X-100 for 10 min and washed three times with PBS. The cells were incubated for 1 h at room temperature in PBS containing 5% (v/v) goat serum and then incubated for 1 h in the same medium containing anti-4-HNE ($10 \mu\text{g/ml}$, R&D Systems, Inc, Minneapolis, MN, USA), COX-2 (1:100, Cayman Chemical, Ann Arbor, MI, USA), p-ERK (1:100, Cell Signaling Technology, Inc, Danvers, MA, USA) or nitrotyrosine antibody (1:100, Molecular Probes, Carlsbad, CA, USA) at room temperature. Staining for nitrotyrosine was used as a surrogate marker for peroxynitrite anions. The cells were washed and bound primary antibodies were detected by the reaction with Alexa Fluor 488 goat anti-mouse or Alexa Fluor 594 goat anti-rabbit IgG antibody diluted 1:200, for 1 h. Cells were thoroughly washed and the polypropylene layer was cut out and placed on a glass slide. A cover glass was placed on top of the polypropylene layer and mounted using VECTASHIELD HardSet Mounting Medium (Vector laboratories, Burlingame, CA, USA). Samples were examined using a Nikon C1 confocal fluorescence microscope (Nikon, Melville, NY, USA). Semiquantitative estimation of fluorescence intensity was obtained using the composite images generated by Image J software (NIH, Bethesda, MD, USA). Relative staining intensity from 100 randomly selected cells was analysed per dish. A total of 300–400 cells were measured from either the control or the irradiated groups.

Mutation assay

After irradiation, cells were trypsinised and plated into a T-25 flask and cultured for 7–9 days. To determine mutant yield, 5×10^4 cells were plated into each of six 60 mm dishes in a total of 2 ml growth medium as described (Hei *et al*, 1998). The cells were incubated for 2 h to allow cell attachment. Then 3% CD59 antiserum and 1.6% freshly thawed complement (v/v) were added to each dish. The cultures were further incubated for 7–10 days before being fixed, stained, and scored for the number of CD59[−] mutants. The cultures from each treatment were tested for mutant yield for two consecutive weeks to ensure the full expression of the mutation. Mutant fractions were calculated as the number of surviving colonies divided by the total number of cells plated after correction for non-specific killing because of complement alone.

Statistics

Statistical analysis was carried out using Student's *t*-test. Differences between means are regarded as significant if $P < 0.05$.

RESULTS

Induction of oxidative stress in cytoplasmic irradiation

To determine whether cytoplasmic irradiation can induce oxidative DNA damage, we used immunoperoxidase staining to detect 8-OHdG levels after irradiating with eight α -particles through the cytoplasm. A representative immunoperoxidase staining for 8-OHdG is shown in Figure 1A. 8-Hydroxydeoxyguanosine was localised mainly in the nucleus of both control and irradiated cells. Although a faint, background staining was evident in the control cultures, cytoplasmic irradiation resulted in a significant induction of 8-OHdG in A_L cells. Quantification of staining in 200 randomly selected cells indicated that there was a 2.7-fold increase in the relative staining intensity of 8-OHdG in cells irradiated with eight α -particles through the cytoplasm when compared with the non-irradiated cells. Concurrent treatment with the free radical scavenger DMSO suppressed formation of 8-OHdG and reduced the staining intensity close to background levels (Figure 1B, $P < 0.05$). Furthermore, cells that were cytoplasmically traversed by α -particles also exhibited higher fluorescence intensity in chloromethyl dichlorodihydrofluorescein diacetate (a cell-permeable fluorescent probe), for enhanced level of intracellular ROS (data not shown).

To evaluate the involvement of RNS in cytoplasmic irradiation, an antibody specific for nitrotyrosine was used as a surrogate marker for peroxynitrite anions to evaluate RNS generation. We detected approximately a two-fold increase in the level of peroxynitrite anion induction in cytoplasmically irradiated cells. The presence of L-NMMA, an inhibitor of nitric oxide synthase, suppressed peroxynitrite levels to control levels (Figure 1C). In addition to overall changes in fluorescence intensity, it was clear that the fluorescence signal in irradiated cells were localised both in nuclei and cytoplasm (Figure 1C). A_L cells pretreated with L-NMMA and irradiated showed detectable staining in the cytoplasm, but to a much less extent (50% less). When we pretreated A_L cells with L-NMMA, the treatment significantly reduced mutation yield by approximately three-fold (Figure 1D, $P < 0.05$). On the other hand, pretreatment of A_L cells with an equivalent dose of the inactive enantiomer, D-NMMA, had no effect on the induction of mutations. These results indicate that RNS are also involved in the induction of mutations as a result of cytoplasmic irradiation.

Cytoplasmic irradiation induces lipid peroxidation

The presence of free radicals and oxidants often results in lipid peroxidation. As the major α , β -unsaturated aldehydes derived

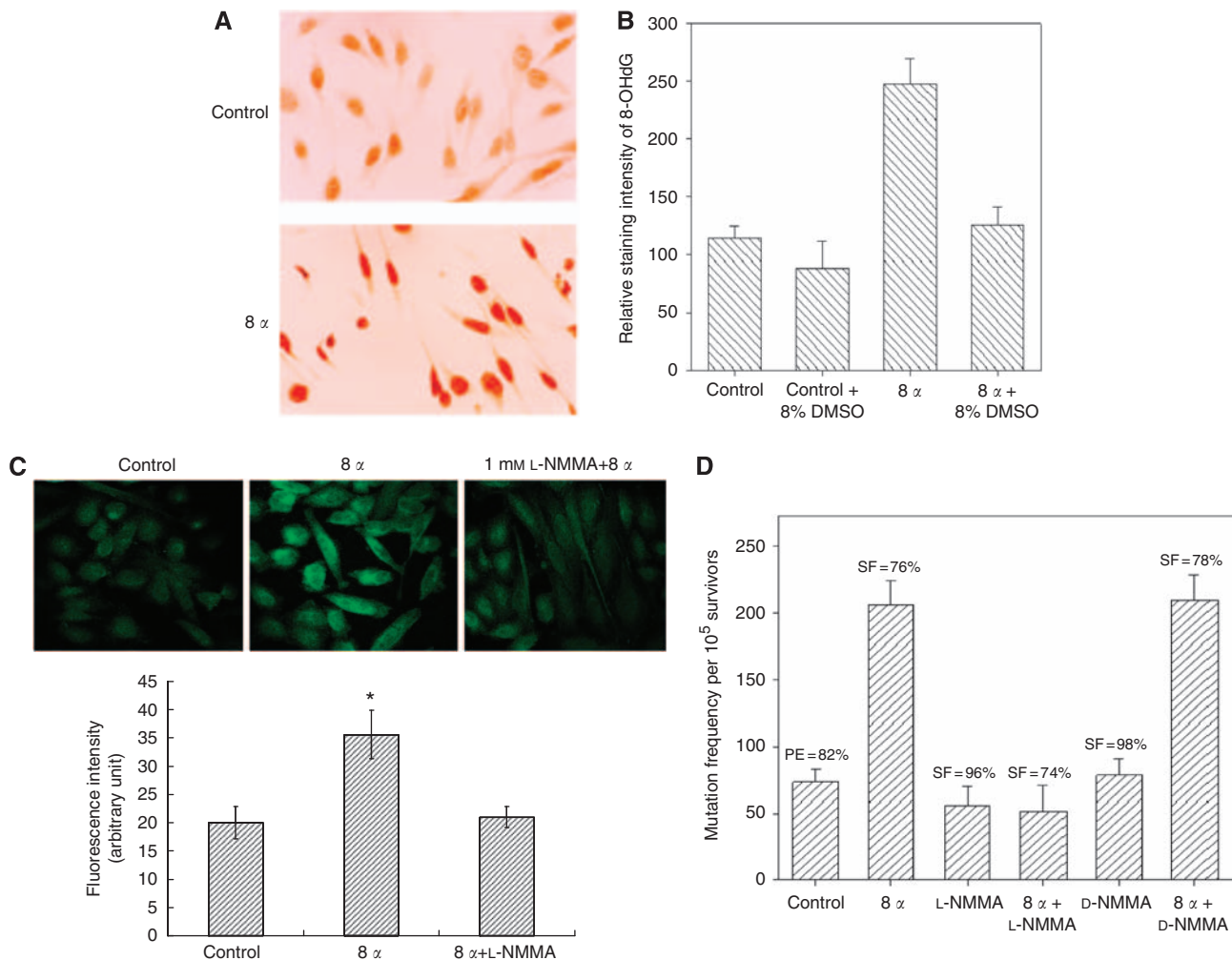


Figure 1 Induced ROS and RNS in cytoplasmic-irradiated A_L cells. **(A)** Immunoperoxidase staining for 8-OHdG in A_L cells. **(B)** Effect of DMSO on the induction of 8-OHdG in A_L cells irradiated with eight α -particles (8 α) through the cytoplasm. **(C)** Nitrotyrosine fluorescent signals in A_L cells after irradiation with 0 (control) or eight α -particles. **(D)** Effect of L-NMMA or D-NMMA on the mutagenic potential of cytoplasmic irradiation at the CD59 locus of A_L cells. Data averaged from three independent experiments. Bars indicate \pm s.e. of sample means (asterisk indicated $P < 0.05$). PE = plating efficiency; SF = surviving fraction.

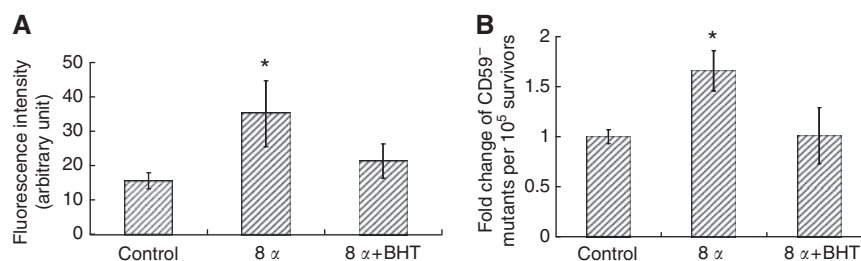


Figure 2 Lipid peroxidation in cytoplasmic-irradiated A_L cells. **(A)** Relative fluorescence intensity of 4-HNE in A_L cells after irradiation with 0 (control) or eight α -particle (8 α). **(B)** Effect of BHT on the mutagenic potential of cytoplasmic irradiation at the *CD59* locus of A_L cells. Data were averaged from three independent experiments. Bars indicate \pm s.e. of sample means (asterisk indicated $P < 0.05$).

from lipid peroxidation, 4-HNE is considered a biomarker of oxidative stress (Poli *et al*, 1987; Esterbauer *et al*, 1991) and has been shown to stimulate cellular proliferation (Dianzani *et al*, 1999), differentiation (Yang *et al*, 2003), and cytoprotective responses (Dickinson *et al*, 2002; Zhang *et al*, 2005) through various signalling pathways. Our next logical step was to analyse the lipid peroxidation level in irradiated cells. As indicated in Figure 2A, cells irradiated with eight α -particles through the cytoplasm showed a significantly higher level of 4-HNE compared with the control, non-irradiated cells ($P < 0.05$). Moreover, pre-treating the cells with BHT, a well known lipid peroxidation chain blocker, suppressed the induction of lipid peroxidation.

To further evaluate the involvement of lipid peroxidation in cytoplasmic irradiation, we compared the *CD59*⁻ mutant yield in A_L cells with or without pre-treatment with BHT. As shown in Figure 2B, A_L cells irradiated with eight α -particles through the cytoplasm showed approximately 1.8-fold higher mutant yield as compared with control, non-irradiated cells, which is consistent with our previous report (Wu *et al*, 1999). However, when A_L cells were pre-treated with 100 μ M of BHT, the *CD59*⁻ mutation fraction was reduced back to a level that was compatible with the non-irradiated control, suggesting a link between lipid peroxidation and the mutagenic effect of cytoplasmic irradiation.

Cyclooxygenase-2 is induced in cytoplasmic-irradiated cells

Cyclooxygenases (COXs) are a group of enzymes that catalyse the conversion of arachidonic acid into eicosanoids. Cyclooxygenase-2 is an inducible isoform of COX and is responsible for the production of high levels of prostaglandin during tissue inflammation and other immune responses. It has been demonstrated that in foamy macrophages within human atheromatous lesions, COX-2 expression was associated with accumulation of lipid peroxidation product 4-HNE, suggesting an inducer function of 4-HNE on COX-2 in this system (Kumagai *et al*, 2004). To evaluate whether there is a similar relationship between lipid peroxidation and COX-2 in our cytoplasmic irradiation system, we measured the COX-2 expression in A_L cells by immunostaining. It was found that COX-2 was significantly up-regulated after cytoplasmic irradiation. Furthermore, cells pre-treated with BHT partially suppressed COX-2 expression in irradiated cells (Figure 3A). Overnight treatment with 50 μ M of COX-2 inhibitor NS-398 before the irradiation significantly reduced the *CD59*⁻ mutant yield to basal levels, thus providing further support of COX-2 involvement in cytoplasmic irradiation-induced mutagenesis (Figure 3B).

Activation of ERK signalling pathway in cellular response to cytoplasmic irradiation

The MAPK pathways are responsible for the various growth factor-mediated cellular events such as proliferation, senescence,

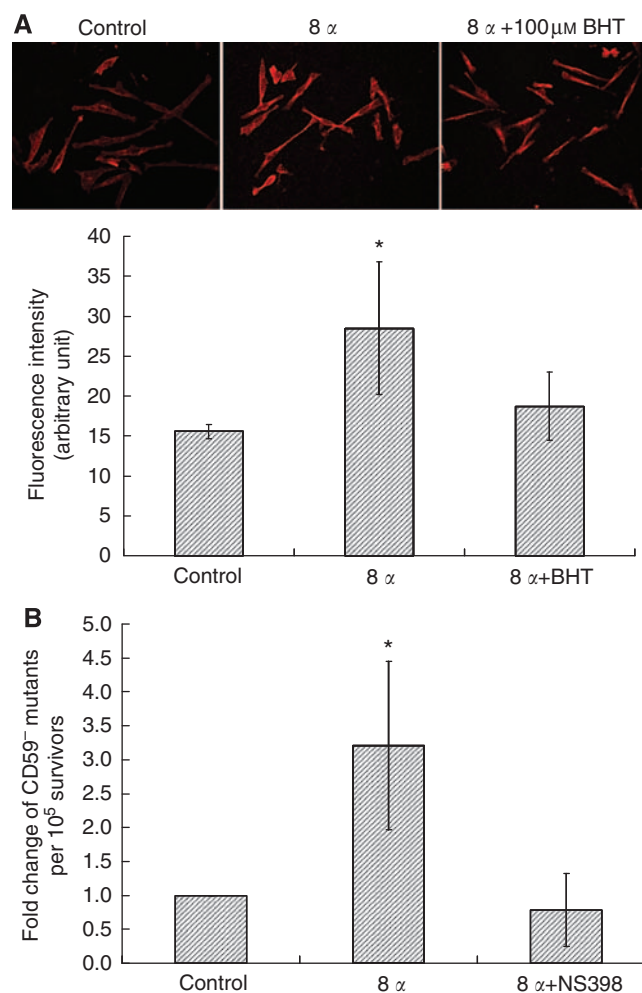


Figure 3 Cyclooxygenase-2 expression in cytoplasmic-irradiated A_L cells. **(A)** Cyclooxygenase-2 fluorescent signal in A_L cells after irradiation with 0 (control) or eight α -particle (8 α) through the cytoplasm with or without co-treatment with 100 μ M BHT. **(B)** Effect of NS-398 on the mutagenic potential of cytoplasmic irradiation at the *CD59* locus of A_L cells. Data were averaged from three independent experiments. Bars indicate \pm s.e. of sample means (asterisk indicated $P < 0.05$).

differentiation, and apoptosis. Numerous studies have shown that exposure of cells to ionising radiation as well as other toxic stresses can induce simultaneous compensatory activation of different MAPK pathways (Dent *et al*, 2003). There is evidence that 4-HNE can act as an inducer for COX-2, possibly through MAPK pathways (Kumagai *et al*, 2004). As shown in Figure 4A, cells irradiated with

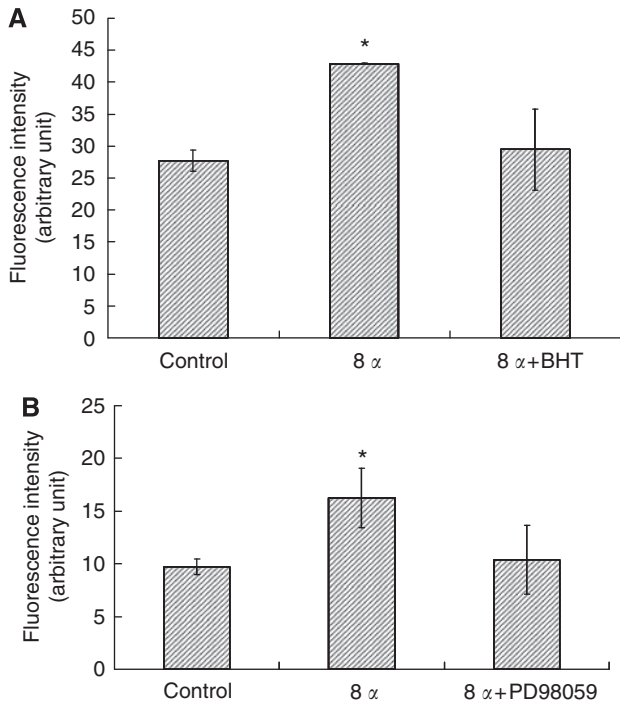


Figure 4 Extracellular signal-related kinase (ERK) activity in cytoplasmic-irradiated A_L cells. **(A)** Relative fluorescence intensity of p-ERK in A_L cells with 0 (control) or after irradiation with eight α -particles (8 α) through cytoplasm with or without 100 μ M BHT treatment. **(B)** Relative fluorescence intensity of COX-2 in A_L cells after irradiated with 0 (control) or eight α -particles through the cytoplasm with or without treatment with 50 μ M PD98059, a specific inhibitor of the MAPK ERK (MEK). Data were averaged from three independent experiments. Bars indicate \pm s.e. of sample means (asterisk indicated $P < 0.05$).

eight α -particles had a higher level of phospho-ERK when compared with that of the control, non-irradiated cells, suggesting that ERK is activated after cytoplasmic irradiation. Treatment of cells with BHT before irradiation reduced phospho-ERK to control levels, indicating that lipid peroxidation may act as an upstream activator of the ERK signalling pathway. If activation of ERK is critical in mediating the irradiation-induced up-regulation of COX-2, it should be possible to mitigate the later response by using a specific inhibitor of the MEK-ERK signalling pathway. Indeed, A_L cells treated with a non-cytotoxic dose of PD 98059 showed suppression of COX-2 expression after ionising irradiation (Figure 4B). This result indicated that the MEK-ERK pathway may have a role in the COX-2 induction after cytoplasmic irradiation.

DISCUSSION

With the availability of a precision microbeam at Columbia University, we previously reported that irradiation of cellular cytoplasm with an exact number of α -particles led to increased mutation yield, whereas inflicting minimal toxicity and that this process is mediated by free radicals. In this study, we used the microbeam setting and A_L cell system to extend the study to provide further evidence that cytoplasmic irradiation leads to elevated levels of ROS and RNS. As only a limited number of cells can be irradiated per day, typically $< 25\,000$, most of the analyses on cellular signalling can only be achieved using semi-quantitative immunostaining methods in this study.

DNA damage induced by ROS has been proposed as a key event relevant to mutagenesis and carcinogenesis. 8-Hydroxydeoxyguanosine

is one of the most abundant oxidised DNA bases and has been shown to be a mutagenic DNA lesion (Gajewski *et al*, 1990). Haegele *et al* (1998) reported that X-radiation induced a small but significant increase in 8-OHdG concentration *in vivo* in mammary gland DNA. High levels of 8-OHdG have been observed in normal human epidermis or purified DNA exposed to ultraviolet radiation (Wei *et al*, 1997). We showed here that cytoplasmic irradiation induced the generation of 8-OHdG in A_L cells. Such an induction can be efficiently suppressed in the presence of DMSO. This result is consistent with the previous mutation data (Wu *et al*, 1999), suggesting generation of free radicals cause oxidative stress in cytoplasmic irradiated cells. The RNS-mediated signalling can often be corroborated with signal transduction associated with oxygen-derived species (Narayanan *et al*, 1997). It has been demonstrated that nitric oxide synthase produces sustained high concentrations of NO in various mammalian cells after exposure to cytokines, hypoxia, heavy metals, crocidolite, and radiation (Chao *et al*, 1996; Zhuang *et al*, 1998; Matsumoto *et al*, 2001). In cytoplasmic-irradiated cells, 3-nitrotyrosine, a nitrosated protein product used as a marker of peroxynitrite anions, was significantly elevated. In addition, the incidence of mutation induced by cytoplasmic irradiation was dramatically inhibited by L-NMMA, implicating a critical role of RNS in the mutagenicity induced by cytoplasmic irradiation.

Consistent with the increase of oxidative stress, lipid peroxidation also increased significantly after transverse of α -particles through the cytoplasm, which was evident by higher level of 4-HNE. Accumulating evidence in recent years has shown that 4-HNE can function as potential signalling molecules in various pathways (Chiarpotto *et al*, 1999; Kumagai *et al*, 2002; Lu *et al*, 2002; Burlando *et al*, 2003). Therefore, the enhanced production of 4-HNE in cytoplasmic-irradiated cells is likely to induce downstream responses that lead to its mutagenic effect. Indeed, in addition to the induction of 4-HNE, we also detected increased COX-2 expression in the irradiated cells. Cyclooxygenase-2 is normally not present or present in very low amounts under basal conditions. However, it is promptly induced by various stimuli including proinflammatory cytokines such as IL-1 β and TNF- α , growth factors and tumour promoters (Uchida and Kumagai, 2003). There have been reports indicating that COX-2 expression may be associated with the accumulation of lipid peroxidation products. Within human atheromatous lesions, COX-2 was colocalised with 4-HNE in foamy macrophages, implicating that COX-2 expression is associated with the accumulation of lipid peroxidation products (Kumagai *et al*, 2004). 4-Hydroxynonenal was also found to be responsible for the induction of COX-2 in 3T3-L1 adipose cells under oxidative stress (Zarrouki *et al*, 2007). We further demonstrated that in cytoplasmic-irradiated A_L cells, ERK activity was elevated and that pre-treatment with lipid peroxidation blocker BHT resulted in reduced ERK phosphorylation (Figure 4), implicating an upstream regulatory role of lipid peroxidation on ERK activation. Moreover, specific MEK1 inhibitor PD98059 was able to suppress COX-2 expression (Figure 4B), further suggesting the involvement of ERK in this event. The p38 kinase activity, however, did not seem to change after cytoplasmic irradiation (data not shown). This may possibly be because of the diverse response of p38 kinase to external stimuli (Zhou *et al*, 2005).

Previous studies in our laboratory have shown that COX-2 is critically linked to the radiation-related bystander effect in normal human lung fibroblasts and that the MAP kinase signalling cascade has an important role in this process (Zhou *et al*, 2005). Our results here provided further evidence to demonstrate that ROS and RNS are both involved in the cytoplasmic irradiation-induced genotoxicity. More importantly, our data suggest that a lipid peroxidation signalling pathway involving 4-HNE and COX-2 may have a critical role in the mutagenic effect induced by cytoplasmic irradiation. Given the similarities in cellular response

and phenotype between cytoplasmic irradiation and bystander effect, these two phenomena are likely to share some signalling pathways. However, this pathway may only present one of the many pathways activated by irradiation because the induction of COX-2 could only partially be inhibited by lipid blocker BHT (Figure 3). Further studies on the cellular and molecular mechanisms of cytoplasmic irradiation will enable us to formulate a more accurate model in assessing the health effects of low dose ionising radiation.

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