

Consequences of Cytoplasmic Irradiation: Studies from Microbeam

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Cytoplasm/Radiation/Microbeam/Bystander Effect/ROS/NF- κ B/ATM.

The prevailing dogma for radiation biology is that genotoxic effects of ionizing radiation such as mutations and carcinogenesis are attributed mainly to direct damage to the nucleus. However, with the development of microbeam that can target precise positions inside the cells, accumulating evidences have shown that energy deposit by radiation in nuclear DNA is not required to trigger the damage, extra-nuclear or extra-cellular radiation could induce the similar biological effects as well. This review will summarize the biological responses after cytoplasm irradiated by microbeam, and the possible mechanisms involved in cytoplasmic irradiation.

INTRODUCTION

Ever since X-rays were shown to induce mutation in *Drosophila* more than 70 years ago, the prevailing dogma has been that the genotoxic effects of ionizing radiation such as mutations and carcinogenesis are attributed mainly to direct damage to the nucleus. This is partially due to the difficulties in selectively targeting the cytoplasm without affecting the nucleus. As such, generations of students in radiation biology have been taught that such heritable biological effects are the consequence of a direct radiation-nuclear interaction. In current radiation risk estimation model, though the possibility of the bronchial or lung cells to get an alpha particle traversal through the cytoplasm is much higher than the nuclei in the environmental radon exposure, the contribution from cytoplasmic traversal is usually ignored based on the dogma that the DNA of the nucleus is the main target for radiation-induced genotoxicity.

The differential biological effects of nuclear versus cytoplasmic irradiation have been of interest to biologists and geneticists for decades. With the availability of precision microbeam to deposit energy to the part of the cells such as cytoplasm or part of nucleus, it is possible to address the differential biological effects of nuclear versus cytoplasmic damage in an unprecedented manner. Accumulating evidences have shown that energy deposit by radiation in nuclear DNA is not required to trigger the damage, extra-

nuclear or extra-cellular radiation could induce the similar biological effects as nuclear radiation.¹⁻⁴⁾

It is of interest to note that the first report of ultraviolet microbeam can be traced back to almost one hundred years ago. In 1912, Chahotin described a method of producing a beam of ultraviolet light of wavelength 2800Å with a few microns in diameter, and reported preliminary experiments using it for cell irradiation.⁵⁾ After that, selective exposure of fractions of intact individual cells to radiation is of interest in radiobiology to gain information about the mechanisms by which radiations produce the damages on living systems, as well as to analyze the normal functions of the various cell parts by selectively altering them.

DIRECT EFFECT OF CYTOPLASMIC IRRADIATION

In early 1950s, Zirkle and Bloom designed their fine proton microbeam which more than 80% of the protons focus a central area 2.5 μ in diameter. Using this microbeam to irradiate the cultured mitotic amphibian heart cells, they found the chromosome aberrations were localized according to the spot of bombardment. A few dozen protons through the middle of a metaphase plate or of a prophase chromosome configuration regularly produced unambiguous abnormalities (chiefly bridges), but hundreds and even a few thousands of protons to extra-chromosomal regions (general cytoplasm, spindle, centriolar region) produced no detectable effects.⁶⁾ In 1973, Johnson *et al.* reported that individual cells of *Saccharomyces cerevisiae* (NCYC 239) were irradiated in either the nucleated parental portion of the cell or the anucleate (but mitochondria-containing) bud with an ultraviolet microbeam. The spontaneous petite frequency was 4/7108 (0.056%) in this experiment. The overall induced petite frequency was 9/540 (1.67%) among nucleus-irradiated cells

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(significantly enhanced) and 2/832 (0.24%) among bud-irradiated cells (insignificantly enhanced), that reflected the difference in the response of the two target areas.⁷⁾ Results from this study indicated that induction of the mutation was apparently associated with the nuclear target. These findings demonstrated that irradiation of cytoplasm was largely innocuous and strongly indicated that DNA was the target for the radiobiological effects of ionizing radiation.

However, using heterochromatic ultraviolet microbeam, Zirkle *et al.* reported that a small portion of the cytoplasm of metaphase newt cells in tissue culture caused the spindle to diminish or disappear even when the site of the irradiation is as much as 30 μ distant from any part of the spindle. It was concluded that some kind of "spindle poison" were probably produced photochemically by the radiation.⁸⁾ Spindle disappearance results in a chromosome derangement, which is followed by a new metaphase arrangement and an orderly but abnormal distribution of chromosome to two daughter cells. Following up this finding, Amenta has observed a nuclear change following irradiation of a small area of the cytoplasm in leucocyte cultures from newt liver. This might also be ascribed to a nuclear poison produced by irradiation of the cytoplasm.⁹⁾ These results challenged the prevailing dogma in radiobiology, and indicated that cytoplasmic irradiation might contribute to biological damages induced by radiation.

Using two microbeams, one of ultraviolet light 3.5 μ m in diameter and one of α -particles 6 μ m in diameter, Dendy and Smith reported in 1964 that irradiation of the cytoplasm with both microbeams produced a progressive reduction in rate of DNA synthesis for several hours after irradiation, although the degree is much less than that of nuclear irradiation with same dose. In the case of ultraviolet irradiation, since the cytoplasm is less absorbing than the nucleus, the effects were seen to be very similar in magnitude when comparing the effects of a 5s cytoplasmic irradiation with a 2s nuclear irradiation. However, in the α -particle experiments, the inhibition in DNA synthesis is smaller than that of in the ultraviolet experiments although it is still significant. Interestingly, irradiation of the surrounding medium by either ultraviolet or alpha particle produced DNA synthesis inhibition in some cases at 2 h. However, DNA synthesis could recover to normal rate in 6 h after irradiation in this case. In addition, in the ultraviolet experiments, this effect was much less significant than that produced after direct irradiation of the cells.¹⁰⁾

Using an α -particle microbeam, Kuzin and Wainson reported a similar findings that irradiation of cytoplasm resulted in a significant (up to 18%) inhibition of DNA synthesis within 1 h after irradiation, but the absorbed dose needed to be ten times larger than that which gave similar results with nuclei. This effect increased dramatically 3 h after cytoplasmic irradiation, and they believe that the inhibition of DNA synthesis after cytoplasmic irradiation mainly resulted from the formation of toxins in damaged cytoplasm

and their diffusion into the nucleus.¹¹⁾ They also predicted that these toxic substances may also influence in some way the activity of DNA polymerase and the enzymes necessary for the formation of essential precursors. Following the hypothesis, Takeda *et al.* reported that nucleolar irradiation or extranucleolar nuclear irradiation caused an inhibition in the rate of DNA synthesis, which developed progressively for several hours (up to 80–90% in 12 h) after ultraviolet microbeam irradiation. However, cytoplasmic irradiation could only cause a diminutive reduction of DNA synthesis (about 10% in 1 h, and 15% in 2 h to 12 h after irradiation). In addition, their results showed that ultraviolet microbeam irradiation at one nucleolar or extranucleolar nuclear site rapidly caused an inhibition in RNA synthesis which is partially restored a short time after the irradiation, but there is no experimental result regarding RNA synthesis alteration caused by cytoplasmic irradiation in this report. Besides the DNA/RNA synthesis, Takeda found only a marginal inhibition of protein synthesis in both cytoplasmic and nuclear irradiation.¹²⁾

Using an ultraviolet microbeam to irradiate HeLa cells in the nucleus, cytoplasm, or whole cells, Usui found that the inhibitions of DNA and RNA synthesis by the solely cytoplasmic irradiation were much less than by nuclear or whole cell irradiation. For example, 0.1 s exposure to ultraviolet light on cytoplasm has almost no effect on DNA and RNA synthesis for up to 8 h, but exposing the nucleus to the same dose can significantly inhibit both DNA and RNA synthesis within 4 h. This finding is consistent with previous studies. However, it is of interest to note in this report that the inhibition of DNA/RNA synthesis is much lower in the solely cytoplasmic irradiation than those in the solely nuclear irradiation, but the lethal effect is higher in the solely cytoplasmic irradiation. It was also found that the nucleus-irradiated cells showed only a slight morphological sign of cell degeneration for several hours after irradiation before the cells were dead by a sudden shrinkage of the cytoplasm in spite of their marked inhibition of nucleic acid synthesis; the solely cytoplasmic irradiation induced vacuolar degeneration developing gradually and lethally in the cytoplasm.¹³⁾

Overall, these previous reports concluded that cytoplasm irradiation do play some roles in alterations in DNA/RNA synthesis, cell killing, and spindle alteration (and possibly chromosome aberrations), though the mechanisms are largely unknown. However, since the effects induced by cytoplasmic irradiation were much less than nuclear irradiation, these findings largely support the prevailing dogma that the genotoxic effects of ionizing radiation are attributed mainly to direct damage to the nucleus.

With the modern cellular/molecular technology and application of computer science recently, the microbeam now has more advantages in studying the biological response after cytoplasmic irradiation. Using a precision charged microbeam in Columbia University, Wu *et al.* reported in 1999 that

irradiation of cellular cytoplasm with an exact number of alpha particles results in gene mutations in the nucleus while inflicting minimal toxicity.¹⁴⁾ For example, 4 alpha particles traversed through the cytoplasm can only kill about 10% of the cells, and more than 70% cells survived even after their cytoplasm was irradiated with 32 alpha particles. In contrast, the survival fractions for nuclear irradiation with the same number of alpha particles result in 35% and less than 0.1% respectively.¹⁵⁾ Assays for mutation induction showed that nuclear irradiation induced 3-4-fold more mutants than cytoplasmic irradiation at equivalent particle traversal. The principal classes of mutations induced by cytoplasmic irradiation are similar to those of spontaneous origin (most are point mutations) and are entirely different from those of nuclear irradiation (most are large deletions). Their results with the radical scavenger, dimethyl sulfoxide (DMSO), and the thiol depleting drug buthionine S-R- sulfoximine (BSO) indicated that reactive oxygen species (ROS) modulate the mutagenic response of cytoplasmic irradiation. Furthermore, they found the induction of 8-OHdG in irradiated cells is consistent with a role of oxidative DNA damage.

Recently, Prise's group found that cytoplasmic-targeted cells showed the p53 binding protein 1 (53BP1) ionizing radiation-induced foci (IRIF) located sparsely throughout the nucleus. Interestingly, when the cell cytoplasm is irradiated, the biological effect appears but to have different kinetics from nuclear irradiation. No change was found 1 h after cytoplasmic irradiation, but the formation of nuclear 53BP1 foci significantly increased 3 h after cytoplasmic targeting. The level of response was found to be identical in hit and non-hit bystander cells ($17 \pm 6\%$ and $16 \pm 8\%$ increase over control, respectively). These data indicate that targeted cytoplasmic irradiations can induce damage at the nuclear level. The use of common reactive oxygen species and reactive nitrogen species (RNS) inhibitors prevent the formation of 53BP1 foci in cytoplasmic irradiated cells. Treatment with filipin to disrupt membrane-dependent signaling does not prevent the cytoplasmic irradiation-induced 53BP1 foci in the irradiated cells, but it does prevent signaling to bystander cells.¹⁶⁾

CYTOPLASMIC IRRADIATION AND BYSTANDER EFFECTS

Radiation-induced bystander effects are defined as the induction of biological effects in cells that are not directly traversed by a charged particle, but have received signals from these irradiated cells. Early investigations of the radiation-induced bystander effect measured the frequency of sister chromatid exchanges (SCE) in populations of cells exposed to very low fluencies of alpha particles. In CHO cells irradiated with low doses of alpha particles where less than 1% of the nuclei were estimated to have been traversed by a particle, an increase in sister chromatid exchanges was

observed in over 30% of the cells.¹⁷⁾ In other words, either cytoplasmic damages or signals received from an extracellular component may have modulated the observed genotoxic response. Results from experiments using microbeam provide evidence that the bystander effects can be demonstrated using a variety of endpoints of biological damage, including micronucleus induction, cell lethality, gene expression and oncogenic transformation in various human and rodent cell lines.¹⁸⁻²⁶⁾ Most of these bystander effects resembled those phenotypes seen in cells that are directly irradiated, albeit in the absence of any dose response relationship. It has been shown, for example, that irradiation of 10% of a confluent human hamster hybrid (A_L) cell population with a single alpha particle per nucleus results in a mutant yield similar to that observed when all of the cells in the population are irradiated.²⁶⁾ A similar observation has also been made using primary human bronchial epithelial cells and incorporating G2 phase premature chromosome condensation as an endpoint.³⁾

Using a charged-particle microbeam to target individual helium ions to individual cells within a population of radioresistant glioma cells cultured alone or in coculture with primary human fibroblasts, Shao *et al.* found that even when a single cell within the glioma population was precisely traversed through its cytoplasm with one alpha particle, bystander responses were induced in the neighboring nonirradiated glioma or fibroblasts so that the yield of micronuclei was increased by 36% for the glioma population and 78% for the bystander fibroblast population. Importantly, the yield of bystander-induced micronuclei was independent of whether the cytoplasm or nucleus of a cell was targeted. The bystander responses were fully eliminated when the populations were treated with 2-(4-carboxyphenyl)-4, 4, 5, 5-tetramethyl-imidazoline-1-oxyl-3-oxide or filipin, which scavenge nitric oxide (NO) and disrupt membrane rafts, respectively. By using the probe 4-amino-5-methylamino-2', 7'-difluorofluorescein, it was found that the NO level in the glioma population was increased by 15% after 1 or 10 cytoplasmic traversals, and this NO production was inhibited by filipin.²⁷⁾ This finding shows that direct DNA damage is not required for switching on important cell-signaling pathways after low-dose irradiation and that, under these conditions, the whole cell should be considered a sensor of radiation exposure.

Following this finding, the same group recently reported that cytoplasmic irradiation induced 53BP1, a member of the BRCT (BRCA1 C-Terminal) repeat family, foci located sparsely throughout the nucleus in bystander cells. The kinetics of 53BP1 focus formation after cytoplasm irradiation (in both directly irradiated and bystander cells) showed a peak at 3 h and decreased after 6 h, and with foci numbers returning close to control values at 18 h. The response was independent of the number of particles used for irradiation, a single helium ion induced the same level of 53BP1 focus

formation as 20 helium ions, both in directly hit and bystander cells following cytoplasmic traversal. Comparison of the bystander response when only one cell or 50% of the cells within the population were targeted through the cytoplasm only showed no significant difference, suggesting bystander responses were independent of the number of cells targeted. Moreover, when compared with the bystander effect induced by nuclear targeting, the level of response was not significantly different at 3 h, implicating that similar mechanisms are involved.¹⁶⁾

MECHANISMS OF BYSTANDER RESPONSES VIA CYTOPLASMIC IRRADIATION

Although the bystander effects have been well described over the past decade, the mechanisms of the process remain unclear. In sub-confluent cultures, there is evidence that reactive oxygen species, nitric oxide, and cytokines such as anti-transforming growth factor (TGF) β are involved in mediating the process.^{21,24,28)} On the other hand, gap junction-mediated cell-cell communications have been shown to be critical in mediating bystander effects in confluent cultures of either human²⁵⁾ or rodent cells.^{23,26)} It is likely that a combination of pathways involving both primary and secondary signaling processes is involved in producing a bystander response. Although existing data suggested that cytoplasmic to nuclear transmission in directly cytoplasmic irradiated cells might play a role in cytoplasmic irradiation induced bystander effect, the mechanisms would be similar in both nuclear and cytoplasmic irradiation induced bystander effect.¹⁶⁾

It is likely that cellular signaling pathways, for example, membrane ligands and the downstream MAPK cascade provide a common link between medium-mediated and gap-junction dependent bystander phenomena. Previous studies by Mitchell *et al.* have shown that when 10% of cells are exposed to alpha particles, a significantly greater number of cells are inactivated when irradiated at high density (> 90% in contact with neighbors) than at low density (< 10% in contact). In addition, the bystander oncogenic transformation frequency is significantly higher in high-density cultures.²⁹⁾ These results suggest that when cells are traversed by alpha particles, the transmission of a bystander signal through cell-to-cell contact results in a higher response compared to those mediated by soluble factors.

Recently evidences indicated that DMSO, Filipin and TGF β 1 could suppress gammaH2AX foci in bystander cells, further confirming that reactive oxygen species (ROS) and membrane-mediated signals are involved in the bystander signalling pathways.³⁰⁾ The presence of ROS in the cytoplasmic compartments has important consequences for the signaling system because ROS can shift the balance between phosphorylated (active) and dephosphorylated (inactive) forms of numerous kinases toward active forms. One target

kinase thus activated is NIK (NF- κ B inducing kinase), a member of the MAPK kinase kinase family with a Ser/Thr kinase activity. NIK participates in the induction of NF- κ B by both TNF- α and interleukin 1 β through phosphorylating IKK α .³¹⁾

NF- κ B directly controls gene expression of cyclooxygenase 2 (COX-2) and iNOS. Ionizing radiation is a strong inducer of the ATM-IKK-NF- κ B signaling pathway,³²⁾ which is further involved in the up-regulation of TNF α gene expression.³³⁾ Our previous studies³⁴⁾ indicated that COX-2 is critically linked to the radiation-induced bystander effect in normal human fibroblasts. There is evidence that NO can induce expression of COX-2 in mouse skin and human cultured airway epithelial cells, and that the NF- κ B pathway is involved in the process.^{35,36)} Bay 11-7082, a specific IKK/NF- κ B inhibitor, can eliminate bystander mutagenesis, highlighting the important role of this transcription factor in the bystander phenomenon. Secreted or membrane forms of TNF α can induce bystander effects in non-irradiated cells via activation of COX-2 gene expression. Inhibitory mAb against TNF partially suppressed NF- κ B activation and the subsequent COX-2 up-regulation in both directly irradiated and bystander cells. Taken together, these data indicated that the NF- κ B/COX-2/PGE2 and NF- κ B/iNOS/NO pathways are critical to the radiation induced bystander effect in mitochondrial functional cells. However, in mitochondrial deficient cells, the contribution of COX-2 to the bystander process is less pronounced, while NF- κ B/iNOS/NO pathway actively operates, although at lower level compared to normal cells.³⁷⁾

Elevated levels of NO have been detected in a variety of pathophysiological processes, including inflammation and carcinogenesis.^{38,39)} Nitric oxide has been postulated to be a potential signaling molecule in radiation-induced bystander effects.^{27,40,41)} Shao *et al.* reported that when only 1% of cell nuclei were individually targeted with a single helium ion, approximately 40% of the cells showed an increase in fluorescence intensity of the NO-sensitive dye, 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM). Moreover, it was found that when only 1 cell in a population of approximately 1,200 cells was targeted with one or five ions, the incidence of micronuclei increased by 20%, and concurrent treatment with c-PTIO significantly reduced the bystander effect.⁴¹⁾ Recently, we found that the NO scavenger had a similar suppressive effect on bystander mutagenesis of both human skin fibroblast (ρ^+) and mitochondrial deficient human skin fibroblast (ρ^0) cells, though the effects were more pronounced in the former.³⁷⁾ Since mitochondria are the main source of reactive radical species in cells, the basal level of free radicals in ρ^0 cells is lower than in mitochondria functional cells. There is evidence that in ρ^0 cells with a complete shut down of the electron transport chain, the decreased level of reactive oxygen species resulted in a down-regulation of the manganese superoxide dismutase,

glutathione, and glutathione peroxidase, an important intracellular antioxidant pool.⁴³) As such, compared with wild type cells, ρ° cells are more susceptible to oxidative stress and express a higher level of bystander mutagenesis. It is likely that one or more other signaling molecules, in addition to NO, are involved in radiation induced bystander effects in mitochondrial deficient cells. This could explain the difference in bystander signaling response in mixed cultures of ρ^{+} and ρ° cells. Alternatively, ρ° cells, by virtue of their reduced apoptotic response (Ivanov and Hei, unpublished observation), may accumulate a higher mutant fraction. This would be consistent with the higher bystander response seen in these cells. It is also possible that signal from one cell line can modulate expression of bystander response in another cell type⁴⁴) and is consistent with the findings with the mixed cell population.

It has been demonstrated that downstream of NO, TGF β 1 plays an important role in the targeted cells induced bystander response to irradiated cells by further causing DNA damage in vicinal fibroblasts through a ROS related pathway.⁴⁵) Iyer and Lernert found the increasing of the concentration of TGF- β 1 in cell supernatants after very low doses of alpha particle irradiation, and TGF- β 1 at concentrations commensurate with those in the supernatants capably induced increases in intracellular ROS in unirradiated cells. Furthermore, the addition of supernatants from alpha-irradiated cells to unirradiated cells decreases cellular levels of TP53 and CDKN1A and increases CDC2 and proliferating cell nuclear antigen in the latter, and this decreased TP53/CDKN1A response can be mimicked by the addition of low concentrations of TGF- β 1 in otherwise untreated cells.²⁴)

A range of evidence has shown that DNA repair status is important for dealing with the consequences of bystander signals. Repair processes related in the processing of double-strand breaks (dsb) appear to be involved suggesting that the bystander response is associated with the delayed or indirect production of dsb-type lesions in bystander cells. Recently, Burdak-Rothkamm reported the importance of ataxia telangiectasia mutated (ATM) / ATM- and rad3-related (ATR) related DNA damage response signaling in bystander cells.⁴⁶) They found a decrease in clonogenic survival in both directly irradiated and non-irradiated bystander ATR/ATM/DNA-PK-proficient cells, but this effect was completely abrogated in ATR and ATM but not DNA-PK-deficient bystander cells. However, inhibition of ATM protein and DNA-PK could not suppress the induction of bystander γ H2AX foci whereas the mutation of ATR abrogated bystander foci induction. Moreover, the induction and colocalization of ATR, 53BP1, ATM-S1981P, p21, and BRCA1 foci in non-targeted cells suggested the involvement of ATM/ATR pathways in bystander DNA damage signaling.⁴⁶) ATM activation in bystander cells was found to be dependent on ATR function, and ATM acted as a further component of the complex signaling network of radiation-induced

DNA damage in non-targeted bystander cells downstream of ATR. Overall the evidence suggest that it is not the production of DNA damage that triggers the bystander response, but DNA damage and repair proficiency play an important role in the downstream consequences in the bystander cells.⁴⁷)

PERSPECTIVES

Recent studies indicated that cytoplasmic irradiation can induce genotoxic effects such as mutation in both directly and indirectly irradiated cells. Furthermore, ROS/RNS played a critical role in this procession, and NF- κ B/COX-2/iNOS/NO pathways as well as ATR/ATM related pathways are involved in cytoplasmic irradiation induced bystander effect. Further studies that would provide addition details regarding the molecule(s)/components involved in cytoplasmic irradiation induced biological response, especially how mitochondrial function regulates such an effect are anticipated.

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