Original Article

DNA Damage and Survival in Bystander Human Intestinal Cells Treated with Conditioned Medium from Tritium-Labeled Cells

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Abstract

Background: Tritium exposure could be one of the radiation hazards in case of accidental exposure with intestine as one of the major target organs. In the cells, low-energy beta emitted from tritium would traverse a very short distance (a few microns). Hence, the intestinal epithelial cells with nuclear localization of tritium would exert its radiobiological effect also through bystander mechanism. In the present study, the effect of conditioned medium obtained from tritiated thymidine-labeled human normal intestinal epithelial (INT407) cells was studied on respective bystander cells in terms of magnitude of survival and induction of DNA damage. **Materials and Methods:** The survival and proliferation of bystander INT407 cells treated with control/irradiated conditioned medium were studied using clonogenic and 5-bromo-2-deoxyuridine (BrdU)-labeling assays. The magnitude of DNA double-strand break was measured by immunofluorescence of γ-H2AX by confocal microscopy. Intracellular nitric oxide (NO) in these cells was measured using 4,5-diaminofluorescein diacetate fluorescent dye. **Results:** Bystander cells treated with conditioned medium from tritiated thymidine-labeled cells showed increased clonogenic survival and BrdU labeling. Cells labeled with tritiated thymidine showed attenuation of γ-H2AX foci at longer period (24 and 48 h) of labeling than at 15 h. Moreover, the bystander cells treated with irradiated conditioned medium showed a higher magnitude of γ-H2AX foci at 24 h. However, compared to 24 h, 48-h treatment of irradiated conditioned medium resulted in a decrease in γ-H2AX foci in the bystander cells. Increased level of intracellular NO was observed in the bystander cells treated with irradiated conditioned medium. **Conclusions:** Bystander cells treated with conditioned medium obtained from tritiated thymidine-labeled cells showed increased clonogenic survival and proliferation, which was correlated with an increase in DNA double-strand break and NO production in these cells.

Keywords: *Bystander effect, DNA damage, intestinal cells, proliferation, tritium*

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Introduction

Tritium (^{3}H) is a beta-emitting radioisotope of hydrogen (average energy: 5.7 keV). In addition to cosmic generation, it is also a by product during nuclear energy production.[1] There is a risk of tritium exposure in case of occupational or accidental exposure.[2] Being a low-energy beta emitter, the health effects of tritium would be implicated only when it would get internalized. In case of ingestion, the tritium would pass through the intestine and its highly proliferating intestinal epithelial cells. Once internalized, cellular effects of radiation would not only limit to target cells but also to bystander cells of the intestinal tissue. The range of beta radiation from tritium in an aqueous cellular system is about 6 μm.[2] Hence, the range of radiation from the tritium localized to the nucleus would be limited only to the cell incorporated. Such irradiation provide conditions for

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bystander interaction from the irradiated cells. Tritiated thymidine has been extensively used as a radiotracer for cellular proliferation/DNA replication studies. However, bystander effects of tritium incorporated/irradiated cells are limited in the literature. Radiation adaptive response in bystander cells in three-dimensional (3D) cultures containing tritium-labeled normal human fibroblasts was studied, which was measured in terms of magnitude of the G1 arrest, micronucleus formation, and changes in mitochondrial membrane potential.[3] Such bystander studies were also found to involve the generation of free radicals and intercellular gap junction communications.[4,5] Bystander interaction after nonuniform distribution of tritium in 3D cultures was studied in human fibroblasts and rat liver epithelial cells (WB-F344).[3,6] There are some studies, which showed a proliferative response of bystander WB-F344 co-cultured with tritiated thymidine-labeled cells.[6,7] The present study is aimed to investigate the effects of conditioned medium obtained from tritiated thymidine-labeled human normal intestinal epithelial cells (INT407) on the counterpart bystander cells. The magnitude of DNA double-strand break and involvement of nitric oxide (NO) was also investigated in bystander cells.

Materials and Methods

Cells and culture

INT407 (human embryonic intestinal epithelial) cell line was obtained from National Centre for Cell Sciences, Pune, India. Cells were cultured in Dulbecco's Modified Eagle Medium (GIBCO-Invitrogen, USA) supplemented with 10% fetal calf serum (FCS; Himedia Laboratories, Mumbai, India) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere of 5% at 37°C. The desired number of cells was harvested from ~95% confluent culture.

Tritium labeling and gamma irradiation

For tritium labeling, cells (0.14×10^6) obtained after trypsinization were plated in P-100 culture dishes in complete culture medium (supplemented with 10% FCS and antibiotics) followed by addition of tritiated thymidine (0.1295 MBq/ml, BRIT, Mumbai). These cells were cultured for 24 h in the standard culture conditions followed by removal of the medium and washing of the cells with prewarmed (at 37°C) Hank's Balanced Salt Solution (HBSS). Cultures were replaced with prewarmed (at 37°C) complete medium and cultured for 48 h. After culture for 48 h, the medium was collected and centrifuged (×2000 g for 5 min) at room temperature. The supernatant (irradiated conditioned medium) obtained was used to treat fresh cultures. For control conditioned medium, INT407 cells were cultured and processed in a similar way except labeling the cells with tritiated thymidine. Both irradiated and control conditioned media were used freshly after collection. Activity in the cells which were directly labeled with tritiated thymidine was calculated to be 1.68 ± 0.58 mBq/cell (average diameter of INT407 cell nuclei: 12.6 ± 0.94 μm). Activity in cells treated with irradiated conditioned medium was calculated as 0.013 ± 0.005 mBq/cell (0.7% of the total activity).[6] Tritium activity in the irradiated conditioned medium was found to be 1.9% of the total activity. For 48-h culture, the absorbed dose was calculated to be 36 cGy at a dose rate of 0.75 cGy/h.[6] For irradiation with gamma radiation, the dishes were sealed with parafilm, wrapped in aluminum foil, and subjected to radiation at room temperature using ^{60}Co gamma teletherapy irradiator (Bhabhatron II, Panacea Medical Technologies, Bengaluru, India; dose rate: 1 Gy/min).

Clonogenic assay

For determination of plating efficiency (PE) of bystander cells, clonogenic assay was carried out. Briefly, INT407 cells (250 per plate) were seeded overnight in a P-100 culture dish followed by replacement with control and irradiated conditioned medium. On the 10th day, the colonies were washed with PBS, fixed with ethanol, followed by staining with crystal violet (0.5% w/v). The colonies with minimum 50 cells were considered to count as a colony. % PE was determined using the formula, % PE = (no. of colonies counted/no. of cells seeded) × 100. The surviving fraction (SF) was determined using the formula, SF = (colonies counted/cells seeded × (PE/100).[8]

BrdU labeling

For BrdU labeling, 50,000 cells were seeded on glass cover slides (placed in P-30 culture dish) for overnight, followed by counting of BrdU-labeled cells after 16 and 22 h of culture in control/irradiated conditioned medium following standard protocol (Abcam) using microscopy.

γ-H2AX immunofluorescence staining

For γ-H2AX immunofluorescence, cells were seeded for overnight on the glass cover slides. After desired treatment, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were blocked with bovine serum albumin (8%) in PBS for 1 h at room temperature and incubated for 1.5 h with the primary antibody (anti-phospho-histone H2AX [Ser139] clone JBW301, EMD Millipore; dilution 1:200). After washing, cells were incubated with secondary antibody (Alexa Fluor 488 Goat Anti Mouse IgG (H + L) Antibody, Life Technologies) (1:500) for 1 h. Cells were washed with PBS and images were

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acquired using confocal microscope (LSM 510 Meta, Carl Zeiss, Germany).[9] To calculate average foci/cell, the number of foci was averaged in randomly selected 200 cells.

Intracellular nitric oxide assay

Fresh cells (one million) treated with control and irradiated conditioned medium for 24 h were labeled with 4,5-diaminofluorescein diacetate (10 μM, Sigma) for 10 min at 37°C followed by washing the cells with HBSS. Fluorescence intensity was measured using a fluorescence spectrometer (Ex: 495, Em: 515 nm, Ex/Em slit: 10/10 nm). One set of control/irradiated conditioned medium-treated cells before trypsinization was observed under a fluorescence microscope (Nikon Eclipse Ti, Japan) with suitable filters.

Statistical analysis

Unless stated, values represent the average of three independent experiments and with error bars as standard deviation. Where ever required, statistical significance was calculated using Origin 6 software.

Results and Discussion

Irradiated conditioned medium resulted in enhanced clonogenicity in INT407 cells

To study the effect of conditioned medium obtained from tritiated thymidine-labeled cells on bystander INT407 cells, the clonogenic assay of bystander INT407 cells was performed [Figure 1a]. Compared to control, an increased (~20%) clonogenic survival was observed in cells cultured in irradiated conditioned medium, suggesting enhanced survival of bystander cells in irradiated conditioned medium. To further confirm cell proliferation in irradiated conditioned medium-treated bystander cells, BrdU labeling was performed. Similar to clonogenic survival, an increased BrdU % labeling was observed in bystander cells treated with irradiated conditioned medium [Figure 1b], suggesting increased proliferation. These results are in agreement with the previous study, which showed increased proliferation in bystander cells co-cultured with tritium-labeled cells.[6,7] The increase in survival may be associated with the presence of growth stimulatory cytokines/chemokines, which may induce cell proliferation pathways.[8]

Tritiated thymidine-labeled cells and cells cultured in irradiated conditioned medium showed enhanced DNA damage

Furthermore, the magnitude of DNA double-strand break was measured in bystander cells treated with control and irradiated conditioned medium. In the first set of studies, the magnitude of DNA double-strand break was determined by γ-H2AX in INT407 cells in the presence of tritiated thymidine for varying labeling periods (15–48 h). It was interesting to observe that the maximum DNA damage was observed at 15 h, which however showed a decrease at 24/48 h of labeling periods [Figure 2a]. It may be important to mention here that cells being cultured in complete medium would undergo cell division and retain the incorporated tritiated thymidine in the nuclei. Over and above tritiated thymidine would get added during the course of cell division. Despite these facts, a decrease in magnitude of DNA damage suggests induction of tritium-emitted radiation-induced adaptive mechanism, which might have resulted in a decrease in DNA damage even if nuclei are labeled with tritiated thymidine. In another set of experiments, the magnitude of DNA damage was measured in bystander cells cultured in the presence of control/irradiated conditioned medium. In this experiment, 2 Gy gamma irradiation was used as a positive control, which showed a higher magnitude of DNA damage compared to irradiated conditioned medium (BT)-treated bystander cells. The results showed that compared to bystander cells treated with control conditioned medium (BC) for 24 h, bystander cells treated with irradiated conditioned medium (BT) showed a substantial increase in DNA

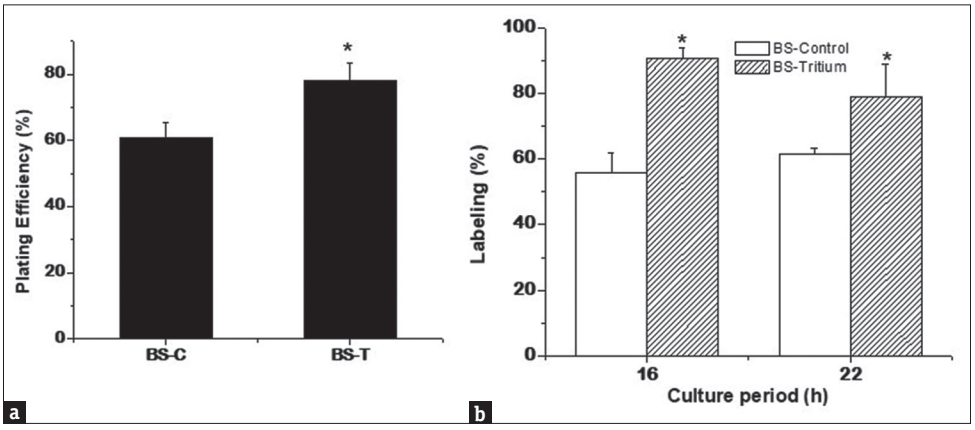

Figure 1: Clonogenic survival and proliferation of INT407 cells cultured in medium obtained from control (BS-C) and tritiated thymidine-labeled (BS-T) cells. (a) Clonogenic assay; (b) BrdU labeling for 16 and 22 h. *Significantly different than control at $P < 0.05$

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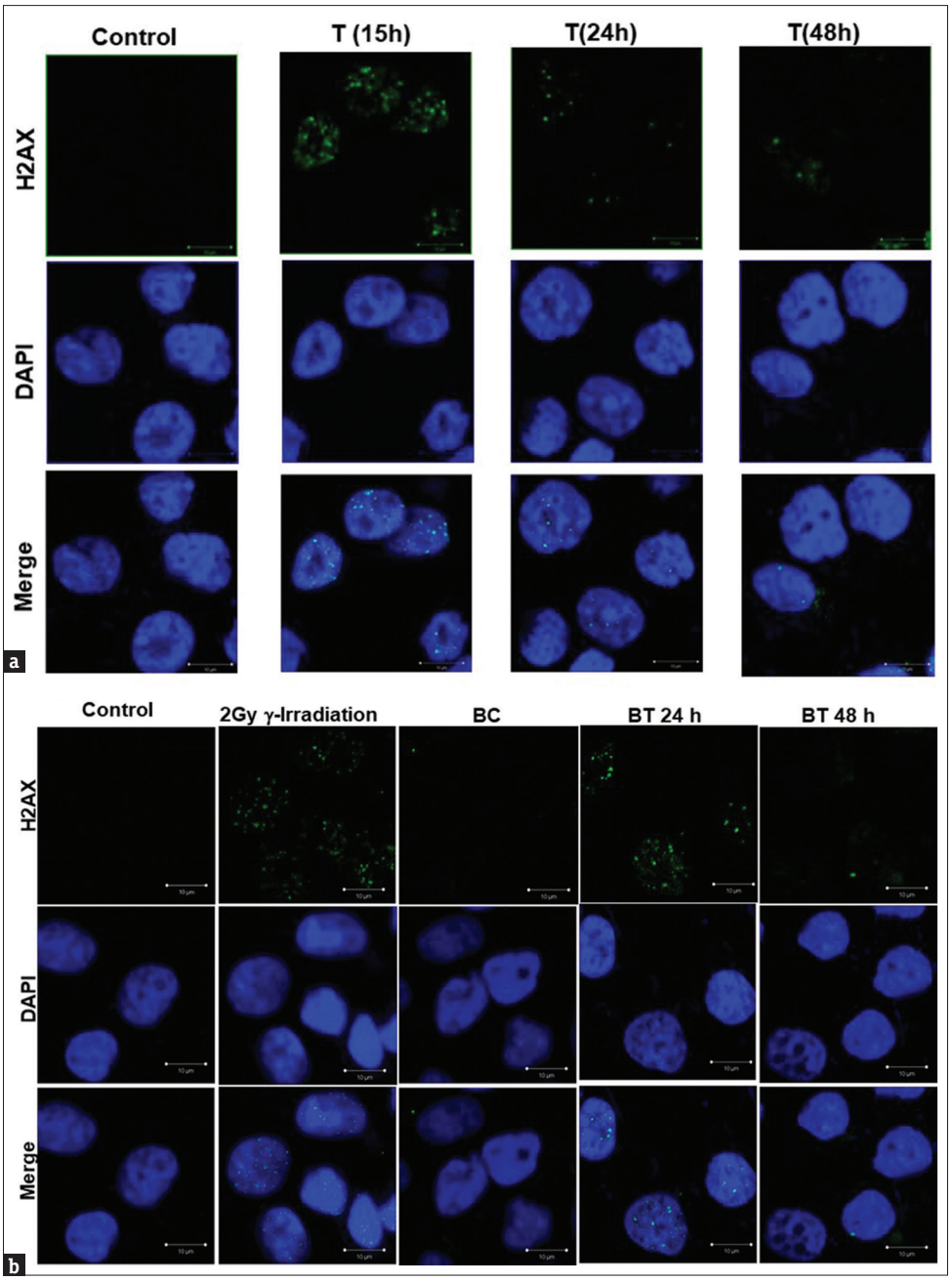

Figure 2: DNA damage measured by γ-H2AX immunofluorescence. (a) Cells were cultured for 15, 24, and 48 h in the presence of tritiated thymidine. (b) Bystander cells treated with 0.5 Gy gamma radiation or cultured in the presence of control (BC) and irradiated conditioned medium (BT) for 24 h and 48 h

damage [Figure 2b]. BC cells at 48 h did not show γ-H2AX foci (data not shown). Interestingly, the magnitude of DNA damage was attenuated when cells were treated for 48 h, suggesting induction of DNA damage repair-induced radiation adaptive response mechanism at longer incubation periods. It seems after induction of DNA damage by tritiated thymidine, cells not only induce adaptive mechanism at cellular level but also secrete cytoprotective factors in the medium, which would induce survival pathways in the neighboring bystander cells.

Irradiated conditioned medium resulted in lower DNA damage after additional gamma radiation but showed slower repair kinetics

Since the irradiated conditioned medium induced higher proliferation and survival [Figure 1a and b], which might be associated with induction of survival pathways due to prosurvival diffusible factors in the conditioned medium, which would prevent DNA damage after exposure of any genotoxic agents like radiation. To validate the hypothesis, bystander cells treated with control/irradiated conditioned medium were treated with 0.5 Gy of gamma

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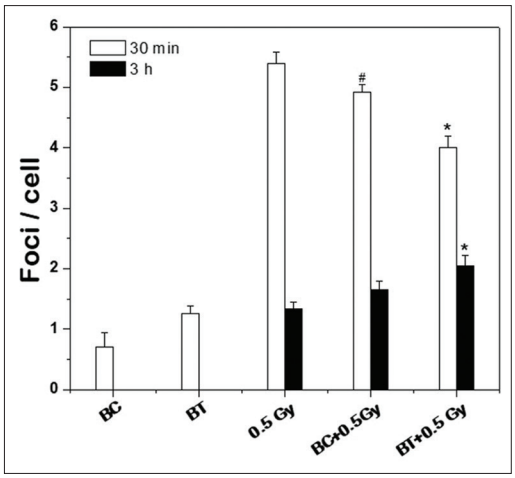

Figure 3: Magnitude of DNA damage measured by γ-H2AX immunofluorescence when cells cultured in control (BC) and irradiated (BT) conditioned medium and were irradiated with a dose of gamma (0.5 Gy). Cells grown in plain culture medium (nonconditioned medium) were used as 0.5 Gy control. γ-H2AX immunofluorescence was done at 30 min and 3 h after gamma irradiation, and DNA damage was expressed as foci/cell. The average of foci in control cells (unirradiated cells cultured in the plain medium) was 0.26 ± 0.16 foci/cell. *Significantly different than BC + 0.5 Gy at $P < 0.05$, #Significantly different than 0.5 Gy at $P < 0.05$

radiation followed by measurement of DNA damage by γ-H2AX at 30 min and 3 h after irradiation. Compared to unirradiated BC, the unirradiated BT cells showed a higher number of average γ-H2AX foci per cell. However, when cells were treated with 0.5 Gy of gamma radiation at 30 min postirradiation, an average of ~5 foci/cell was observed, which was decreased in BC and BT cells irradiated with gamma radiation. Interestingly, γ-irradiated (0.5 Gy) BT cells showed lower γ-H2AX foci/cell than 0.5 Gy γ-irradiated BC cells. These results suggest the prevention of DNA damage in the bystander cells treated with conditioned medium obtained from tritiated thymidine-labeled cells [Figure 3]. On the other hand, when the results were analyzed for 3 h postirradiation, a significant decrease in γ-H2AX foci/cell was observed, suggesting repair in DNA damage. It was interesting to observe that compared to 0.5 Gy and γ-irradiated BC cells, γ-irradiated BT cells showed higher γ-H2AX foci/cell suggesting lower recovery of DNA damage in these cells. It seems that diffusible factors in the conditioned medium of tritiated thymidine-labeled cells prevent induction of gamma radiation-induced DNA damage at shorter period (30 min) but not able to induce repair pathways (at 3 h), which needs further investigation.

Irradiated conditioned medium resulted in higher nitric oxide production in bystander cells

NO is one of the reactive nitrogen species, which has been known to cause radiation-induced DNA damage and play a crucial role in manifestation of radiation-induced bystander effects.[10-12] Hence, the magnitude of intracellular NO

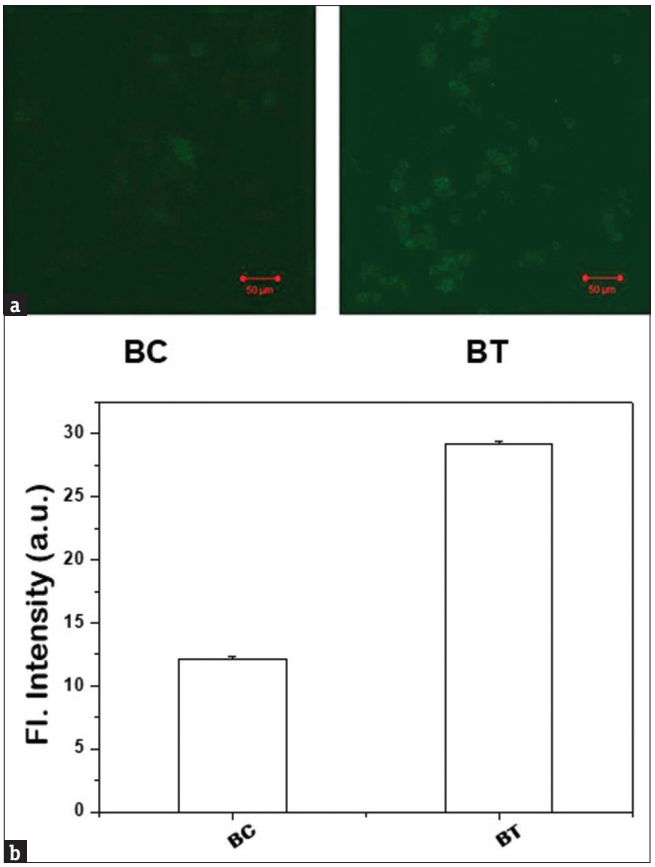

Figure 4: Intracellular nitric oxide in bystander cells treated with control (BS) and irradiated (BT) conditioned media. (a) Before harvesting the cells, fluorescence images were acquired using fluorescence microscopy. Scale bar.: 50 µM. (b) Cells were harvested by trypsinization and fluorescence intensity was determined by fluorimetry. a.u.: Arbitrary unit

was measured in bystander INT407 cells treated with control and irradiated conditioned mediums. Compared to control (BC), BT cells showed a higher magnitude of NO oxide [Figure 4a]. These results were also correlated with higher fluorescence intensity of BT cells [Figure 4b].

Conclusions

Bystander cells treated with conditioned medium obtained from tritiated thymidine-labeled cells showed increased clonogenic survival and proliferation, which was correlated with an increase in DNA double-strand break and NO production in these cells.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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