Radiation quality effects alteration in COX-2 pathway to trigger radiation-induced bystander response in A549 lung carcinoma cells

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ABSTRACT

This study aimed to determine whether the radiation-induced bystander effect (RIBE) is affected by radiation quality. To mimic the different radiation qualities of the direct action \((D)\)/indirect action \((ID)\) ratio, A549 cells were exposed to X-rays, with either 100 mM of the radical scavenger, thio-urea (TU⁺), or null (TU⁻). Biological responses in irradiated and bystander cells were compared at equal lethal effects of a 6% survival dose, which was estimated from the survival curves to be 8 Gy and 5 Gy for TU⁺ and TU⁻, respectively. Cyclooxygenase-2 (COX-2) expression in TU⁻ irradiated cells increased up to 8 h post-irradiation, before decreasing towards 24 h. The concentration of prostaglandin E2 (PGE2), a primary product of COX-2 and known as a secreted inducible factor in RIBE, increased over 3-fold compared with that in the control at 8 h post-irradiation. Conversely, COX-2 expression and PGE2 production of TU⁺ irradiated cells were drastically suppressed. These results show that the larger \(D/ID\) ratio suppressed COX-2 expression and PGE2 production in irradiated cells. However, in contrast to the case in the irradiated cells, COX-2 expression was equally observed in the TU⁻ and TU⁺ co-cultured bystander cells, which showed the highest expression levels at 24 h post-irradiation. Taken together, these findings demonstrate that radiation quality, such as the \(D/ID\) ratio, may be an important factor in the alteration of signalling pathways involved in RIBE.

Keywords: radiation-induced bystander effect; COX-2; DNA damage complexity; indirect action; thio-urea; radiation quality, radical scavenger

INTRODUCTION

In classical radiation biology, DNA damage induced by the direct energy deposition of radiation is the main cause for the variety of radiobiological consequences. Hence, DNA is considered to be the primary target of radiation [1, 2]. Radiation-induced bystander effect (RIBE) [3] is a phenomenon observed in cells that have not been exposed to radiation, but have been affected through bidirectional intercellular signalling from irradiated cells [4, 5]. In the past several decades, multiple inter- and intracellular signalling pathways have been reported to be involved in RIBE, and the propagation of these signals involves gap junctional intercellular communication (GJIC) [6, 7] and media-mediated intercellular communication (MMIC) [8–12]. Several studies have clarified the major players in bystander signals, such as nitric oxides [9, 12, 13], cytokines [11, 14], exosomes [15], and inflammatory products, such as prostaglandin E2 (PGE2) [16, 17]. In addition, evidence has accumulated showing that...
RIBE exists within different types of radiation, such as photons of low linear energy transfer (LET) \[6, 14, 18\], neutrons \[19\], protons \[5\], \(\alpha\) particles \[3, 20\], and high-LET heavy ions \[6, 13, 14, 21\]. However, it is still unclear whether RIBE is affected by radiation quality \[6, 13, 14\], or is only affected by the accumulated dose \[21\]. The biological effects of radiation are principally derived from DNA damage caused by both direct action (D) (in which atoms of the DNA are ionized or excited directly by the radiation) and indirect action (ID) (interaction between the radiation and atoms of other molecules to produce free radicals that are able to diffuse far enough to reach and damage the DNA) \[22, 23\]. The radiation qualities with larger D/ID ratios are known to induce a larger degree of complex and irreparable DNA damage \[24\]. These observations, whether in irradiated cells or RIBE cells, show that DNA damage can occur in various ways.

In this study, we mimicked differences in radiation quality in terms of the difference in D/ID ratio by suppressing the ID. This was achieved by using the hydroxyl (OH) radical scavenger, thio-urea (TU) \[25\] in the cell culture. We investigated the cyclooxygenase-2 (COX-2)/prostaglandin E\(_2\) (PGE\(_2\)) pathway \[17, 26–28\] as an indicator for cellular responses in irradiated and bystander cells to clarify whether the RIBE and its signalling pathways would be affected by radiation quality.

**MATERIALS AND METHODS**

**Cell cultures and treatments**

Human lung adenocarcinoma cells, A549 (RCB0098), were obtained from the RIKEN Bioresource Center. The cells were maintained in Dulbecco’s Modified Eagle’s Medium (D-MEM; 041–30081, Wako Pure Chemical Industries, Ltd, Osaka, Japan) supplemented with 10% foetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 \(\mu\)g/ml streptomycin) at 37°C in a 5% CO\(_2\) and humidified atmosphere.

**X-ray irradiation**

For irradiation, an X-ray generator (TITAN, Shimazu Co., Kyoto, Japan) set at 200 kVp and 20 mA was employed, and irradiation was made through a copper and aluminium filter with a thickness of 0.5 mm, producing an effective energy of ~83 keV. The samples were irradiated through a co-culture insert dish (BD Falcon No. 3099, MA, USA) and co-cultured for up to 1, 4, 8 and 24 h post-irradiation.

**Measurement of OH radical production by X-ray irradiation**

The production of OH radicals and other reactive oxygen species (ROS) by X-ray irradiation was measured by the oxidant-sensing fluorescent probe 2,7’-dichlorodihydrofluorescein diacetate (DCFH-DA) \[27\]. DCFH-DA solutions (100 \(\mu\)M DCFH-DA in PBS, 044–28241, WAKO, Osaka Japan) were prepared under two sets of conditions: null (TU\(^-\)), and with an OH radical scavenger, 100 mM thio-urea (TU\(^+\)) (33812–92, Nacalai Tesque Inc., Kyoto, Japan). A total of 1 ml of DCFH-DA solutions were irradiated with various doses (Fig. 1). After irradiation, 200 \(\mu\)l of DCFH-DA solutions were transferred per into each well of 96-well plate (164588, Thermo Fisher Scientific, MA), and the DCF fluorescence was quantified with a fluorescent image scanner (FLA-5100, FUJIFILM, Tokyo).

**Colony formation assay**

At 1 day prior to irradiation, 1 \(\times\) 10\(^6\) cells were seeded in T25 flasks (353014, Falcon, NY) with 5 ml of medium. Thirty minutes before irradiation, media were changed with freshly prepared media containing 100 mM thio-urea or null. Immediately after irradiation, the irradiated cells and controls were harvested using 2.5 g/l trypsin solution (35555–54, Nakarai Tesque Inc., Kyoto, Japan), and plated in triplicate to obtain ~200 surviving cells per dish. After 12 days, the cells were fixed with 5% formalin in PBS, and stained with 1% methylene blue. Colonies that contained >50 cells were counted as survivors.

**Measurement of prostaglandin E\(_2\) in conditioned medium**

The concentrations of prostaglandin E\(_2\) were measured by a PGE\(_2\) monoclonal ELISA kit (514010, Cayman Chemical, MI) according to the manufacturer’s instructions. Briefly, 5 \(\times\) 10\(^5\) cells in 35 mm dishes were exposed to X-rays of 3, 5 or 8 Gy. At 8 h post-irradiation, the medium was collected and centrifuged, and an ELISA assay was performed. PGE\(_2\) concentration within the samples was measured using a microplate reader (iMark\textsuperscript{TM} plate reader, Bio-rad, CA).

**Co-culture of irradiated and bystander cells**

To investigate COX-2 expression through the RIBE, a cell co-culture insert dish (BD Falcon No. 3099, MA, USA) and companion TC plate well (BD Falcon No. 3052) were applied, as demonstrated by others \[28\]. The membrane bottom of the insert dish has 0.4 \(\mu\)m pores with a density of 1 \(\times\) 10\(^6\)/cm\(^2\) to allow for the passage of biomolecules. One day before irradiation, cells were seeded (5.0 \(\times\) 10\(^5\) cells/well in 2 ml medium) into 6-well companion plates to be 80–90% confluent at the time of irradiation. Simultaneously, the same number of cells was seeded on insert dishes. Prior to irradiation, the media of inserts were replaced with fresh media containing 100 mM thio-urea (TU\(^+\)) or null (TU\(^-\)). Cells were exposed to X-ray radiation at a 6% survival dose of 5 Gy for TU\(^-\) and 8 Gy for TU\(^+\), which were estimated from survival curves (Fig. 2). Media of both inserts and the co-culture plates were changed to fresh medium and then they were co-cultured for up to 1, 4, 8 and 24 h post-irradiation.

**Western blot analysis for COX-2 expression**

Cells were washed with PBS, then collected into SDS sample buffer [4% (w/v) sodium dodecyl sulfate (SDS), 125 mM Tris-HCl (pH 6.8)] and heated at 95°C for 20 min. Each lysate (20 \(\mu\)g protein) was mixed with 4 \(\times\) loading buffer solution (250 mM Tris-HCl, 0.02% bromo-phenol blue, 8% SDS 40% glycerol, 20% 2-mercaptoethanol pH 6.8; 191–13272, WAKO, Osaka Japan), loaded into wells of 10% SDS-PAGE gels for electrophoresis and then transferred to PVDF membranes. After blocking in 5% skim milk in Tris-buffered saline-Tween 20 (TBST), the PVDF membrane was incubated with the primary antibody at 4°C overnight. The antibodies for COX-2 (#12282), HRP-linked secondary antibody (#3683) and GAPDH (#7074) were purchased from Cell signalling...
Technology, MA. Anti–COX-2 was labelled with HRP-linked secondary antibody, and anti-GAPDH was used as a loading control. All antibodies were diluted to 1/1000 in TBST and the membrane was washed three times with TBST between every step after incubation. Protein bands were visualized using a chemi-luminescence substrate (Pierce™ ECL Plus Western Blotting Substrate, Thermo Fisher Scientific, MA) and chemi-luminescence imager (EZ-capture MG, ATTO, Tokyo, Japan).

To measure COX-2 expression by PGE2, cells were cultured in media supplemented with 0.1 ng/ml PGE2 for 1, 4 or 8 h and lysates were collected as mentioned above.

**Statistical analysis**

A Student’s t-test was performed by Sigma Plot 12 (Systat Software, Inc, CA) to analyse differences in data between TU– and TU+. Differences with P < 0.05 were considered statistically significant.

**RESULTS AND DISCUSSION**

There is currently debate regarding the dependency of bystander effects on radiation quality. Generally, increasing LET would reduce OH radical production per dose, resulting in a larger D/ID ratio [23, 29, 30]. To mimic the differences in radiation quality, we set up a simplified model by reduced the ID by using TU to increase the D/ID ratio. We used a DCFH-DA fluorescent probe to estimate their production of ROS by X-rays as well as their reduction by 100 mM thio-urea (TU+) to quantify the ID. As shown in Fig. 1, the slope of TU+ was decreased to that of 35% of TU–, indicating that 65% of the OH radicals per dose were scavenged by TU+ compared with TU–. The survival curves shown in Fig. 2 clearly show that TU+ irradiated cells were protected compared with TU– cells. Generally, the D/ID ratio for X-ray–induced cell killing is known to be at ~0.5. Hirayama et al. reported that 76% of X-ray–induced cell killing is from ID [23], resulting in a D/ID ratio of 0.315. The IDTU– was reduced to 35% of IDTU+, thus, TU+ can be estimated to be DITU+/DITU– = 0.902. Hence, our experimental system mimicked two radiation types of 2.86-fold different D/ID ratios.

Next, we measured the production of PGE2, the primary product of COX-2, from the irradiated cells at 8 h post-irradiation (Fig. 3A). COX-2 is known as prostaglandin-endoperoxide synthase 2 and is a key regulatory enzyme not only responsible for multiple inflammatory mitogenic, and angiogenic activities, but also strongly involved in the development and progression of cancer [27]. PGE2 is known to be a potent endogenous molecule, which binds to E-series prostanoid (EP) receptors [26, 27]. In other words, PGE2 functions as a trigger signal to activate the expression of COX-2 in bystander cells [16, 17]. As shown in Fig. 3B, we have confirmed that PGE2 activates COX-2 expression in A549 cells at concentrations as low as 0.1 ng/ml. The PGE2 concentration of TU– irradiated cells increased in a dose-dependent manner up to 5 Gy, which was the highest of all doses, and then decreased at 8 Gy. This trend was not observed with TU+ cells, even at the iso-effect dose for cell killing of 8 Gy, in which the PGE2 concentration was ~60% of the 5 Gy–exposed TU– cells. The trend in the COX-2 expression was consistent with that of the PGE2 expression (Fig. 4). COX-2 expression of TU+ cells was 2-fold higher than that of the control at 4 h post-irradiation, whereas TU+ cells only showed a slight, insignificant increase. These results indicate that a higher D/ID ratio may reduce COX-2 expression, therefore suppressing PGE2 production.

In contrast, COX-2 expression of TU+ bystander cells increased to nearly 2-fold of the control toward 24 h post-irradiation (Fig. 4A-2)

**Fig. 1.** Measurement of ROS production by X-ray exposure. Plots were fitted by linear regression after background subtraction. The slopes (rfu/Gy) of the graphs for TU– and TU+ were 5931 and 2059, respectively. DCF = dichlorofluorescein, rfu = relative fluorescence units.

**Fig. 2.** Plots were fitted according to the linear quadratic model, S = exp (−α × D − β × D2), where S is the survival fraction, D is the dose in Gy, and α [Gy−1] and β [Gy−2] are the fitting parameters. The α, β, 10% survival dose (D10) and 1% survival dose (D1) for TU– and TU+ were calculated to be TU–: 0.38, 0.037, 4.27 Gy and 7.14 Gy and TU+: 0.175, 0.022, 6.96 Gy and 10.9 Gy, respectively.
and B-2). Surprisingly, this trend was very similar to that in the TU-bystander cells, and there was no significant difference between the two. COX-2 expression and PGE2 concentration were greatly reduced in the TU+ irradiated cells, indicating that the PGE2 did not play a dominant role in inducing COX-2 signalling in TU+bystander cells. These results suggest that COX2 expression in TU+bystander cells was activated by pathways other than the COX-2/PGE2 pathway, contrary to the situation in TU− bystander cells. It is known that COX2-derived PGE2 can initiate a positive feedback loop to activate epidermal growth factor receptor (EGFR), resulting in enhanced expression of COX-2 and increased synthesis of prostaglandins. However, EGFR and its downstream effectors can be activated independently of COX-2/PGE2 [27]. The NFκB/COX-2 pathway is also independent of the COX-2/PGE2 pathway, and can be activated through hypoxia-inducible factor, HIF-1 [8]. In addition, other secreted diffusible factors, such as ROS [12, 13] and exosomes [15], which trigger medium mediated RIBE may contribute to COX-2 expression in bystander cells.

In conclusion, we demonstrated lower COX-2 expression and PGE2 production in irradiated cells with a higher D/ID ratio. The pathway responsible for COX-2 expression as a bystander response could be an altered from the COX-2/PGE2 pathway to others due to the radiation quality of higher D/ID ratios. Further investigation will be necessary in order to identify the alternative pathways for COX-2 expression and to understand the correlation between radiation quality and the pathways responsible for RIBE.

Fig. 3. Panel A shows the PGE2 level of irradiated cells measured at 8 h post-irradiation with various X-ray doses. PGE2 concentrations of 5 Gy-irradiated TU− cells and TU+ cells were 93.4 pg/ml and 38.1 pg/ml, respectively. PGE2 concentration for 8 Gy TU+ irradiated cells was 59.5 pg/ml, which was lower than 5 Gy TU− irradiated cells (*P < 0.05). Panel B shows COX-2 expression after the addition of 0.1 ng/ml PGE2 (#P = 0.1).

Fig. 4. Comparison of COX-2 expression in TU− and TU+ irradiated and bystander cells. Panels A-1 and B-1 are representative images of western blot analyses of irradiated cells and bystander cells, respectively. Panels A-2 and B-2 show the COX-2 expression for each hour post-irradiation of irradiated cells and bystander cells, respectively. *P < 0.05.
SUPPLEMENT DATA
Supplementary data are available on Journal of Radiation Research online.

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CONFLICT OF INTEREST
The authors state that there are no conflicts of interest.

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