Effects of irradiated cell conditioned medium on the response of human lung cancer cells to anticancer treatment in vitro

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Abstract. Ionizing radiation induces a cellular response not only in the irradiated cells, but also in the neighboring non-irradiated cells, and this effect is known as the radiation-induced bystander effect. The irradiated cells transmit signals to the non-irradiated cells via gap junctions or soluble factors, exerting biological effects on the neighboring non-irradiated cells. In this study, we investigated the effects of irradiated cell conditioned medium (ICCM) on the response of human lung cancer cells (A549 and H1299) to anticancer treatment. First, we analyzed the effects of ICCM on the induction of apoptosis by anticancer treatment (ionizing radiation or gefitinib). Human lung cancer cells were cultured on the induction of apoptosis by anticancer treatment (ionizing radiation, bystander effect, irradiated cell conditioned medium, gefitinib, apoptosis, migration)

Introduction

Ionizing radiation exerts biological effects, such as cell death and chromosomal aberration due to the direct radiation of the cells. However, evidence suggests that this radiation affects not only the cells irradiated directly, but also the surrounding non-irradiated cells (1-3). This response, known as the non-targeted effect, includes genomic instability and other radiation-induced bystander effects. Genomic instability refers to biological effects, such as delayed gene mutations and chromosomal aberrations that occur in the progeny of the irradiated cells (3), whereas radiation-induced bystander effects are caused by the transmission of signals from the irradiated cells to the non-irradiated cells via gap junctions or soluble factors (1,2). Various factors, such as transformation growth factor-β (TGF-β), tumor-necrosis factor-α, and reactive oxygen species have been reported to be possible candidate bystander factors (4). In general, to examine the bystander effects mediated by soluble factors in vitro, non-irradiated cells are co-cultured with irradiated cells or cultured in the presence of irradiated cell conditioned medium (ICCM). Non-irradiated cells co-cultured with irradiated cells or treated with ICCM have been reported to undergo various biological effects, such as DNA double-strand breaks and apoptosis, generally observed in irradiated cells (1,3).

Radiation therapy is widely used in the treatment of various types of cancer. Although radiation therapy is considered to control the tumor cells locally, there is evidence to indicate additional systemic antitumor effects of this therapy (5,6); these effects have been referred to as the abscopal effect. In the abscopal effect, the reduction or disappearance of tumors occurs not only in the irradiated lesions, but also in the non-irradiated lesions, suggesting that signals from irradiated tissues can affect the unirradiated tissues outside of the irradiated volume. There is recent evidence to suggest the involvement of the immune system in the abscopal effect (7). Briefly, irradiated tumors release immunostimulatory molecules, such as inflammatory cytokines and damage-associated molecular patterns, which are endogenous molecules released...
due to cellular damage. The released signals activate the innate immune system, leading to T-cell-mediated cytotoxicity against tumors in non-irradiated lesions (8).

The factors released from irradiated cells exert biological effects, such as the induction of apoptosis and activate antitumor immunity, which may prove beneficial for the treatment of cancers (5,9,10). However, little is known about the effects of factors released from irradiated cells on the response of cancer cells to anticancer treatment. Therefore, the present study investigated the effects of ICCM on the response of human lung cancer cells to anticancer treatment in terms of the induction of apoptosis and cellular migration.

Materials and methods

Reagents. Dimethyl sulfoxide (DMSO) and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gefitinib was purchased from Selleckchem (Houston, TX, USA).

Cells and cell culture. Human lung cancer cell lines A549 and H1299 cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and cultured in RPMI-1640 medium (Gibco®, Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1% penicillin/streptomycin (Gibco®) and 10% heat-inactivated fetal bovine serum (Japan Bioserum Co., Ltd., Nagoya, Japan).

In vitro irradiation. The cells were irradiated (150 kVp; 20 mA; 0.5 mm Al and 0.3 mm Cu filters) using an X-ray generator (MBR 1520R 3; Hitachi, Ltd., Tokyo, Japan) at a distance of 45 cm from the focus and a dose rate of 1.01-1.07 Gy/min.

Medium transfer experiments. Medium transfer experiments were performed, as previously reported (11). A schematic illustration of the medium transfer experiments is presented in Fig. 1. Approximately 2.4x10⁵ cells were seeded onto 35-mm culture dishes (Iwaki, Chiba, Japan) and cultured for 5 h at 37°C to promote their adherence to the dish. The cells were then exposed to 8 Gy X-ray and cultured for 24 h at 37°C.

The conditioned medium was then collected by centrifugation (180 x g for 5 min at room temperature) wherein, the supernatant was filtered using a 0.45-μm syringe filter (2053-025; Iwaki) to remove cells and debris. The filtrated cell layers were then exposed to 8 Gy X-ray and cultured for 24 h at 37°C to allow for their adherence.

Results

Effects of ICCM on the induction of apoptosis by gefitinib treatment. Inhibitors of the epidermal growth factor receptor (EGFR) tyrosine kinase, such as gefitinib have been used in the treatment of lung cancer (14). Therefore, in this study, we examined the effect of ICCM on the induction of apoptosis by gefitinib in the A549 and H1299 cells. As shown in Fig. 3, gefitinib treatment significantly increased the proportion of Annexin V⁻ apoptotic

Apoptosis assay. Apoptosis was analyzed using Annexin V-FITC, PI and Annexin V binding buffer (all BioLegend Inc., San Diego, CA, USA), as reported previously (12). The stained cells were analyzed by performing flow cytometry (Cytomics FC500 with CXP software version 2; Beckman Coulter, Inc., Brea, CA, USA).

Scratch assay. The A549 cells were cultured in a 24-well plate (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) until they reached approximately 90% confluence. The cell monolayer was scratched using a yellow tip. After the culture medium containing floating cells was aspirated, ICCM (500 μl) was added to the plate. The cell-free scratched area was measured using an Olympus LX71 microscope and DP2-BSW software version 2.1 (both Olympus, Tokyo, Japan) immediately, and at 24 and 48 h after scratching, and the percentage of wound closure area was calculated. In some experiments, the cells were exposed to 8 Gy X-ray at 2 h following the addition of ICCM.

Statistical analysis. Data are presented as the means ± standard deviation (SD). Comparisons between the control and experimental groups were performed using a two-sided Student’s t-test or a two-sided Mann-Whitney U test depending on the data distribution. Multiple data were analyzed using one-factor analysis of variance followed by the Tukey-Kramer test. Differences were considered statistically significant at P<0.05. All statistical analyses were performed using Excel 2016 software version 1903 (Microsoft, USA), with an add-on software Statcel 4 (OMS Publishing, Inc., Tokyo, Japan).
Figure 1. Schematic illustration of medium transfer experiments. The procedures for the collection of ICCM and treatment of cells with ICCM. ICCM, irradiated cell conditioned medium.

Figure 2. Effects of ICCM on apoptosis in cells exposed to X-ray. (A) A549 and (B) H1299 cells cultured in the presence of ICCM were exposed to 8 Gy X-ray irradiation. After 3 days of culturing, the cells were harvested for apoptosis assay. (Left panels) Representative cytograms of Annexin V/PI staining with inset numbers indicating the percentage of Annexin V+/PI- and Annexin V+/PI+ cells. (Right panels) Percentages of Annexin V+ cells (sum of Annexin V+/PI- and Annexin V+/PI+ cells) are presented as the mean ± standard deviation from at least 3 independent experiments. *P<0.01, compared with 0 Gy; †P<0.05. ICCM, irradiated cell conditioned medium.
A549 and H1299 (P<0.05) when compared with the cells treated with DMSO. However, in contrast to the results obtained with X-ray irradiation (Fig. 2), no significant differences in the proportion of Annexin V+ apoptotic cells were noted between ICCM obtained from non-irradiated cells and that from irradiated cells (Fig. 3).

**Effect of ICCM on cellular migration.** As shown in Fig. 4, both ICCM from non-irradiated cells and that from 8 Gy-irradiated cells exerted minimal effects on the migration of the non-irradiated A549 cells. Additionally, no significant difference in the wound closure area was observed between the non-irradiated cells and 8 Gy-irradiated cells. Despite this, ICCM obtained from irradiated cells suppressed the migration of H1299 cells, with a significant difference observed (P<0.01, compared with non-irradiated cells).

In summary, ICCM can modulate the response of cancer cells to anticancer treatments, with effects on both apoptosis and migration. The specific mechanisms by which ICCM exerts these effects are likely to be complex and may involve signaling pathways that are not yet fully elucidated.
cells (ICCM 0 Gy in Fig. 4B vs. ICCM 0 Gy in Fig. 4C). However, as shown in Fig. 4C, the wound closure area in the 8 Gy-irradiated A549 cells at 24 h after the scratch was made was significantly greater in the cells treated with ICCM from irradiated cells when compared with those from non-irradiated cells (P<0.01).

Discussion

The factors released from irradiated cells induce various biological effects, such as cell death and inflammatory responses, and these effects may be preferential for cancer treatment. In this study, we investigated the effects of ICCM from irradiated human lung cancer cells on the response to anticancer treatment (ionizing radiation or gefitinib). Although ICCM did not induce the apoptosis of non-irradiated cells in the current study, it attenuated the induction of apoptosis by ionizing radiation, but not by gefitinib, depending on the cell type. We also demonstrated that ICCM enhanced the migration of 8 Gy-irradiated cells, but not that of non-irradiated cells. Taken together, these results suggest that cancer cells treated with ICCM exhibit resistance to ionizing radiation in terms of apoptosis and cellular migration. In line with our results, Iyer and Lehnert reported that clonogenic survival after migration. In line with our results, Iyer and Lehnert reported that cell type specific-effect of ICCM, which is not that of non-irradiated cells. Taken together, these results suggest that cancer cells treated with ICCM exhibit resistance to ionizing radiation in terms of apoptosis and cellular migration. In line with our results, Iyer and Lehnert reported that clonogenic survival after migration. In line with our results, Iyer and Lehnert reported that clonogenic survival after migration. In line with our results, Iyer and Lehnert reported that clonogenic survival after migration.

In conclusion, although the present study is limited in terms of the in vitro nature of the analysis, our results suggest that cancer cells treated with ICCM exhibit resistance to ionizing radiation, which may be unfavorable for cancer treatment. Therefore, further studies clarifying the underlying mechanisms involved are required to achieve an effective treatment strategy for cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HY and IK initiated the research. HY, MN and KM performed the experiments, and collected and analyzed the data. HY and IK wrote, reviewed, and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
References


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