Epithelial–mesenchymal transition in non-targeted lung tissues of Kunming mice exposed to X-rays is suppressed by celecoxib

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ABSTRACT

Lung cancer is one of the highest health risks caused by ionizing radiation, which induces both direct effects and non-targeted effects. However, whether radiation-induced non-targeted effects result in epithelial–mesenchymal transition, a critical process during tumorigenesis, in non-targeted lung tissues remains unknown. In the present study, Kunming mice were subjected to whole-body, cranial or local abdominal irradiation of single-dose or fractionated 4 Gy X-rays, and the expressions of epithelial–mesenchymal transition markers in non-targeted lung tissues were assessed by both qRT-PCR and immunofluorescent staining. It was found that the epithelial marker was downregulated while the mesenchymal markers were upregulated significantly in non-targeted lung tissues of the irradiated mice. Local abdominal irradiation was more efficient in inducing epithelial–mesenchymal transition than whole-body or cranial irradiation when the fractionated irradiation method was adopted. In addition, the intraperitoneal administration of celecoxib suppressed epithelial–mesenchymal transition in the non-targeted lung tissues. In conclusion, our findings suggest that epithelial–mesenchymal transition is induced in non-targeted lung tissues, but can be suppressed by inhibition of cyclooxygenase-2 by celecoxib.

Keywords: radiation-induced non-targeted effect; mouse; epithelial–mesenchymal transition; cyclooxygenase-2

INTRODUCTION

The dogma that the radiobiological effects are restricted to the cells directly targeted by ionizing radiation (IR) has been challenged by the discovery of the radiation-induced bystander effect (RIBE), which suggests that the cells in close proximity to the directly irradiated cells can manifest various kinds of biological effects, including transformation, chromosomal aberrations, apoptosis, etc. [1]. Up till now, most knowledge about RIBE comes from the conventional in vitro cell culture system. It has been identified that both gap junctional intercellular communication (GJIC) and soluble factors generated by directly hit cells play important roles in the RIBE in cell cultures [2]. However, in vivo studies about RIBE/non-targeted effects are limited, which has important implications for both the precise assessment of radiation risk and the accurate evaluation of existing radiotherapy models. It has been found by several researchers that the RIBE exists in both 3D human tissue models and animal models, with endpoints including DNA damage, gene expression dysregulation, cell death, tumorigenesis, etc. [3–6]. The RIBE can also be considered as abscopal effects in clinical radiotherapy and contributes to secondary carcinogenesis [7].

Epithelial–mesenchymal transition (EMT), an important process through which epithelial cells transform into mesenchymal cells, is involved in development, inflammation, tumorigenesis, tumor...
development, and metastasis, etc. [8, 9]. It was found that overexpression of EMT-related transcriptional factors in mammary epithelial cells induced EMT and resulted in both the acquisition of mesenchymal traits and expression of stem cell markers, which hinted that EMT functions in the early stage of tumorigenesis [10]. However, whether the RIBE plays a role in the induction of EMT in non-targeted tissues remains unknown.

It has been reported that the lung was the second-most common cancer site among the atomic bomb survivors, and lung cancer accounted for 10.9% of all solid cancer cases [11]. However, the underlying mechanisms remain to be elucidated. In addition, lung cancer shows one of the highest morbidities and mortalities of all kinds of cancers throughout the world [12–14]. Since most lung cancer patients need to be treated with radiotherapy, the potential for a second carcinogenesis in non-targeted tissues induced by radiotherapy cannot be ignored. It was found that cyclooxygenase-2 (COX-2) was significantly upregulated in non-targeted lung tissues, and that inhibition of COX-2 by Nimesulide reduced the RIBE-induced oxidative DNA damage, implying that it may be a target for inhibition of the RIBE in lung tissues [15]. In this study, to investigate whether EMT was induced in non-targeted lung tissues in vivo, we examined expression of the EMT marker genes in the non-targeted lung tissues of mice receiving partial body irradiation, and determined the inhibitory effect of celecoxib (a specific inhibitor of COX-2) on EMT, in the hope of revealing the role of the RIBE in radiation-induced tumorigenesis in lung cancer, as well as of developing the related countermeasures.

MATERIALS AND METHODS

Animal and irradiation treatment
Male Kunming mice (4–5 weeks old), housed in a virus-free facility and given food and water ad libitum, were randomly divided into several groups according to different treatments. Each group consisted of ten animals. Animals were subjected to either whole-body irradiation (WBIR), cranial irradiation (CIR) or local abdominal irradiation (LAIR) of 4 Gy X-rays by using a cabinet X-ray generator (Faxitron, Wheeling, IL, USA) operated at 100 kVp and 5 mA with a dose rate of 1.2 Gy/min. The thickness of the beryllium filter used for either CIR or LAIR, and a set of 4-mm-thick lead shields were laid upon the animals in a manner allowing only the needed areas to be exposed. As for LAIR, a 1-cm² (1 cm × 1 cm) area of the lower abdomen of the mouse was chosen and subjected to irradiation (Fig. 1). The protection of shielded tissues was successful (0.61 mGy/min under the 4-mm-thick lead shields), as verified by radiation dosimetry using a calibrated semiconductor dosimeter (PTW-DIADOS, Freiburg, Germany). To measure whether scattering contributed to the EMT observed in the bystander tissues, a Monte Carlo simulation was employed to determine the scatter radiation dose resulting from X-ray scattering within the mouse itself. The absorbed dose to the lung tissues after LAIR with a single 4 Gy dose of 100 kVp X-rays was estimated to be ~5.3 mGy. Thus WBIR with 5.3 mGy of X-rays was used as a scatter radiation control in another cohort of 10 male Kunming mice. Animals were euthanized at 48 h after irradiation. All animal studies were reviewed and approved by the Soochow University Institutional Animal Care and Use Committee, and performed in compliance with the institutional regulations and guidelines for the welfare and use of animals in cancer research [16].

qRT-PCR analysis
At 48 h post-irradiation, mice were euthanized for lung tissue collection without perfusion. Total RNA extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was quantified by NanoDrop™ ND-2000 (Thermo Fisher Scientific, Sunnyvale, CA, USA) and reverse-transcribed (RT) using an All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia, Rockville, MD, USA). The real-time PCR analysis was carried out using a SYBR Premix Ex Taq II Kit (Takara, Japan) on a Chromo4 Real-Time PCR system (Bio-Rad, Hercules, CA, USA). The expression levels of mRNAs were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed with the C(t) value comparison method.

Immunofluorescent staining
Lung tissues were submerged in Tissue Freezing Medium (Leica, Wetzlar, Germany) and frozen at −80°C before frozen section

Fig. 1. Sketch of the cranial irradiation (CIR) and local abdominal irradiation (LAIR) set-ups of the mice. (A) CIR set-up. A 4-mm-thick lead shield was used to cover the mouse body except the head. (B) LAIR set-up. A 1-cm² (1 cm × 1 cm) area of the lower abdomen of the mouse was irradiated with 4 Gy of X-rays. A set of 4-mm-thick lead shields were used to cover the mouse body in a manner that allowed only the needed area to be exposed.
production. The frozen tissues were cut into 5 μm sections, fixed in 4% paraformaldehyde for 15 min, then permeabilized in 0.5% Triton-X 100 for 1 min. After blocking with 5% milk for 1 h, slides were incubated with a mixture of antibodies against E-cadherin and Vimentin (CST Biotech, Danvers, MA, USA) for 2 h at room temperature. After three washes with 1 × phosphate-buffered saline (PBS), slides were incubated with a mixture of Alexa Fluor® 594 anti-mouse IgG and Alexa Fluor® 488 anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After another three washes with 1 × PBS, DAPI mounting medium (Invitrogen, Carlsbad, CA, USA) was used for nuclear staining. Slides were then examined under an Olympus FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). The immunofluorescence assay was conducted in the lung tissues from at least three mice from each group.

Statistical analysis
All data are presented as means ± SE. Statistical significance was determined by Student’s t-test between the indicated groups. P < 0.05 was considered to be statistically significant.

RESULTS
To determine whether WBIR, CIR or LAIR was capable of inducing EMT in non-targeted lung tissues of mice exposed to single-dose or fractionated irradiation, lung tissues of the irradiated mice were collected 48 h post-irradiation and the expressions of several EMT marker genes were examined by both qRT-PCR and immunofluorescent staining.

Relative transcript expression levels of EMT markers (E-cadherin, N-cadherin, Fibronectin and Vimentin) in non-targeted lung tissues were determined by qRT-PCR. As shown in Fig. 2, all three kinds of exposure methods were found to be able to induce significant expression changes of EMT markers in non-targeted lung tissues of the irradiated mice. The epithelial marker E-cadherin was downregulated significantly, while the mesenchymal markers, N-cadherin, Fibronectin, and Vimentin, were upregulated significantly (P < 0.05). While whole-body exposure to 5.3 mGy of X-rays did not result in downregulated E-cadherin or upregulated mesenchymal markers (Supplementary Fig. 1), indicating that the observed changes in the expressions of EMT markers were not likely to be caused by scatter radiation. It was also found that all three exposure methods had comparable abilities to induce EMT when the single-dose irradiation method was adopted. LAIR was more efficient in inducing the downregulation of E-cadherin in non-targeted lung tissues than WBIR or CIR when the fractionated irradiation method was used. In addition, COX-2 expression was upregulated significantly in the non-targeted lung tissues of the irradiated mice with both exposure methods (Supplementary Fig. 2). The administration of celecoxib significantly suppressed the expression changes of EMT markers, as well as COX-2, in non-targeted lung tissues of the irradiated mice (P < 0.05), regardless of the exposure method or dose delivery method (Fig. 2, Supplementary Fig. 2). Though the induced upregulation of Vimentin was decreased significantly by the celecoxib administration when both dose delivery methods were used (P < 0.05), it didn’t recover to the control level. The other three EMT markers (E-cadherin, N-cadherin and Fibronectin) recovered to the control levels in irradiated mice with celecoxib administration (no significant difference from the control levels).

Similar results were obtained in the immunofluorescent staining assays of E-cadherin and Vimentin. E-cadherin was reduced while Vimentin was decreased significantly in the non-targeted lung tissues of the irradiated mice with celecoxib administration (Fig. 3).

DISCUSSION
Millions of people die from lung cancer every year, for which radiotherapy is one of the main treatments. It is estimated that 75% of patients with non–small cell lung cancer need to be treated with radiotherapy [17, 18]. In addition to the beneficial effects of radiotherapy, several side effects threatening normal tissues of patients...
after treatment need to be considered, especially the high incidences of second primary cancers [19]. It has been suggested that ~8% of secondary solid tumors could be attributed to radiotherapy, though the estimate varies by tumor type [20]. The RIBE/non-targeted effect, referring to a complicated response in non-irradiated cells caused by the release of clastogenic factors from irradiated cells, is thought to be associated with secondary cancers following radiotherapy.

Most of the RIBE reports derived from previous in vitro studies didn’t consider the microenvironment and immune response as playing essential roles in carcinogenesis. An in vivo RIBE has been found in various tissues and organs of animals [21]. However, it has not been determined whether EMT plays a role in RIBE-induced tumorigenesis in vivo, EMT has been shown to be a key process in the tumorigenesis and development of various kinds of cancer [22]. In this study, we found that expression changes of EMT markers were significantly induced in the non-targeted lung tissues of the mice subjected to either CIR or LAIR, suggesting EMT may be an early step in the lung tumorigenesis induced by the in vivo non-targeted effect. In addition, inhibition of COX-2 by celecoxib suppressed the RIBE-induced expression changes of EMT markers in lung tissues. COX-2 is a key enzyme catalyzing the metabolism of arachidonic acid into prostaglandins and finally contributes to cellular inflammation and carcinogenesis, also involved in RIBE, both in vitro and in vivo [23, 24]. Increased COX-2 expression and/or activation in distant non-targeted tissues will cause oxidative DNA damage and elevated cancer risk [25]. It has been found that COX-2 plays a key role in EMT of cancer cells. Hepatocyte growth factor promoted carcinogenesis and EMT in hepatocellular carcinoma via the COX-2 pathway [26]. Sulforaphane inhibited EMT via the COX-2/MMP2/9/ZEB1 pathway in human bladder cancer cells [27]. Xian et al. also reported that WIN could inhibit the EMT of gastric cancer cells through downregulation of COX-2 [28]. However, it hasn’t been figured out whether COX-2 plays a role in the EMT as well as carcinogenesis in non-targeted tissues of mice exposed to ionizing radiation, which is of significance for the understanding of the mechanisms underlying the radiotherapy-induced secondary cancer risk. In our study, the inhibition of COX-2 by celecoxib suppressed the RIBE-induced EMT in non-irradiated lung tissues, implying that RIBE-induced EMT in non-targeted lung tissues depends on COX-2, and inhibition of COX-2 is promising in reducing the secondary cancer risk induced by radiotherapy. Moreover, it was found that LAIR was more efficient in inducing EMT of non-targeted lung tissues compared with WBIR or CIR in mice exposed to fractionated irradiation, implying that the lower abdomen is crucial in the induction of non-targeted effects in vivo, and non-targeted effects depends on the type of irradiated organs. However, the underlying mechanisms are yet to be further elucidated.

In conclusion, the present work indicates that the expression changes of EMT markers are induced by non-targeted effects in abscopal lung tissues, and inhibition of COX-2 exerts a suppressive effect.
function, which are of great significance in the mechanistic studies of lung tumorigenesis induced by RIBE, as well as in the assessment of cancer risks related to accidental and clinical exposures to radiation.

SUPPLEMENTARY DATA
Supplementary data are available at Journal of Radiation Research online.

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CONFLICT OF INTEREST
The authors declare no potential conflicts of interest.

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