Extracellular vesicles released from irradiated neonatal mouse cheek tissue increased cell survival after radiation

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(Received 28 July 2020; revised 17 September 2020; editorial decision 1 October 2020)

ABSTRACT

Alopecia is one of the common symptoms after high-dose radiation exposure. In our experiments, neonatal mice that received 7 Gy X-ray exhibited defects in overall hair growth, except for their cheeks. This phenomenon might suggest that some substances were secreted and prevented hair follicle loss in the infant tissues around their cheeks after radiation damage. In this study, we focused on exosome-like vesicles (ELV) secreted from cheek skin tissues and back skin tissues, as control, and examined their radiation protective effects on mouse fibroblast cell lines. We observed that ELV from irradiated cheek skin showed protective effects from radiation. Our results suggest that ELV from radiation-exposed cheek skin tissue is one of the secreted factors that prevent hair follicle loss after high-dose radiation.

Keywords: radiation protection; extracellular vesicles; skin tissue; animal model

INTRODUCTION

Alopecia is one of the effects of acute radiation syndrome, caused by an exposure to a high-dose of ionizing radiation. It is also a side effect of radiotherapy of various cancers [1, 2]. The possible mechanisms of radiation damage in hair follicles were elucidated in previous studies of radiation-induced alopecia [3–6]. Recently, Huang et al. reported that hair follicles induce the accumulation of radio-sensitive ectopic progenitors from distinct compartments of transit-amplifying cells, allowing regeneration in places of dystrophy caused by ionizing radiation [7].

Exosome-like vesicles (ELV) have been functionally identified as extracellular mediators of cell-to-cell communication which contain mRNA, miRNA, DNA and proteins [8]. Interestingly, several studies reported that ELV from mesenchymal stem cells rescued hematopoietic cells from radiation damage [9–11]. In our current work, we observed that the colony-forming efficiency of un-irradiated mouse fibroblast cell lines [murine fibroblast (m5S) cells and mouse embryonic fibroblast (MEF) cells] was reduced after treatment of ELV from cheek or back skin from un-irradiated and 7 Gy-irradiated mouse. However, after radiation exposure to m5S and MEF cells, ELV from irradiated cheek skin showed radiation-protective effects. Hence, the γ-H2AX foci assay revealed faster repair of DNA double-strand breaks in m5S and MEF cells treated with ELV from irradiated cheek skin compared with m5S and MEF cells treated with ELV from un-irradiated cheek skin. These results suggest that ELV from irradiated cheek skin acted as a radiation-protective factor only when ELV-receiving mouse fibroblast cell lines were damaged by radiation.
MATERIALS AND METHODS

Animals
C3H/He male and female mice were purchased from Charles River Japan Inc, and were bred and maintained at the Institute for Animal Experiments in Hirosaki University. The mice were housed three to an autoclaved cage. Mice were fed with a standard diet (MB-1) and were given water ad libitum. The animal experiments were conducted according to Hirosaki University Guidelines for animal experimentation and the research protocol was approved in advance by the Ethics Committee (approval number: G17001).

Irradiation and tissue collection
Male mice of 1 week of age (n = 4) were irradiated to 7 Gy (1 Gy/min) with an X-ray generator (MBR-1520R-3; Hitachi Medical). Tissues were then collected from their cheeks and backs immediately after irradiation.
Fig. 2. Effect of ELV from mouse skin on the colony-forming ability of mouse fibroblast cell lines m5S and MEF. (A) Detection of CD9 and CD63 proteins in the ELV from un-irradiated and irradiated (7 Gy) cheek and back tissue by western blot. (B) Representative images of PKH67-labeled and unlabeled ELV derived from un-irradiated back skin. Relative colony-forming ability of m5S cells (C) and MEF cells (D) treated with ELV released from skin collected immediately after mouse exposure to 7 Gy. The five groups of treatment include: (I) un-treated, (II) treated with ELV from un-irradiated back skin, (III) treated with ELV from 7 Gy-irradiated back skin, (IV) treated with ELV from un-irradiated cheek skin, and (V) treated with ELV from 7 Gy-irradiated cheek skin. Relative colony-forming ability of m5S cells (E) and MEF cells (F) treated with ELV released from skin collected 6 h after mouse exposure to 7 Gy. Data are expressed as mean ± SD (n = 4). * Differences were significant based on Student’s t-test (P < 0.01).
irradiation or 6 h after irradiation. The tissues were washed with 5 x wash medium (DMEM containing 500 U/mL penicillin and 500 μg/mL streptomycin). Tissues from un-irradiated mice were also processed the same way.

Cell culture
mSS and MEF cells were cultured in 2 mL of D-MEM with 10% exosome-depleted fetal bovine serum (SBI), 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific) and cultured in a 6-well plate at 37°C in a humidified atmosphere of 5% CO₂.

ELV collection from mouse tissue culture and labeling.
Immediately after irradiation or 6 h after irradiation, mouse skin tissues were cultured for 6 h in D-MEM medium at 37°C in a humidified atmosphere of 5% CO₂. ELV were collected from cultured medium as described before [12]. Detection of ELV protein by western blotting with anti-CD9 (1:500; ab92726, Abcam) and anti-CD63 (1:1000; MAB5417, R&D systems) antibodies was as described previously [12]. To check for the uptake of ELV, a PKH-67 labeling kit (Sigma-Aldrich) was added to the medium of mSS cells (2 μg/mL final). After 6 h incubation, cells were fixed with 4% formaldehyde for 15 min, then mounted with Vectashield with DAPI (Vector Laboratories). Images were captured using a fluorescent microscope and CCD camera (Olympus).

Colonies formation assay
A schematic diagram of the experimental procedure for the colony formation assay is shown in Fig. 1A. mSS and MEF cells were irradiated with 0, 3 G and 6 Gy X-rays. Two hours after irradiation, mSS and MEF cells were divided into five groups; (I) un-treated, (II) treated with ELV from un-irradiated back skin, (III) treated with ELV from 7 Gy-irradiated back skin, (IV) treated with ELV from un-irradiated cheek skin, and (V) treated with ELV from 7 Gy-irradiated cheek skin. At 6 h after ELV treatment (final ELV concentration of 2 μg/mL in cell medium), mSS cells were seeded into 100 mm dishes and incubated for 1 week. The colonies were washed with PBS, fixed with 100% methanol (Wako) and stained with 1% Giemsa solution (Millipore). A colony was defined as a group of >50 cells and colonies were counted as survivors.

Immunofluorescence staining
mSS and MEF cells grown on coverslips were irradiated with 2 Gy X-rays. Two hours after irradiation, mSS and MEF cells were divided into three groups; (I) un-treated, (II) treated with ELV from un-irradiated back skin, and (III) treated with ELV from 7 Gy-irradiated cheek skin (final ELV concentration of 2 μg/mL in cell medium). Immunofluorescence staining was performed as described previously [13].

Statistical analysis
The statistical significance of the difference between groups was assessed using the statistical tests indicated in the figure legends. All the analyses were performed using StatMate III software, ver. 3.08 (ATMS). Statistical significance was set at P < 0.01 unless otherwise noted.

RESULTS
We first observed that hair growth in the neonatal mice was hindered after irradiation (Fig. 1B). However, normal hair growth was observed around the cheeks 2 weeks after irradiation (Fig. 1B). We then investigated the possibility of ELV playing a role in preventing hair follicle loss.
The overall experimental procedure is illustrated in Fig. 1A. First, we examined the immunoblot analysis of ELV markers (CD9 and CD63) (Fig. 2A), and observed labeled ELV inside the treated mSS cells after 6 h of treatment (Fig. 2B). ELV of all groups were labeled and their incorporation in mSS and MEF cells was confirmed 6 h after treatment (data not shown). After the treatment of ELV from tissues that were immediately cultured after irradiation, the relative colony-forming ability of un-irradiated mSS and MEF cells was significantly reduced in four groups: (II), (III), (IV) and (V) compared with (I) (Fig. 2C, D). However, no effect was seen on the colony-forming ability of 3 or 6 Gy-irradiated mSS and MEF cells in four groups: (II), (III), (IV) and (V) compared with (I) (Fig. 2C, D). Next, after the treatment of ELV from tissues cultured 6 h after radiation exposure, the relative colony-forming ability of un-irradiated mSS and MEF cells was also reduced in four groups: (II), (III), (IV) and (V) compared with (I) (Fig. 2E, F). However, a significant increase was observed in (V) compared with (I) in both 3 and 6 Gy-irradiated mSS and MEF cells (Fig. 2E, F). These results suggest that ELV released 6 h after irradiation from cheek skin supported the growth of damaged mSS and MEF cells. To investigate the effect of ELV to DNA repair dynamics, we used the Y-H2AX foci assay to analyze the repair kinetics of DNA double-strand breaks in mSS and MEF cells treated with ELV. We observed faster repair kinetics in mSS and MEF cells treated with ELV from irradiated cheek skin compared with mSS and MEF cells treated with ELV from un-irradiated cheek skin (Fig. 3A, B).

**DISCUSSION**

The mechanism of the radiation-protection effect of ELV still remains unclear. Mrowczynski et al. showed radiation-derived ELV from cancer cell lines contain elevated oncogenic miRNA, oncogenic mRNA and proteins of the ubiquitin–proteasome pathway and several important cell signaling pathways, and had decreased levels of tumor-suppressive miRNA and multiple tumor-suppressive mRNA, which can affect cancer cell survival after radiation [14]. Interestingly, Boelens et al. reported that stromal cells interacted with breast cancer cells via exosomes, modifying transcriptional responses to various cell signaling pathways and resulting in increased radio-resistance and tumor growth [15]. In addition, Ni et al. suggested that radiation can affect the composition and abundance of ELV, as well as their potential impact on the recipient cells [16]. Indeed, our results suggest that ELV from un-irradiated cheek skin showed no effect. However, ELV from irradiated cheek skin showed radiation-protective effects (Fig. 2E, F), potentially because of up-regulation of DNA damage repair activity (Fig. 3A, B). There is no direct evidence of link between radiation survival of MEF cell lines and radiation survival of cheek hair follicle cells. However, a recent study reported that ELV from dermal fibroblasts stimulate hair follicle proliferation and regulate hair growth [17], suggesting that different types of cells in organs communicate with each other via ELV. These lines of evidence support that the composition of ELV from cheek skin was affected by radiation exposure, and the state of ELV recipient cells which were irradiated or un-irradiated was also involved in expressing radio-protective effects (Fig. 4).
ACNOWLEDGEMENTS
We would like to thank all the lab members for technical and secretarial assistance.

CONFLICT OF INTEREST
None declared.

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