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Radiation increases the cellular uptake of exosomes through CD29/CD81 complex formation

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ABSTRACT

Exosomes mediate intercellular communication, and mesenchymal stem cells (MSC) or their secreted exosomes affect a number of pathophysiologic states. Clinical applications of MSC and exosomes are increasingly anticipated. Radiation therapy is the main therapeutic tool for a number of various conditions. The cellular uptake mechanisms of exosomes and the effects of radiation on exosome–cell interactions are crucial, but they are not well understood. Here we examined the basic mechanisms and effects of radiation on exosome uptake processes in MSC. Radiation increased the cellular uptake of exosomes. Radiation markedly enhanced the initial cellular attachment to exosomes and induced the colocalization of integrin CD29 and tetraspanin CD81 on the cell surface without affecting their expression levels. Exosomes dominantly bound to the CD29/CD81 complex. Knockdown of CD29 completely inhibited the radiation-induced uptake, and additional or single knockdown of CD81 inhibited basal uptake as well as the increase in radiation-induced uptake. We also examined possible exosome uptake processes affected by radiation. Radiation-induced changes did not involve dynamin2, reactive oxygen species, or their evoked p38 mitogen-activated protein kinase-dependent endocytic or pinocytic pathways. Radiation increased the cellular uptake of exosomes through CD29/CD81 complex formation. These findings provide essential basic insights for potential therapeutic applications of exosomes or MSC in combination with radiation.

1. Introduction

Exosomes, bilipid membrane vesicles (30–100 nm in diameter) that originate in multi-vesicular bodies and are released into the extracellular milieu upon fusion with the plasma membrane, are attracting increased attention [1]. Exosome secretion is a cellular mechanism for delivering cargo to mediate intercellular communication and to affect biologic function by the exchange of proteins and lipids, or the delivery of genetic materials to recipient cells [2]. Exosomes are also involved in various other cellular functions and pathophysiologic states, and thus could potentially provide a new approach for detecting noninvasive disease and predicting disease progression [3]. Moreover, exosomes have properties that can be exploited for therapeutic interventions as a new drug delivery system and a novel therapeutic tool in various conditions, including cancer, inflammation, ischemia, and regeneration [4].

Tumor cells and the cancer-associated microenvironment, comprising fibroblast-like cells, extracellular matrix, and inflammatory cells, secrete exosomes between them, allowing for crosstalk that leads to the promotion or inhibition of tumor progression, but the precise mechanism of communication is poorly understood [5,6]. Mesenchymal stem cells (MSC), clusters of multipotential fibroblast-like cells present in every organ as well as in the tumor stromal microenvironment, have regenerative and protective effects for injured tissues, and inhibit or promote tumor metastasis with their secreted exosomes, but the underlying mechanism is not clearly understood [6]. Potential applications of MSC and their secreted exosomes are currently attracting attention in a number of medical fields, such as oncology, immunology, and radiation therapy [7,8].

Radiation and drug therapy are currently the main therapeutic tools for a number of diseases. Radiation therapy not only acts on target cells, but also affects the stromal microenvironment. Thus, understanding how radiation affects cellular uptake and the secretion of exosomes between target cells and stromal cells is crucial.

Recent studies of exosome biogenesis revealed that exosomes originate from endosomal proteins involved in membrane transport and fusion in processes requiring heat shock proteins,

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integrins, and tetraspanins, and that the source of exosomes defines their function [7]. For therapeutic application of exosomes, especially those derived from MSC, the target cells must effectively internalize the exosomes. Several mechanisms of exosome uptake involving their surface molecules have been described and two distinct modes of internalization have been suggested [1]. In monocytes and macrophages, exosome internalization depends on the actin cytoskeleton and phosphatidylinositol 3-kinase regulated by dynamin2, and non-phagocytic cells require an energy-dependent pathway, including caveolae, macropinocytosis, and clathrin-coated vesicles [9,10]. The effects of radiation on exosome uptake processes, however, remain unknown. More detailed knowledge of the mechanisms of cellular uptake and the effects of radiation on these processes is needed to promote the effective use of exosomes and MSC as potential therapeutic tools. A better understanding of the processes involved will be instructive for modifying exosomes to be preferentially targeted in pathologic conditions by bioengineering. Here, we address several essential questions relating to the basic cellular uptake of exosomes and how radiation regulates that process, with a focus on target cell ligands. Our findings revealed that radiation leads to the colocalization of integrin (CD29) and tetraspanin (CD81) and increases the cellular uptake of exosomes.

2. Materials and methods

2.1. Reagents

Antibodies were obtained from Cell Signaling Technology (glyceraldehyde 3-phosphate dehydrogenase, phospho-p38 mitogen activated protein kinase [MAPK] at Thr180/Tyr182, phos-heat shock protein [Hsp] 27 [Ser82], and integrin (β1/CD29), EPITOMICS (integrin αV/CD51), abcam (CD9), Santa Cruz Biotechnology (dynamin2, CD63, CD81, CD151), BD Bioscience (CD29), or Invitrogen (Alexa Fluor 488, 568, 633). Small interference RNAs (siRNAs) were obtained from Santa Cruz Biotechnology (dynamin2, CD81, CD151, CD29) and Invitrogen (integrin αV/CD51). The p38 MAPK inhibitor (SB203580) was purchased from CST. N-Acetyl-L-cysteine (NAC) was obtained from Sigma–Aldrich.

2.2. Cells

Human bone marrow-derived MSC (immortalized cells) were purchased from the Health Science Research Resource Bank and cultured in modified Eagle's medium-α supplemented with 15% heat-inactivated fetal bovine serum (FBS). Rat small intestinal epithelial cells (IEC6) were maintained in Dulbecco's modified Eagle's medium supplemented with 5% FBS, and insulin. Human umbilical cord vein-derived normal endothelial cells (HUVEC) were cultured as previously described [17]. Exosomes were depleted of FBS by centrifugation at 100,000×*g* for 2 h to eliminate contaminating bovine exosomes.

2.3. γ-Ray irradiation

Cells were irradiated by a cesium-137 (Cs137) gammator (model M Gammator, Irradiation Machinery, Parsippany), at a dose rate of 8.0 Gy/min on a rotating platform.

2.4. Exosome isolation and labeling

Exosomes were isolated from the cell culture medium by ultracentrifugation. Briefly, collected culture medium was centrifuged at 1000×*g* for 20 min at 4 °C, and further filtered using a 0.22-μm filter (NALGENE) to remove cells and debris. Exosomes were then pelleted at 100,000×*g* for 2 h and washed with phosphate-buffered saline (PBS) at 100,000×*g* for 2 h using a swinging-bucket rotor SW28 (Beckman), all at 4 °C. Exosomes were resuspended in PBS and aliquoted, and kept at −80 °C. Purified exosomes were labeled with a PKH67 fluorescent labeling kit (Sigma–Aldrich) or a CellVue® claret far red fluorescent cell linker kit (Sigma–Aldrich). After labeling exosomes according to the manufacturer's recommendations, exosomes were washed twice with PBS at 100,000×*g* for 2 h using a swinging-bucket rotor SW28 (Beckman), all at 4 °C. Exosomes were suspended in PBS and filtered using a 0.22-μm filter (NALGENE) before adding to the medium.

2.5. Exosome uptake assay

Cells (2×10^5) were exposed to 8 Gy irradiation and fluorescence-labeled exosomes were added to culture medium. At 16 h or determined incubation time, cells were intensively washed twice with PBS. Harvested cells were washed and re-suspended in PBS. The fluorescence intensity of cells was detected using a fluorescence cell analyzer (FACSCalibur, Becton Dickinson).

2.6. Effect of exosomes on irradiated-cell viability

Cells (2×10^5) were exposed to 8 Gy of irradiation and then the purified exosomes (20 μL) were added to the culture media (2 mL) 10 min later. The culture was continued for 24 h. The cultured cells were harvested, and their viability was examined using the trypan blue exclusion method. All experiments were repeated three times.

2.7. siRNA transfection

Cells were transfected with each specific siRNA described above using TransMessenger Transfection Reagent (QIAGEN) according to manufacturer's recommendation. The cells were used for exosome uptake assay at 24 or 48 h after transfection. The siRNA efficiency was tested by Western blot or FACSCalibur.

2.8. Immunoblotting

Harvested cells were lysed directly in Laemmli sample buffer and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were blocked and probed with primary antibody and peroxidase-conjugated secondary antibodies followed by detection using an ECL Western blotting substrate (Millipore). Images were detected using Image Reader LAS-400 Fugifilm.

2.9. Cell surface expression analysis using flow cytometry

Cells were cultured for 6 h after 8 Gy irradiation. Harvested cells were washed once in PBS, re-suspended in 100 μL of diluted (1:100 in PBS with 1% vol/vol bovine serum albumin) primary antibody or fluorescein isothiocyanate-coupled antibody, and incubated for 30 min at 4 °C in the dark. The cells were washed once and incubated with anti-rabbit or mouse Alexa Fluor 488 secondary antibody (1:100 in PBS with 1% vol/vol bovine serum albumin) for 30 min at 4 °C in the dark. The cells were washed again and re-suspended in PBS. The fluorescence intensity of 10,000 cells for each sample was then analyzed using FACSCalibur.

2.10. CD29 and tetraspanin family colocalization assay

MSC were exposed to irradiation and incubated for 6 h. The cells were fixed in 4% formaldehyde PBS solution and stained for CD29 and tetraspanins (CD9, CD63, CD81, or CD151) and Alexa Fluor