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Role of the MAPK pathway in the observed bystander effect in lymphocytes co-cultured with macrophages irradiated with γ-rays or carbon ions

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ABSTRACT

Aims: The radiation-induced bystander effect (RIBE) has potential implications in cancer risks from space particle radiation; however, the mechanisms underlying RIBE are unclear. The role of the MAPK pathway in the RIBEs of different linear energy transfer (LET) was investigated.
Main methods: Human macrophage U937 cells were irradiated with γ-rays or carbon ions and then co-cultured with nonirradiated HMy2.CIR (HMy) lymphocytes for different periods. The activation of MAPK proteins and the generation of intracellular nitric oxide (NO) and reactive oxygen species (ROS) in the irradiated U937 cells were measured. Micronuclei (MN) formation in the HMy cells was applied to evaluate the bystander damage. Some U937 cells were pretreated with different MAPK inhibitors before irradiation.
Key findings: Additional MN formation was induced in the HMy cells after co-culturing with irradiated U937 cells, and the yield of this bystander MN formation was dependent on the co-culture period with γ-ray irradiation but remained high after 1 h of co-culture with carbon irradiation. Further investigations disclosed that the time response of the RIBEs had a relationship with LET, where ERK played a different role from JNK and p38 in regulating RIBEs by regulating the generation of the bystander signaling factors NO and ROS.
Significance: The finding that the RIBE of high-LET radiation could persist for a much longer period than that of γ-rays implies that particle radiation during space flight could have a high risk of long-term harmful effects. An appropriate intervention targeting the MAPK pathway may have significant implications in reducing this risk.

Introduction

During space exploration, the human body is at great risk for exposure to γ-rays, high energy protons, and high energy heavy ions that originate primarily from the Van Allen belts, galactic cosmic rays and solar particle events [1]. These types of radiation exposure could lead to significant health risks including carcinogenesis and degenerative diseases that could occur long-term after returning to earth following successful space exploration [2–4]. The quality of radiation, termed linear energy transfer (LET), has a great influence on the biological effects of radiation. High-LET radiation (normally > 10 keV/μm [5]) has a particular depth-dependent energy deposition "Bragg" peak [6] and can produce more complex and serious biological effects, e.g., more DNA double strand breaks, DNA fragments, and clustered DNA damage [7]; thus, space particle radiation is one of the greatest health concerns for astronauts [8].

Accumulating experimental evidence has shown that cells in the vicinity of directly targeted cells can respond to radiation; this response is termed the "radiation induced bystander effect" (RIBE) [9,10]. A series of biological endpoints could be induced by RIBE, including increased micronuclei (MN) formation, sister chromatid exchanges [9], and carcinogenesis [11] and reduced cell survival.

The MAPK pathway is an important pathway that is involved in radiation responses, that plays a role in eliciting specific biological responses through transmitting stress signals from the cell surface to the nucleus [12,13] and that is linked to the growth factor-mediated regulation of diverse cellular events such as proliferation, senescence, differentiation and apoptosis [14–16]. Three primary MAPK families have been widely characterized, i.e., the ERK1/2 pathway, C-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, and p38 kinase pathway. MAPK can be activated in response to radiation-induced DNA damage [13], and bystander MAPK signaling factors can be activated by tumor growth factor and ROS released from activated macrophages.

Radiation-induced blood injury is one of the most important health risks of space radiation. Lymphocytes and macrophages are two important components in the blood that not only interact with each other but also affect other organs in the human body via blood flow. In irradiated tissues, macrophages are key drivers of bystander signaling factors [17]

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and, therefore, play a profound role in RIBE. Additionally, lymphocytes are extremely sensitive cells that often interact with vicinal macrophages in the body [18]. In this work, the stress responses of the MAPK family in macrophage cells irradiated with low-LET γ-rays and with high-LET carbon (^{12}C) ions and the subsequent effects on their bystander lymphocytes were investigated.

Materials and methods

Cell lines

The macrophage cell line U937 was cultured in suspension in RPMI 1640 medium (HyClone, Beijing, China) supplemented with 10% fetal bovine serum (Gibco Invitrogen, Grand Island, NY, USA), 100 U/ml penicillin and 100 U/ml streptomycin. The human B lymphoblast cell line HMy2.CIR (HMy) was cultured in Iscove's modified Dulbecco's medium (HyClone) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin. All cell cultures were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C. Both cell lines were obtained from Shanghai Cell Bank of China. The human macrophage cell line U937 has been extensively used as an in vitro model to investigate the molecular mechanisms of radioprotection [19], and the HMy cell line has also been used to examine the influence of space radiation on human immune cells [20].

Cell irradiation

Exponentially growing suspensions of U937 cells in 35-mm dishes (2×10^6 cells) were exposed to γ-rays or ^{12}C ions at room temperature. The γ-rays were generated by a ^{137}Cs irradiator (Gammacell-40, Nordion International Inc., Ontario, Canada) with a LET of approximately 0.23 keV/μm at a dose rate of 0.75 Gy/min. The ^{12}C ions were generated by the accelerator at the Heavy Ion Research Facility (HIRFL) of the Institute of Modern Physics, Chinese Academy of Sciences. Carbon ions were accelerated to 300 MeV/u with a dose rate of 1 Gy/min. To better simulate space radiation, the LET was set at 30 keV/μm for cell irradiation, i.e., the cells were targeted in the plateau region of the Bragg curve but not at the Bragg peak [20,21]. When the cells were irradiated with 30 keV/μm carbon beams, the average particle number in the population was 18.91/μm^2/Gy according to the formula established by Tsuruoka et al. [22]. Because the relative biological effect (RBE) range of carbon ions is from 0.2–9.6, with a mean value of 2.2 ± 1.20 [23], and the RBE of 30 keV/μm carbon beams is approximately 2–4 [23–27], the doses of 3 Gy for γ-rays and 1 Gy for ^{12}C ions were selected for the experiments and were be expected to cause equivalent radiation responses. Additionally, our previous studies have shown that these doses are optimal for the induction of bystander responses [28,29].

Drug treatment and cell co-culture

U937 cells were treated with different inhibitors of the MAPK pathway for 1 h before irradiation. The ERK inhibitor U0126 (Cell Signaling Technology, Danvers, MA, USA), JNK inhibitor SP600125 (Sigma Co., St. Louis, MO, USA) and p38 inhibitor SB203580 (Cell Signaling Technology), with a widely used concentration of 10 μM, were applied to this cell treatment. After irradiation, the cells were washed three times with PBS and resuspended in fresh culture media for further analysis.

To investigate the influence of irradiated U937 cells on the HMy cells, these two cell lines were co-cultured in a set of Transwell insert dishes. After irradiation, 1×10^6 U937 suspension cells were reseeded in the insert dish (Nunc., Roskilde, Denmark) with a semipermeable polycarbonate membrane bottom with many pores (0.4 μm, 8×10^5 pores/cm^2) to allow medium and biomolecules to transfer through. Then, this inserted dish was placed in a companion TC plate well where 1×10^6 HMy cells in log-phase were already being cultivated. After being co-cultured from 30 min to 24 h, these cells were harvested for further measurements via MN assay.

MN assay

The cytokinesis-block MN assay technique was applied to detect chromosomal damage [30] in the bystander HMy cells. In brief, HMy cells were treated with 3.5 μg/ml cytochalasin B (CB, Sigma) for 30 h to provide sufficient time for dividing cells to form MN. Then, the cells were subjected to 0.075 M KCl hypotonic solution for 20 min, fixed in methanol with acetic acid (8.5:1.5), and dropped onto glass slides. After the slides were air-dried, the cells were stained with Giemsa solution (Sigma) and observed under a microscope (Olympus, Tokyo, Japan). MN were scored in at least 500 binucleated cells for each treatment, and the MN yield Y_{MN} was calculated as the ratio of the number of MN to the number of scored binucleated cells.

Western blot analysis

Western blot analysis was used to measure the expression of ERK, JNK, p38 and p-ERK, p-JNK and p-p38 total proteins in the U937 cells. Approximately 1.8×10^7 cells were transferred to a 100-mm dish immediately after irradiation. Then, at each indicated time point, 2×10^6 U937 cells were collected into a pre-cooled Eppendorf tube, washed three times with ice-cold PBS, and lysed with 200 μl of RIPA lysis buffer (Beyotime Biotechnology, Jiangsu, China) supplemented with the protease inhibitor PMSF (Sigma). Next, the cells were denatured at 100 °C for 10 min and centrifuged at 12,000 × *g* for 5 min at 4 °C. The clarified cell lysates were used for Western blot analysis. Protein samples with 1× loading buffer (62.5 mM Tris–HCl (pH 6.8), 2.2% w/v SDS, 10% v/v glycerol, 50 mM DTT and 0.01% w/v bromophenol blue in deionized water) were loaded onto preparative gels, subjected to 10% SDS-PAGE, and blotted onto nitrocellulose membranes (Millipore, Bedford, MA, USA). After the membranes were blocked in 5% non-fat dry milk powder in TBST (TBS containing 0.05% Tween 20) for 1 h to reduce non-specific protein binding, they were incubated overnight at 4 °C with the following antibodies: anti-p-ERK (CST., 1:1000 dilution in non-fat milk), anti-p-JNK (CST, 1:500 dilution in non-fat milk), anti-p-p38 (CST, 1:1000 dilution in non-fat milk), anti-ERK, anti-JNK, anti-p38 (Bioworld, Nanjing, China, 1:500 dilution in blocking buffer), and anti-β-Actin (Beyotime, 1:2000 dilution in blocking buffer). After washing four times with TBST, the membranes were incubated with secondary antibody (Beyotime) for 1.5 h at room temperature. Then, the membranes were incubated with ECL Plus (Millipore), and the protein bands were visualized using a ChemiDox XRS system (Bio-Rad Laboratories, Hercules, CA, USA). The results were analyzed using Quantity One software (Bio-Rad Laboratories).

Measurements of intracellular NO and ROS

The intracellular NO and ROS levels in the U937 cells after different drug treatments with or without irradiation were detected using the fluorescence probes DAF-FM-DA and DCFH-DA (Beyotime), respectively. Briefly, at the indicated period after irradiation (2 h and 24 h), 1×10^6 U937 cells were treated with 5 μM DAF-FM-DA or 3 μM DCFH-DA for 20 min at 37 °C, rinsed three times with PBS and resuspended in 600 μl PBS. Then, the levels of intracellular NO and ROS were measured using flow cytometry (Gallios, Beckman Coulter, USA). The mean fluorescence intensity of each sample was normalized to that of the control and analyzed using FCS Express V3 software (De Novo Software Co., Canada).

Statistical analysis

The data, which are presented as the mean ± SE, were obtained from at least three independent experiments with three to five replicates each and analyzed using SPSS software (SPSS Inc., Chicago, IL, USA). A