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Nitric oxide-mediated bystander signal transduction induced by heavy-ion microbeam irradiation

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ABSTRACT

In general, a radiation-induced bystander response is known to be a cellular response induced in non-irradiated cells after receiving bystander signaling factors released from directly irradiated cells within a cell population. Bystander responses induced by high-linear energy transfer (LET) heavy ions at low fluence are an important health problem for astronauts in space. Bystander responses are mediated via physical cell–cell contact, such as gap-junction intercellular communication (GJIC) and/or diffusive factors released into the medium in cell culture conditions. Nitric oxide (NO) is a well-known major initiator/mediator of intercellular signaling within culture medium during bystander responses. In this study, we investigated the NO-mediated bystander signal transduction induced by high-LET argon (Ar)-ion microbeam irradiation of normal human fibroblasts. Foci formation by DNA double-strand break repair proteins was induced in non-irradiated cells, which were co-cultured with those irradiated by high-LET Ar-ion microbeams in the same culture plate. Foci formation was suppressed significantly by pretreatment with an NO scavenger. Furthermore, NO-mediated reproductive cell death was also induced in bystander cells. Phosphorylation of NF-κB and Akt were induced during NO-mediated bystander signaling in the irradiated and bystander cells. However, the activation of these proteins depended on the incubation time after irradiation. The accumulation of cyclooxygenase-2 (COX-2), a downstream target of NO and NF-κB, was observed in the bystander cells 6 h after irradiation but not in the directly irradiated cells. Our findings suggest that Akt- and NF-κB-dependent signaling pathways involving COX-2 play important roles in NO-mediated high-LET heavy-ion-induced bystander responses. In addition, COX-2 may be used as a molecular marker of high-LET heavy-ion-induced bystander cells to distinguish them from directly irradiated cells, although this may depend on the time after irradiation.

1. Introduction

Astronauts in space are affected constantly by radiation and microgravity (Yatagai and Ishioka, 2014). Space radiation includes protons, heavy ions with high charge and energy, and secondary radiation, including neutrons and the recoil nuclei generated from reactions with spacecraft walls or within tissues (Cucinotta and Durante, 2006; Cucinotta et al., 2008). High-linear energy transfer (LET) heavy ions are major contributors to the total dose equivalent (Held, 2009). Previously, high-LET heavy ions have been demonstrated to have higher relative biological effectiveness (RBE) than low-LET photons (γ-rays and X-rays) for a variety of biological endpoints (Held, 2009; Asaithamby and Chen, 2011). If these high-LET heavy ions hit cell nuclei, they induce multiple local DNA lesions. Complex clustered DNA damage exhibits strong LET dependence (Goodhead, 1999), and thus it is a good candidate as a prime determinant of the LET-RBE relationship. The complex clustered DNA damage induced by high-LET heavy ions is more difficult to repair by cells than simple individual damage (Asaithamby and Chen, 2011). Recently, it was suggested that the determining factor for a high RBE value in the presence of high-LET heavy ions

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may be the inefficacy or inefficiency of DNA double-strand break (DSB) repair via non-homologous end-joining (NHEJ) (Takahashi et al., 2014). In addition, it is considered that mitochondria are a potential cytoplasmic target of high-LET α particles that mediate cellular damage (Zhang et al., 2014).

In the space environment, it is also important to evaluate the biological effects induced by low fluence in low fluence-rate irradiation conditions to accurately estimate the human health risk (Held, 2009; Yatagai and Ishioka, 2014). The heterogeneity of the absorbed dose within the irradiated tissues or cells is more relevant for high-LET heavy ions than for low-LET photons because the absorbed dose per single hit (one nucleus traversal) in the former case is much greater than that in the latter. Therefore, directly irradiated and non-irradiated cells co-exist within tissues exposed to the low fluence of high-LET heavy ions. Non-targeted effects include the direct consequences of radiation-induced initial lesions produced in cellular DNA, as well as intra- and inter-cellular communication involving both targeted and non-targeted cells (Matsumoto et al., 2011; Tomita and Maeda, 2015), which mainly comprise radiation-induced adaptive responses, low-dose hypersensitivity, genomic instability, and radiation-induced bystander response (RIBR). In general, RIBR is defined as a cellular response induced in a non-irradiated cell that receives bystander signals from directly irradiated cells within an irradiated cell population (Matsumoto et al., 2011; Tomita and Maeda, 2015). RIBRs are mediated mainly by physical cell–cell contact, such as gap-junction intercellular communication (GJIC) and/or diffusive factors released into the medium during cell culture. The connexin 43 (Cx43)-mediated GJIC is involved in α-particle-induced bystander signaling in confluent cell cultures (Azzam et al., 2001) and the induction of Cx43 has been observed after mean α-particle doses as low as 1.6 mGy (Azzam et al., 2003). The mean propagation distance of the bystander signal ranges from 20 to 40 μm around the intranuclear α-particle impact point (Gaillard et al., 2009). In the bystander response elicited via cell culture medium, nitric oxide (NO) is a well-known major initiator/mediator of intercellular signaling molecules (Matsumoto et al., 2011; Tomita and Maeda, 2015). Therefore, the modes of action of reactive nitrogen species (RNS) in bystander signaling could help to elucidate the mechanism of RIBRs (Matsumoto et al., 2011).

The heavy-ion microbeam system at the Takasaki Ion Accelerators for Advanced Radiation Application (TIARA) in the Japan Atomic Energy Agency (JAEA, Gunma, Japan) is a pioneering collimated heavy-ion microbeam system, which can provide targeted irradiation of several types to biological materials, where heavy-charged particles are accelerated using an azimuthally varying field (AVF) cyclotron with a minimum beam diameter of 5 μm (Funayama et al., 2008). An additional excellent feature of the TIARA facility is that the same particles with similar energy in the broadbeams and microbeams can be used to expose biological materials, thereby contributing significantly to the understanding of heavy-ion-induced bystander responses (Shao et al., 2003; Funayama et al., 2005; Kanasugi et al., 2007; Hamada et al., 2008; Iwakawa et al., 2008; Harada et al., 2009; Fournier et al., 2009; Hino et al., 2010; Mutou-Yoshihara et al., 2012; Autsavapromporn et al., 2013 and so on). The significant role of NO in the bystander response was determined in studies using heavy-ion microbeams (Kanasugi et al., 2007; Mutou-Yoshihara et al., 2012). The contribution of NO to the high-LET heavy-ion-induced bystander response and radioadaptive response was also identified using other broadbeam heavy-ion irradiation facilities (Matsumoto et al., 2000; Shao et al., 2001, 2002, 2004; Yang et al., 2007).

In our previous studies using X-ray microbeams, bystander cell killing was mainly initiated/mediated by NO in normal human lung fibroblast WI-38 cells, cultured confluent, exponentially growing human non-small-cell lung cancer H1299 cells expressing wild-type or mutated *p53*, and Chinese hamster V79 cells (Tomita et al., 2010, 2012, 2013; Maeda et al., 2010, 2013). However, the biological effects and signal transduction events induced by NO-mediated high-LET heavy-ion-induced bystander responses are still unclear. In this study, we determined the contribution of NO to foci formation by DSB repair-related proteins and the induction of reproductive cell death in bystander cells of normal human fibroblasts using a 13 MeV/u Ar^{14+}-ion microbeam. We also demonstrated the activation of NF-κB, Akt, and cyclooxygenase-2 (COX-2, also known as prostaglandin endoperoxide synthase-2) by bystander signaling. The activation of these proteins depended on the incubation time after irradiation and the presence of NO.

2. Materials and methods

2.1. Cell culture

Normal human lung fibroblast WI-38 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in D-MEM/F-12 medium (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin, and they were maintained at 37 °C in a humidified incubator with a 95% air/5% CO_2 atmosphere.

2.2. Heavy-ion broadbeam irradiation

WI-38 cells were cultured on a 25-mm cover glass in a six-well culture plate for 1 week until they formed confluent monolayers. The cover glass was then placed in a 60-mm dish and 2–3 ml fresh medium was added at least 2 h before irradiation. Immediately before irradiation, the cell culture medium was removed and the dish was covered with 8-μm thickness polyimide film (Du Pont-Toray, Tokyo, Japan) to maintain hydration during irradiation (approximately 10 min). The cells were irradiated with 5 Gy of 13 MeV/u Ar^{14+} ions delivered from the AVF cyclotron at TIARA, JAEA (Gunma, Japan), at room temperature. The LET value at the cell surface was 1370 keV/μm, which was calculated according to the kinetic energy loss, assuming water equivalence. The absorbed dose (Gy) was calculated as the fluence (number of ion particles/cm^2) × LET (keV/μm) × (1.6×10^{-9}).

2.3. Heavy-ion microbeam irradiation

To study the formation of 53BP1 and γ-H2AX foci, the cell suspension concentration was adjusted to 1×10^6 cells/ml. The cell suspension (5 μl) was spotted onto the 25-mm cover glass in the 60-mm dish where five spots (one at the center and four satellites) were placed on the cover glass, as shown in Figs. 1A and 1B. The distance between the midpoint of the central and satellite colonies was 5 mm. After incubation for 1 h, 3 ml of fresh medium was added to the dish and the cells were incubated for 1 day to form colonies. Immediately before irradiation, the medium was removed and the cover glass was covered with 8-μm thickness polyimide film. A single cell nucleus at the midpoint of the central colony was irradiated with an average of five particles of collimated Ar^{14+}-ion microbeam (13 MeV/u) delivered from the AVF cyclotron at the TIARA. The set-up and irradiation procedures were described previously (Funayama et al., 2008). The LET value on the cell surface was 1130 keV/μm. Irradiation was performed at room temperature. When the WI-38 cell nucleus was irradiated with five Ar ions, the estimated absorbed dose was 5 Gy.

In the cell survival and western blot analyses, WI-38 cells were cultured on a 25-mm cover glass for 1 week, as described above. On average, five particles of the Ar-ion microbeam were irradiated on five points every 400 μm along a straight line in the center of the cover glass, as shown in Fig. 2A.