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Alteration in the expression of signaling parameters following carbon ion irradiation

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Abstract

Ionizing radiation induces DNA damage, which generates a complex array of genotoxic responses. These responses depend on the type of DNA damage, which in turn can lead to unique cellular responses. High LET radiation results in clustered damages. This evokes specific signaling responses, which can be cytotoxic or cytoprotective in nature. In the present study the effect of carbon ion irradiation on p44/42 MAPK and NF-κB, which are essentially survival factors, have been studied. Moreover, the effect of inhibition of DNA-PK, which is an important component of DNA repair mechanism, with wortmanin on these signaling factors has been studied. The expression of p44/42 MAPK was different at 0.1 Gy and 1 Gy and wortmanin was found to inhibit its expression. NF-κB expression was higher at 1 Gy than at 0.1 Gy and its expression is unaffected by inhibition of DNA-PK. The notable findings of this study are that the responses to high and low dose of high LET radiation are essentially different and the 6 h time point post irradiation is crucial in deciding the response and needs further investigation. (Mol Cell Biochem **276**: 169–173, 2005)

Key words: high LET radiation, signaling, p44/42 MAPK, NF-κB, wortmanin

Introduction

Environmental exposure to α-particles produced by radon and its decay products are responsible for approximately 50% of the average annual effective dose from natural sources of ionizing radiation worldwide [1]. Moreover, it has been estimated that as much as 15% of all lung cancer may be caused by exposure to residential radon [2]. In some cases, artificial radionuclides such as Plutonium 239 or Americium 241 associated with nuclear industry make some small additional contribution. Ionizing radiation induces DNA damage, which generates a complex cascade of events leading to cell cycle arrest, transcriptional and post-transcriptional activation of a subset of genes including those associated with DNA repair and triggering apoptosis. Probably the most dangerous of all the types of DNA damage are double strand breaks (DSBs) and their repair is complex. The amount of induction of DSBs is weakly dependent on linear energy transfer (LET) of the radiation [3]. However, the degree of lesion complexity increases with increasing LET [4]. The cellular genotoxic response depends on the type of DNA damage [5], which in turn can evoke unique cellular response. The genotoxic effects of heavy ion irradiation are not well understood. There is consensus, however, that it causes clustered DNA damage, which are manifested as abasic sites, strand breaks or oxidized bases. This initiates a cellular genotoxic response and signal

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transduction pathways that differ with the quality of radiation, e.g., UV radiation induces primarily point mutations while ionizing radiation induces deletion mutations through error-prone rejoining of broken DNA ends. The signal transduction pathways that ensue could be different for each kind of radiation. There are several reports on the mechanism of cell cycle arrest following high LET irradiation [5, 6]. DNA Dependent Protein Kinase (DNA-PK) and ataxia-telangiectasia (ATM) gene product recognize Dsbs, but the actual repair is initiated by BRCA2 because cells that lack BRCA2 show decreased ability to survive [7]. The expression levels of BRCA and XRCC are also dependent on MAPK [8]. A few early reports indicated that murine skid cells deficient in DNA-PK catalytic subunit were highly sensitive to γ-rays but not to α-particles [9, 10] and highly clustered damages were not substrates for the ATM pathway either [10]. Thus if DNA-PK pathway does not participate in the repair of DNA damaged by heavy ion, it is likely that the MAPK pathway might participate in the repair process because MAPK can also activate BRCA and XRCC. Cell lines deficient in DNA-PK (MO59J) displayed elevated levels of apoptosis following low dose of high LET radiation when compared to its counterpart that has DNA-PK (MO59K) [11]. However, in the same study with high dose of high LET radiation there was no difference between the two cell lines. Thus clearly, there may be two different pathways of signaling one for low dose (high and low LET) radiation and the other for low LET radiation. Not only does the signaling course taken depends on the quality of radiation, it also depends on the dose delivered. Very high doses of high LET radiation may lead to larger percentage of necrotic cells and the signaling parameters activated may not represent a true picture. The dose of high LET radiation employed should be of lesser magnitude if the signaling pathways have to be clearly defined.

In the present work, the response of the MAPKs, which play crucial role in cell-cycle arrest, has been investigated. In general, stress stimuli strongly activate p38 MAPK and SAPK while only weakly activating p44/42 MAPK [12]. Contrarily, ionizing radiation strongly activates the p44/42 MAPK pathway [12]. This pathway is essential for G2/M arrest in cells exposed to ionizing radiation [13]. Since DNA_PK is not involved in cell cycle arrest following high LET radiation [12] and MAPK plays a role in DNA repair and is known to activate XRCC [8]. We have simulated conditions where DNA-PK is completely inhibited and then looked at the expression of p44/42 MAPK and NF-κB. Another prevalent idea about high LET radiation is that the formation of Reactive Oxygen Species (ROS) is minimal due to recombination of the radicals [14]. If so, how is this reflected in the expression of NF-κB, which is a known redox-regulating factor and is also activated by DNA-PK, has been investigated.

Materials and methods

Cell culture

Chinese hamster V79 cells were cultured in 75 cm^2 culture flasks in alpha MEM (Hyclone, USA.) supplemented with 10% fetal calf serum (Sigma, USA.) and kept at 37 °C in humidified atmosphere with 5% CO_2.

Modulators

Wortmanin (Sigma Chemicals, USA) was used at a concentration of 10 μM and was added to the medium 1 h prior to irradiation.

Irradiation

Heavy ion irradiation ($^{12}C^{5+}$) was carried out using Radiation Biology beam line of 16 MV 15 UD Pelletron at Nuclear Science Centre, New Delhi. The primary heavy ion beam from the Pelletron was diffused using a gold foil and low flux beam was obtained at the exit window made of 6.25 mg/cm^2 thick aluminum foil. A silicon surface barrier (SSB) detector was positioned at the same position where samples were irradiated and the beam energy was measured. The positive current signal from the diffuser foil was integrated using a current integrator. The ratio of the current signal to that of the flux measured using SSB detector at the sample position was measured by multiple trials and the calibration factor was obtained. The current signal was also fed to a preset controller to terminate the beam after irradiation using a Faraday cup. Specially fabricated stainless steel rings (2.5 cm diameter) were used as petri plates for sample irradiation. A 6 μm thick polypropylene film was attached to the ring to form the base on which 0.5×10^6 cells were seeded 24 h before irradiation. Immediately before irradiation, the medium was drained leaving a thin film of medium on the monolayer of cells attached to the polypropylene. The cells were irradiated under sterile conditions, at atmospheric pressure and were exposed to the ions through the polypropylene film. Fresh medium was added following irradiation and cells were kept back in the CO_2 incubator for different periods of time (up to 8 h) before they were lysed.

Western blotting

Cells (2×10^6) were lysed in 150 μl of 1 × SDS gel-loading buffer (50 mMTris.Cl, pH 6.8; 100 mM dithiothreitol; 2% SDS; 0.1% bromophenol blue and 20% glycerol). The lysate

(10 μl) was run on 8% SDS Polyacrylamide Gel followed by transfer to Nitrocellulose membrane (Amersham, USA). Antibodies against NFκB (Transduction Laboratories, USA) and ERK (Cell Signaling Technology, USA) were used at dilution of 1:1000 whereas anti-β-Actin (Sigma Chemicals, USA) was used at a dilution of 1:5,000. The membranes were then probed with horseradish peroxidase conjugated secondary antibody against mouse/rabbit (Roche Molecular Biochemicals, Germany) at a dilution of 1:3000 and developed using Roche Molecular Biochemicals Chemiluminiscence Western Blotting Kit (Roche Molecular Biochemicals, Germany). Densitometry was done using Shimadzu CS 9000 Dual wavelength flying spot scanner. Statistical analysis was done using ANOVA.

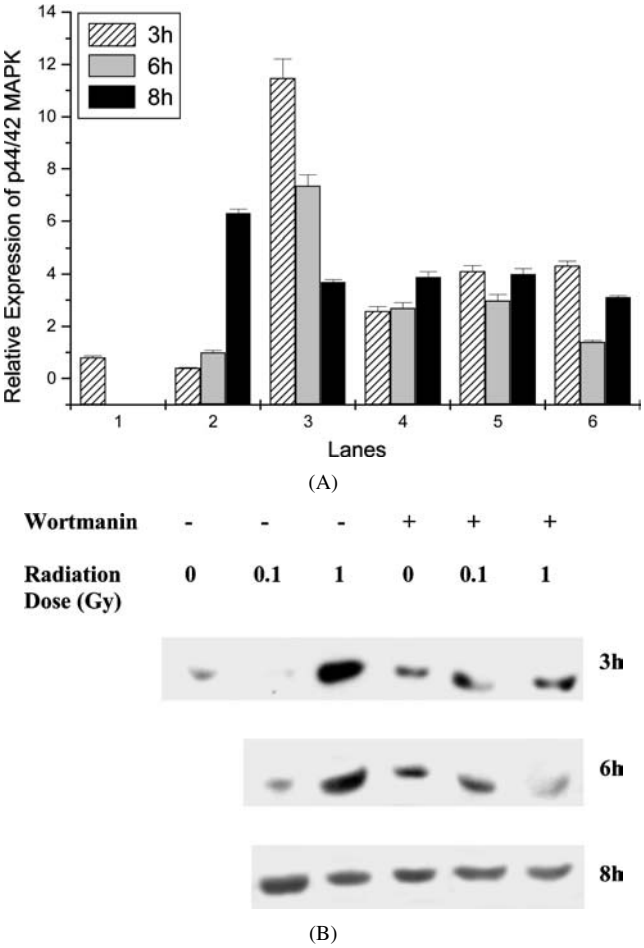

Fig. 1. (A) Expression of p44/42 MAPK at 3, 6 and 8 h following heavy ion irradiation: $^{12}C^{+5}$ ion irradiation of V79 cells was carried out and 2×10^6 cells were loaded per well. SDS-PAGE and immuno-blotting was carried out followed by detection by chemiluminescence and densitometry. *Lane 1*: Unirradiated control; *Lane 2:* 0.1 Gy; *Lane 3:* 1 Gy; *Lane 4:* Treated with wortmanin; *Lane 5:* Wortmanin + 0.1 Gy; *Lane 6:* Wortmanin +1 Gy. (B) Corresponding western blots for p44/42 MAPK at 3, 6 and 8 h post-irradiation.

Results

The temporal response of p44/42 MAPK and NF-κB at 3, 6 and 8 h post-irradiation was studied at 0.1 and 1 Gy of $^{12}C^{+5}$ ion irradiation. The effect of wortmanin (DNA-PK, PI3 kinase inhibitor) on these signaling factors was studied. For a dose of 0.1 Gy the p44/42 MAPK expression was around control levels at 3 and 6 h but increased many-fold at 8 h (Fig. 1A and B). The trend was very different for 1 Gy where there is a strong increase at 3 h, which gradually decreases by 8 h. When cells were treated with wortmanin alone, an increase in p44/42 MAPK was observed. When wortmanin was combined with radiation, a significant decrease in p44/42 levels was observed as compared to radiation alone, for both 0.1 Gy and 1 Gy.

NF-κB expression peaked strongly at 8 h following a radiation dose of 0.1 Gy (Fig. 2A and B). Although an overall

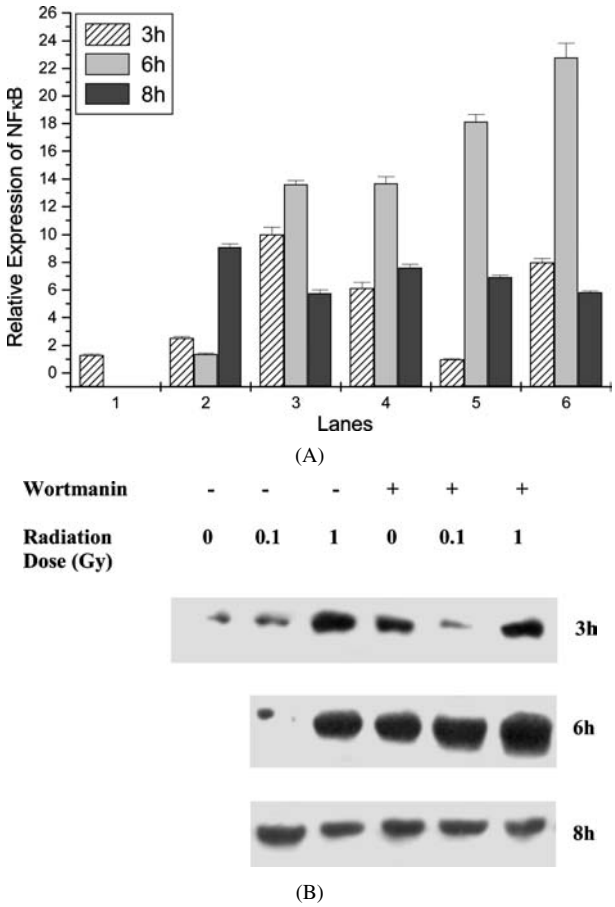

Fig. 2. (A) Expression of NF-κB at 3, 6 and 8 h following heavy ion irradiation: $^{12}C^{+5}$ ion irradiation of V79 cells was carried out and 2×10^6 cells were loaded per well. SDS-PAGE and immuno-blotting was carried out followed by detection by chemiluminescence and densitometry. *Lane 1:* Unirradiated control; *Lane 2:* 0.1 Gy; *Lane 3:* 1 Gy; *Lane 4:* Treated with wortmanin; *Lane 5:* Wortmanin +0.1 Gy; *Lane 6:* Wortmanin +1 Gy. (B) Corresponding western blots for p44/42 MAPK at 3, 6 and 8 h post-irradiation.

increase in NF-κB was observed at all time points following 1 Gy, maximum expression was at 6 h. Wortmanin treatment alone had a very similar effect with a peak at 6 h. A combination of wortmanin with radiation resulted in a strong increase in NF-κB at 6 h for both 0.1 and 1 Gy.

Discussion and conclusions

The expression of MAPK at 0.1 Gy and 1 Gy was found to be different. With the lower dose there was a gradual increase in expression with time. The expression reaches a maximum at 8 h of irradiation. However with higher dose, there was a drastic increase at 3 h followed by a decline at 6 and 8 h. The response of MAP kinase is different at low (0.1 Gy) and high (1 Gy) doses probably because at very low doses, damage is less, hence all the alarm signals (DNA-PK, PARP etc.) are not activated so activation of MAP kinase takes time to reach the peak. At 1 Gy all the alarm signals are activated due to extensive DNA damage leading to many signals that converge on the MAP kinase cascade so a robust activation is observed. The alarm signals, however, are soon turned off and MAP kinase activity declines. MAP kinases are known to respond differentially to low and high dose of low LET radiation [15, 16]. Moreover, transient activation of MAP kinase leads to increased proliferation while prolonged activation results in decreased DNA synthesis potentially through super-induction of CDKN1A [17]. It may be noted that MAPK is essentially a survival signal and is required to overcome radiation induced G_2M block [18]. At the lower doses it may be a part of the signaling process and a clear dose response is seen over the time periods observed.

Wortmanin was found to inhibit the expression of MAPK (ERK1/2) completely. Even with doses as high as one gray the expression was very meager as compared to the one irradiated without wortmanin. When DNA-PK is completely inhibited, cell cycle arrest could be affected via MAPK as ERK1/2 is known to participate in cell cycle arrest. Growth factor induced MAPK signaling has been proposed to regulate both proliferation and differentiation and recently, cell cycle arrest Transient activation of MAPK is known to increase proliferation potentially via coordinated increase in the expression of cyclin molecules and cyclin dependent kinase inhibitor p21 [19]. Prolonged activation leads to inhibition of DNA synthesis potentially via super-induction of p21 and inhibition of cyclin dependent kinases [19]. In the present study, however, the lack of activation of ERK1/2 by wortmanin (which inhibits DNA-PK) after heavy ion irradiation would argue against the involvement of ERK1/2 in cell cycle arrest following heavy ion irradiation.

The expression of NF-κB was higher at 1 Gy than at 0.1 Gy. NF-κB has been reported to be activated in various cells following irradiation [20, 21] and the activation has been implicated in mediating the radiation-induced expression of various cytokines. NF-κB was also found to be inhibited by wortmanin at 3 and 8 h post irradiation as compared to radiation alone. This may be due to the fact that inhibition of DNA-PK prevents phosphorylation of IκB by DNA-PK. DNA-PK has recently been implicated in the activation of NFκB, although activation of NF-κB may be by other pathways as well [22]. The cells irradiated with 1 Gy also show a significant activation of NFκB at 3 h, peaking at 6 h. Activation of NF-κB 3 h after irradiation was lesser at 0.1 Gy as compared to 1 Gy. This was contrary to our expectations since ROS production following high is supposedly lesser at higher doses. The reason being that dense ionizations produced in the heavy ion tracks lead to a high rate of radical-radical recombination. This effectively lowers the sparsely ionizing component (free radicals including ROS) of the high LET radiation [14, 23]. But the fluctuating pattern of NF-κB does suggest that ROS production and signaling at the 2 doses and at various times are different. In the present study the lesser activation of NF-κB at 0.1 Gy may be due to the fact that at this dose the number of cells traversed by the radiation tracts is very few as against the higher doses. Therefore, the cumulative damage is significantly less. This in turn will result in lesser activation of upstream signaling factors like PKC which lead to NF-κB activation.

The two notable facts in this study are that the response to high dose and low doses may be entirely different where heavy ion irradiation is concerned and secondly, that the 6 h time point after heavy ion irradiation may be a crucial point where the cell decides whether it has undergo apoptosis or not. NF-κB acts as a survival factor [24] and its activation at 6 h and activation in spite of wortmanin are suggestive of the fact that many other pathways other than DNA-PK lead to its activation following heavy ion irradiation. The differential dose response and the significance of the 6 h time point need to be further investigated.

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