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Protein kinase C epsilon is involved in ionizing radiation induced bystander response in human cells

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ABSTRACT

Our earlier study demonstrated the induction of PKC isoforms (βII, PKC-α/β, PKC-θ) by ionizing radiation induced bystander response in human cells. In this study, we extended our investigation to yet another important member of PKC family, PKC epsilon (PKCε). PKCε functions both as an anti-apoptotic and pro-apoptotic protein and it is the only PKC isozyme implicated in oncogenesis. Given the importance of PKCε in oncogenesis, we wished to determine whether or not PKCε is involved in bystander response. Gene expression array analysis demonstrated a 2–3-fold increase in PKCε expression in the bystander human primary fibroblast cells that were co-cultured in double-sided Mylar dishes for 3 h with human primary fibroblast cells irradiated with 5 Gy of α-particles. The elevated PKCε expression in bystander cells was verified by quantitative real time PCR. Suppression of PKCε expression by small molecule inhibitor Bisindolylmaleimide IX (Ro 31-8220) considerably reduced the frequency of micronuclei (MN) induced both by 5 Gy of γ-rays (low LET) and α-particles (high LET) in bystander cells. Similar cytoprotective effects were observed in bystander cells after siRNA mediated silencing of PKCε suggestive of its critical role in mediating some of the bystander effects (BE). Our novel study suggests the possibility that PKC signaling pathway may be a critical molecular target for suppression of ionizing radiation induced biological effects in bystander cells.

1. Introduction

Experimental data obtained thus far have demonstrated the existence of ionizing radiation induced "bystander effects" (BE) in diverse eukaryotic cell systems. Bystander cells exhibit DNA damage recognition/repair responses due to the transmission of signal elicited from directly irradiated cells. The transfer of signal from the directly targeted cells to the bystander cells may occur either through gap junction intercellular communications (GJIC) or secretion of soluble factors from the targeted cells into the medium (Mothersill and Seymour, 2001; Ballarini et al., 2002; Little, 2003; Hall and Hei, 2003; Chaudhry, 2006; Hei et al., 2008; Iwakawa et al., 2008). Bystander response is assessed by different biological endpoints such as chromosomal aberrations, sister chromatid exchanges, mutations, apoptosis, changes in the expression of genes and proteins (Mothersill and Seymour, 2001; Nagasawa and Little, 1999; Zhou et al., 2000; Azzam et al., 2001, 2004; Hamada et al., 2007). Although many of these end points are due to DNA damage induction, the precise molecular nature of the signal for BE remains enigmatic. Understanding the signaling mechanism(s) for BE is critical not only for radiation risk assessment but also for the development of radiotherapy protocols for tumors such that the damage to the normal healthy bystander cells can be prevented or minimized.

BE can be induced both by low- and high-linear energy transfer (LET) radiations. Using microbeam irradiation, micronuclei (MN) induction (Belyakov et al., 2001; Ponnaiya et al., 2004), mutations (Zhou et al., 2000) and oncogenic transformation (Sawant et al., 2001) have been observed in bystander cells. Additionally, treatment of cells with irradiated conditioned medium (ICM) collected from directly targeted cells with low LET radiation also resulted in BE. Transfer of ICM from γ-irradiated epithelial cells to non-targeted cells reduced their clonogenic survival by increasing their apoptotic potential (Mothersill and Seymour, 1997). Delayed radiation induced apoptosis and neoplastic transformation have been documented in bystander human HeLa cells and skin fibroblast hybrid CGL1 cells (Lewis et al., 2001). Available evidence supports both modes of signal transfer from the directly irradiated cells to bystander cells: (I) GJIC and (II) secretion of soluble factors from the irradiated cells into the medium (Azzam et al., 2002; Hei et al., 2008 and references therein). Inhibitors of GJIC have been shown

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to reduce the mutations in bystander cells (Zhou et al., 2000). In addition to genetic effects, epigenetic effects involving histone and DNA methylation changes have been observed recently in bystander cells (see Kovalchuk and Baulch, 2008 and references therein).

Ionizing radiation induced cellular response is complex involving the activation of multiple signal transduction pathways. The delicate balance between cell survival and cell death after ionizing radiation depends on the efficiency with which these multiple signaling cascades are activated. Studies on signal transduction pathways that are specifically activated in bystander cells may provide clues to understand the nature of molecular signal as well as the underlying mechanism(s) with which the bystander response is triggered by ionizing radiation. Protein kinase C, a family of serine/threonine kinases, is one of the earliest responders to ionizing radiation and inhibition of PKC activity increases the cell killing by ionizing radiation (Choi et al., 2001). PKC, a calcium-dependent and phospholipid dependent kinase, is activated *in vitro* by tumor promoting agents and the data suggest that the PKC plays a key role in signal transduction and in various aspects of neoplastic transformation, tumor promotion and progression (see Koivunen et al., 2006). PKC family consists of 12 isozymes whose concerted roles regulate the cell growth and differentiation. A role for PKC in radio adaptive response has been demonstrated (Lee et al., 2000; Sasaki et al., 2002). Our earlier study has demonstrated the isoform-specific activation of protein kinase C (p-PKC-α/β, θ, βII) in bystander cells (Baskar et al., 2008).

PKCε, an important member of PKC family, is found to be activated in multiple cell types through second messengers such as diacylglycerol (DAG), fatty acids, and phosphatidylinositol 3,4,5-triphosphate (PIP3). PKCε is believed to function both as a pro-apoptotic and an anti-apoptotic factor in different mammalian cell systems (Nakajima, 2006). Further, PKCε is the only isozyme that has been associated with cancer development processes (Basu and Sivaprasad, 2007). Earlier studies reported the decreased clonogenic survival and increased apoptotic potential in bystander cells (Mothersill and Seymour, 2001; Lyng et al., 2002; Belyakov et al., 2002). In contrast, increased frequency of cellular transformation events that are critical for cancer development was also observed in bystander cells (Lewis et al., 2001; Mitchell et al., 2004). Given the importance of PKCε both in cell survival and apoptosis, we wished to determine whether PKCε participates in mediating some of the radiation induced BE in normal human dermal fibroblasts (NHDF). Our results indicate that the expression of PKCε was elevated in bystander cells. Further, suppression of PKCε expression either by small molecule inhibitor or siRNA in NHDF cells significantly reduced the MN frequency both in directly targeted and non-targeted bystander cells. Collectively, our novel study indicates that the activation of PKCε may be involved in mediating some of the radiation induced BE in human cells.

2. Materials and methods

2.1. Cell culture

NHDF cell line was obtained from Clonetics, USA. Cells were routinely cultured in FGM-2 Bullet Kit (FBM plus Single Quotes of growth supplements, Clonetics, USA).

2.2. α-Particles irradiation

NHDF cells in exponential growth phase were co-cultured on double-sided Mylar dishes as described previously (Geard et al., 2002). Double-sided Mylar dishes with approximately 60% confluent populations (0.5×10^6 cells) of NHDF cells on both surfaces were either sham treated (0 Gy) or irradiated with 5 Gy of 6.1 MeV α-particles using the track segment facility at RARAF. The LET of charged particle is estimated to be 120 keV/μm (dose rate 0.4 Gy/s) through the cells and α-particles penetrate less than 100 μm through the 9000 μm of medium (Ponnaiya et al., 2004). Two sided Mylar dishes (prepared the same way as described before) with cells that were not subjected to α-particles irradiation served as sham treated control groups for targeted and non-targeted bystander cells. The two cell populations attached to the lower and upper Mylar surfaces of each dish were carefully separated 3 h after irradiation. Cells from each side were harvested and then the total cellular RNA was isolated from both directly targeted and bystander cells. For MN determination, cells were reseeded in 2-well chamber slides.

2.3. Gene array and quantitative real time PCR

Total RNA was isolated from irradiated (lower Mylar surface) and bystander (upper Mylar surface) cells 3 h after α-particles irradiation. Commercially available Signal Transduction Pathway Finder array (GEArray QE Series, Super Array, Frederick, MD, USA) was used for gene expression analysis. These arrays contain 96 marker genes that are involved in 18 signal transduction pathways. Procedures for cDNA labeling with biotin, array hybridization and post-hybridization washings were essentially the same as described in the manufacturer's protocol. Gene expression analysis was performed using ScanAlyze (version 2.42) software developed at the Lawrence Berkeley National Laboratory, USA. This software is freely available to the scientific community. This software program measures the pixel intensity of hybridization spots on the array. In the Super Array, each gene is represented by 4 identical spots in close proximity so that reliable and consistent pixel measurements can be made. The gene expression profiles were normalized to two of the house keeping genes (β-actin and GAPD). The absolute data (signal intensity, detection call and detection *P*-value) were subjected to ANOVA approach to find differentially expressed genes ($P < 0.05$). In our study, genes that showed an increase of 2-fold and more were considered to be up regulated. Genes that showed the expression value below 1 were considered to be down regulated.

For quantitative real time PCR, cDNA synthesis was performed using 5 μg of total RNA using the cloned AMV first stand cDNA synthesis kit (Invitrogen, USA). 1 μl of cDNA was used for each quantitative real time PCR. All the real time PCR reactions were performed in triplicates by using three reaction wells for each cDNA sample. The expression of PKCε detected in different treatment groups (sham treated control, irradiated and bystander) was normalized to β-actin. The gene expression was estimated by ΔΔCt method (Livak and Schmittgen, 2001).

2.4. Inhibition of PKCε induction by Bisindolylmaleimide IX and knock down by PKCε siRNA

Exponentially growing NHDF cells were treated with different concentrations (1–10 μM) of Bisindolylmaleimide IX (Ro 31-8220, EMD Biosciences, USA) 1.5 h prior to radiation treatment. Treated and non-treated cells were irradiated with 5 Gy of γ-rays radiation and the total cellular proteins were extracted 1 h after radiation treatment essentially as described earlier (Balajee and Geard, 2001; Balajee et al., 2004). The optimum concentration required for complete inhibition of PKCε protein level was determined by Western blot. To directly assess the role of PKCε in bystander response, PKCε was silenced by siRNA transfection following the manufacturer's instruction (Invitrogen, USA). PKCε specific siRNA and control siRNA duplexes were purchased from Santa Cruz (USA) and reconstituted in RNAase-free water. Total cellular proteins were isolated 72 h after transfection and the level of inhibition of PKCε expression was monitored by Western blot analysis using a commercially available antibody (Santa Cruz Biotechnology, USA).