Published in final edited form as:

Int J Biochem Cell Biol. 2009 December ; 41(12): 2413–2421. doi:10.1016/j.biocel.2009.06.012.

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

Burong Hu^{*}, Bo Shen^{*}, Yanrong Su, Charles R. Geard[#], and Adayabalam S. Balajee[#]

Center for Radiological Research, Department of Radiation Oncology, College of Physicians and Surgeons, 630 West, 168th Street, VC-11, Room 239, Columbia University, New York, NY 10032

Abstract

Our earlier study demonstrated the induction of PKC isoforms (beta II, PKC-alpha/beta, PKC-theta) 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 (PKC ϵ). PKC ϵ functions both as an anti-apoptotic and pro-apoptotic protein and it is the only PKC isozyme implicated in oncogenesis. Given the importance of PKC ϵ in oncogenesis, we wished to determine whether or not PKC ϵ is involved in bystander response. Gene expression array analysis demonstrated a 2-3 fold increase in PKC ϵ 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 PKC ϵ expression in bystander cells was verified by quantitative real time PCR. Suppression of PKC ϵ 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 PKC ϵ 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.

Keywords

bystander effects; protein kinase C ϵ ; ionizing radiation; signal transduction pathway

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;

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[#]Author for Correspondence Adayabalam S. Balajee, Ph. D., Center for Radiological Research, Columbia University, VC 11-239, 630 W. 168th ST New York, NY 10032 Tel: (212) 342-6838 Fax: (212) 305-3229 Adayabalam S. Balajee (ab836@columbia.edu) and Charles R. Geard (crg4@columbia.edu).

^{*}These authors have contributed equally to this work.

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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; Azzam et al., 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., 2003; Hei et al., 2008 and references therein). Inhibitors of GJIC have been shown 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 , β II) in bystander cells (Baskar et al., 2008).

PKC- ϵ , 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). PKC ϵ is believed to function both as a pro-apoptotic and an anti-apoptotic factor in different mammalian cell systems (Nakajima, 2006). Further, PKC ϵ 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 PKC ϵ both in cell survival and apoptosis, we wished to determine whether PKC ϵ participates in mediating some of the radiation induced BE in normal human dermal fibroblasts (NHDF). Our results indicate that the expression of PKC ϵ was elevated in bystander cells. Further, suppression of PKC ϵ 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 PKC ϵ may be involved in mediating some of the radiation induced BE in human cells.

Materials and Methods

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).

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 (0Gy) or irradiated with 5 Gy of 6.1 MeV alpha-particles using the track segment facility at RARAF. The LET of charged particle is estimated to be 120 keV/ μ m (dose rate 0.4Gy/sec) through the cells and α -particles penetrate less than 100 μ m through the 9000 μ m 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.

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 μ g of total RNA using the cloned AMV first stand cDNA synthesis kit (Invitrogen, USA). 1 μ l 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 PKC ϵ

detected in different treatment groups (sham treated control, irradiated and bystander) was normalized to β -actin. The gene expression was estimated by $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

Inhibition of PKC ϵ induction by Bisindolylmaleimide IX and knock down by PKC ϵ siRNA

Exponentially growing NHDF cells were treated with different concentrations (1-10 μ M) 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 1h after radiation treatment essentially as described earlier (Balajee et al., 2001 and Balajee and Geard, 2004). The optimum concentration required for complete inhibition of PKC ϵ protein level was determined by western blot. To directly assess the role of PKC ϵ in bystander response, PKC ϵ was silenced by siRNA transfection following the manufacture's instruction (Invitrogen, USA). PKC ϵ 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 PKC ϵ expression was monitored by western blot analysis using a commercially available antibody (Santa Cruz Biotechnology, USA).

γ -rays irradiation and medium transfer treatment

NHDF cells treated with and without the PKC ϵ inhibitor (Ro 31-8220), were irradiated with 5 Gy of γ -rays using a ^{137}Cs source at a dose rate of 0.82Gy/Min (Gamma cell 40, Atomic Energy of Canada, Canada). Irradiated cells (donor cells) were incubated for 1 hr at 37°C and the medium was carefully collected from the irradiated cells and unirradiated cells. The conditioned medium collected from irradiated and sham-treated cells were either centrifuged at 1000 rpm for 5 min to remove the floating cells or filtered through 0.2 μ M filters (Millipore, USA). Both these approaches were interchangeably used without any noticeable effect on bystander response. In case of cells transfected with control scrambled or PKC ϵ siRNA, cells were collected by trypsinization 48 h after transfection. The siRNA transfected cell suspension was split into two portions. One portion of the cell suspension was placed in 60mm culture dishes. Other portion of cells was seeded onto 2-well glass chamber slides (Nunc, USA) at a density of 2.5×10^4 for MN assay. Control and PKC ϵ siRNA transfected cells 8 h after replating in 60mm culture dishes were irradiated or sham-irradiated with 5 Gy γ -rays and then returned to the incubator for additional 1.5 h incubation.

MN assay

For MN determination after α -particles radiation, cells after sham and radiation treatments were seeded in 2-well chamber slides in the presence of Cytochalasin B (final concentration 1.5 μ g/ml) as described earlier (Fenech, 1993). After 48 h, the slides were rinsed once with PBS and fixed in methanol: acetic acid (3:1) for 20 min. The fixed cells were stained either with DAPI or 0.1% (w/v) acridine orange before microscopic observation. MN frequency was assayed by fluorescence microscopy and identified morphologically following the standard criteria (Fenech, 1993). At least 500 randomly chosen binucleate cells were scored for each treatment.

Statistical Analysis of Data

Experimental data were presented as the mean with standard deviations of the mean for at least three independent experiments. Statistical significance level was assessed using the Student's t-test. Statistical significance was accepted at a p -value of <0.05 between sham treated control and treatment groups.

Results

Induction of PKC ϵ in bystander cells after α -particles irradiation

Our earlier study demonstrated the induction of different PKC isoforms in low LET radiation induced human bystander cells (Baskar et al., 2008). In this study, gene expression analysis was performed using commercially available signal transduction pathway finder array (Super Array, Gaithersburg, MD, USA). This array contains a total of 96 genes including PKC ϵ that are involved in 18 signal transduction pathways. Gene expression analysis was performed on bystander NHDF cells that were co-cultured for 3 h with cells directly targeted with 5 Gy of alpha particles. The representative pictures showing the expression profiles of different genes in directly targeted and bystander cells together with sham treated control cells are given in Fig.1a and b. In our study, the expression levels of genes were normalized to β -Actin and GAPD. Compared to sham treated control cells, cells directly targeted with 5 Gy of α -particles showed elevated expression of 2 fold or more in 25 genes (ATF2, Bax, BCL2, BCL2A1, BCL2L1, BIRC1, BIRC2, BIRC3, BMP4, CD5, CDK2, CDKN1A, CDKN1C, CDKN2B, CDX1, CEBPB, EN1, FASN, FLJ12541, GADD45A, HK2, IGFBP3, MDM2 and KLK2 and PIG3, Fig 1a). All of these genes, with the exception of CDKN1A, showed 2.27-4.66 fold increase in expression in directly targeted cells as compared to sham treated control cells. CDKN1A showed the highest increase (7.39 fold increase over sham treated control) in expression after α -particle irradiation. As many as 15 genes were found to be down regulated in directly targeted cells as compared to sham treated control cells (IL2RA, PRKCE, PTCH1, PTGS2, RBP1, SWIP1, TFRC, TMEPA1, TNFA, TNFSF6, TP53, VCAM1, WISP1 and WISP3). Interestingly, expression of PKC α (PRKCA) and PKC β 1 (PRKCB1) was found to be 1.86 and 1.20 fold more than sham treated cells respectively (Fig. 1a). The expression levels for the rest of 54 genes varied from 1.01 to 1.89 in directly targeted cells.

In contrast to directly targeted cells, bystander cells showed only 3 genes (IGFBP3, NFKBIA and PKC ϵ) whose expression levels showed a >2 fold increase (2.26-2.85) in comparison to sham treated control cells (Fig 1b). In sharp contrast to directly targeted cells which did not show any elevation, bystander cells showed a 2.86 fold increase in PKC ϵ expression. To verify the result of the PKC ϵ expression in bystander cells, quantitative real time PCR was carried out using PKC ϵ specific primers (Fig. 2). Consistent with gene array results, quantitative real time PCR revealed a modest increase in PKC ϵ expression (1.74) in bystander cells. Although elevated PKC ϵ expression was not detected in directly targeted cells by gene array approach, quantitative real time PCR detected a 2.40 fold increase in PKC ϵ expression in directly irradiated cells compared to sham treated control cells.

Effect of PKC ϵ inhibition on radiation induced bystander response

Both gene expression array and quantitative real-time PCR analyses demonstrated the activation of PKC ϵ expression in bystander NHDF cells. To directly assess the importance of PKC ϵ expression in bystander response, we suppressed PKC ϵ expression using a small molecule inhibitor (Ro 31-8220) in the donor cells. Initial experiments were performed to determine the optimum concentration required for the effective suppression of PKC ϵ . NHDF cells irradiated with 5 Gy of γ -rays showed an efficient activation of PKC ϵ at 30 min after exposure. However, the PKC ϵ activation was abolished when NHDF cells were pretreated with Ro 31-8220 at 1 μ M concentration for 1.5 h prior to 5 Gy of α - particles radiation. Although a modest inhibition of PKC ϵ was observed at 10 μ M concentration at 30 min, the inhibition was found to be consistent at both sampling time (30 min and 60 min) in cells treated with 1 μ M concentration of Ro 31-8220 (Fig. 3). Interestingly, this inhibitor seems to be far more effective in inhibiting the radiation induced activation of PKC ϵ than the basal level of PKC ϵ in sham treated control cells. In the subsequent experiments, 1 μ M concentration of Ro 31-8220 was used.

For the assessment of PKC ϵ on bystander response, we chose to analyze MN as it is one of the well known biological endpoints used by several laboratories. For this purpose, double sided Mylar dishes with approximately 60% confluent populations of NHDF cells on both surfaces were utilized. Cells were pre-treated with PKC ϵ inhibitor for 1.5 h prior to α -particles radiation. Increased MN frequency was observed in directly targeted cells as compared to sham treated cells in the absence of PKC ϵ inhibitor. Interestingly, suppression of PKC ϵ expression by the inhibitor Ro 31-8220 considerably reduced the MN frequency in both directly targeted and bystander cells (Fig.4a and b). Representative pictures of Cytochalasin B blocked binucleated cells with MN in directly targeted and bystander cells are shown in Fig.4c and d. Similar to α -particles radiation, considerable reduction in MN frequency was also observed in directly targeted and bystander cells following exposure to 5 Gy of γ -rays radiation (Data not shown). Collectively, our data indicate that PKC ϵ expression is critical for the induction of BE after low and high LET radiations.

Effects of siRNA mediated silencing of PKC ϵ expression on directly targeted and bystander cells after low LET radiation

Ro 31-8220 is not only a selective and ATP-competitive PKC inhibitor but also an inhibitor of PKA function (Davis et al., 1992; Wilkinson et al., 1993). Further any chemical inhibitor can have non-specific effects on targets other than PKC and PKA. Therefore, we wished to verify our results by utilizing the siRNA technique to suppress the expression of PKC ϵ . The specificity of the siRNA on PKC ϵ expression was verified using two different concentrations (25 nM and 50 nM) and the PKC ϵ expression was efficiently reduced by more than 80% by 50 nM of PKC ϵ siRNA (Fig. 5). Similar level of PKC ϵ inhibition was observed in directly targeted and bystander cells (Data not shown). Effect of PKC ϵ on bystander response was assessed by the determination of MN frequency. MN frequency was compared between directly targeted (5 Gy of γ -rays) and bystander cells that were either transfected with scrambled siRNA or PKC ϵ siRNA. The frequency of MN was significantly increased in bystander cells (1.7 fold over sham control) that received the conditioned medium from irradiated cells transfected with scrambled siRNA (Fig.6). Interestingly, bystander cells treated with ICM of PKC ϵ specific siRNA transfected cells did not show elevated micronuclei. This observation clearly indicates that activation of PKC ϵ is involved in mediating some of the bystander effects.

Discussion

PKC ϵ , an important member of novel PKCs, has been shown to be activated in multiple cell types by second messengers such as diacylglycerol (DAG), fatty acids, and phosphatidylinositol 3, 4, 5-triphosphate (PIP3). Studies have shown that the signals that stimulate G-protein coupled receptors, receptor tyrosine kinases and non-receptor tyrosine kinases can cause generation of DAG (Nishizuka, 1992; Newton, 1995). Several PKC isoforms are activated by phospholipase C (PLC) and phosphatidylinositol 3kinase (PI-3) pathway (Keranen et al., 1995). Activation of PKC ϵ is achieved by platelet derived growth factor involving either the PLC γ or PI-3 pathway (Moriya et al., 1996). In this study, we have demonstrated an efficient transcriptional activation of PKC ϵ both in directly targeted and bystander cells. Further, we demonstrate that abolition of PKC ϵ expression considerably reduced the MN frequency in both directly targeted and bystander cells.

PKC was originally identified as a phospholipid-calcium dependent protein kinase (Takai et al., 1979). PKC belongs to serine/threonine family of kinases. As many as 12 different PKC isoforms that function in cellular growth and differentiation have been described in the literature (Mackay and Twelves, 2003). PKC activation has been demonstrated after cellular exposure to ionizing radiation (Hallahan et al., 1991; Woloschak et al., 1990; Kim et al., 1992; Hasan et al., 1996). The finding that PKC inhibition leads to increased radiation

sensitivity clearly demonstrates its role in cellular response to radiation (Choi et al., 2000). Our previous study (Baskar et al., 2008) utilizing the medium transfer technique showed the activation of phosphorylated PKC isoforms in bystander human fibroblast cells (Baskar et al., 2008). Activation of different isoforms of PKC in bystander cells indicates that the PKC pathway may be critical for radiation induced bystander response.

MN formation has been found to be one of the useful biological endpoints for the assessment of bystander response. MN are formed during cell division due to inefficient repair of DNA double strand breaks when the nuclear envelope is reconstituted around chromosomes or chromosome fragments. As compared to directly targeted cells, bystander cells showed only a modest increase in MN frequency after low and high LET radiations. The moderate increase in MN frequency observed in bystander cells suggests that MN formation is probably due to mis-repair or lack of repair of DNA lesions. It is not clear whether the differences in MN frequency between directly targeted and bystander cells are due to the extent of DNA damage induction. Earlier studies have documented the lack of radiation dose dependency on many of the biological endpoints studied in bystander cells (see Morgan and Sowa, 2007 and references therein). It is likely that the DNA damage response elicited by bystander cells may be distinctly different from directly targeted cells. Nevertheless, suppression MN frequency in both directly targeted and bystander cells after inhibition of PKC ϵ expression clearly indicates that PKC ϵ is critical for the potentiation of bystander response. The downstream signaling events after PKC activation are not yet clearly established. Available evidence suggests the activation of MEK-ERK pathway by PKC α , δ and ϵ via Raf1 (Kolch et al., 1993; Ueda et al., 1996; Cai et al., 1997). In corroboration, activation of PKC isoforms as well as ERK1/2 has been demonstrated in our earlier study (Baskar et al., 2008). Li et al. (2006) showed that PKC δ and PKC ϵ have the potential to modulate cell survival through dephosphorylation of Akt. Collectively, these studies point out that PKC isoforms can significantly modify the cellular signal transduction pathways. This notion is partly supported by our finding that PKC ϵ inhibition modulates the extent of bystander response (as judged by the MN frequency) triggered by low and high LET radiations. It is likely that the suppression of PKC ϵ expression may modify the cellular signal transduction pathways leading to the reduction of MN frequency both in directly targeted and bystander cells. Although the exact molecular link between PKC ϵ expression and MN formation remains to be identified, our finding indicates that PKC pathway may be an ideal molecular target for suppressing the harmful effects of radiation in bystander cells.

In this study, a total of 25 genes were up regulated (≥ 2 fold in expression) in directly targeted cells in contrast to only 3 genes in bystander cells. Unique patterns of the gene expression profiles observed in directly targeted and bystander cells may be due to intrinsic differences in the nature and extent of molecular signals (intra- and extracellular) that are elicited and transmitted from directly targeted cells to bystander cells. Previous studies have also reported differential expression of genes in directly targeted and bystander cells (Chaudhry, 2006; Iwakawa et al., 2008). Similar to our study, Chaudhry et al. (2006) reported the induction of some of the genes such as Bcl2, Bcl2L1 and CDKN1A after low LET radiation in bystander normal human fibroblast cells (NHLF; Chaudhry, 2006) using the Affymetrix human U133A array. Gene expression profiles greatly vary as a function of post-recovery time and cell type. These variables significantly contribute to gene expression profiles reported in various studies. Further, the gene expression profiles also vary between quiescent and proliferating cells. The differences observed in gene expression patterns between directly targeted and bystander cells reinforce the suggestion that the DNA damage/stress response mechanisms in bystander cells may be distinctly different from directly targeted cells. In support, Sokolov et al. (2005) demonstrated that γ -H2AX formation was considerably delayed in bystander cells and the cells with multiple γ -H2AX foci increased by 3.7 fold after 18 h of treatment. Further, p53 phosphorylation was also shown to be delayed in bystander cells (Hamada et al., 2008). In

addition to p53 phosphorylation, the time course of apoptotic response also greatly differed between directly targeted and bystander cells (Hamada et al., 2008).

Radiation dose dependent induction of PKC at the mRNA level was demonstrated in an earlier study in Syrian hamster embryo (SHE) fibroblasts after by low doses of low LET radiation (Woloschak et al., 1990). Consistent with this study, induction of PKC isoforms at the protein level was reported earlier by us in human primary fibroblasts (Baskar et al., 2008). Although the precise nature of DNA damage inflicted in the bystander cells in this study remains unclear, observation of MN formation in bystander cells indicates the induction of DNA double strand breaks. Our earlier studies demonstrated that the enhanced clonogenic survival and proliferation in radiation induced bystander cells correlate with the activation of PKC isoforms (Baskar et al., 2007; Baskar et al., 2008). The question of whether radiation induced bystander response is beneficial or harmful is open to debate as available evidences support both of these options (See Morgan and Sowa, 2007). The harmful side of the bystander response was demonstrated recently by employing the radiosensitive *Patched-1* heterozygous mouse model system (Mancuso et al., 2008). Irradiation of the head shielded *Ptch-1* mice with 3Gy of X-rays resulted in the induction of double strand breaks and apoptotic cells in the cerebellar region of the brain. Like wise, cranial X-ray radiation of mice resulted in the increased DNA damage, altered cellular proliferation and apoptosis in the shielded spleen (Koturbash et al., 2008). Nevertheless, one of the serious concerns of the bystander response is the probability whereby the normal healthy cells adjacent to tumor become neoplastically transformed by radiation injury. In this respect, our observation of elevated expression of PKC isoforms in general (Baskar et al., 2008) and PKC ϵ in particular (this study) in radiation induced bystander cells deserves special attention. Firstly, PKC isoforms are implicated in cell growth and differentiation and secondly PKC isoforms are constitutively over expressed in many cancer cell types. A recent study showed that PKC ϵ is over expressed in prostate cancer cells and the elevated PKC ϵ expression correlated with tumor aggressiveness, while over expression of PKC ϵ in androgen sensitive LNCaP prostate cancer cells not only enhances their proliferation rate but also render the cells resistant to androgen (Aziz et al., 2007). It is likely that the elevated PKC ϵ expression confers proliferative advantage to the directly targeted and bystander cells and renders them susceptible for neoplastic transformation. Also, elevated PKC ϵ expression can regulate the signal transduction pathways that are linked with cell survival thereby increasing the radiation induced mutational load and genomic instability. As mentioned earlier, PKC δ and PKC ϵ have the potential to modulate cell survival through dephosphorylation of Akt (Li et al., 2006). Future experiments will verify whether PKC is required for the radiation induced neoplastic transformation. Collectively, this study demonstrates that the PKC ϵ signaling pathway is activated in bystander cells and that PKC ϵ may be a potential molecular target for suppressing the biological effects of radiation in healthy bystander cells during the radio therapy of tumor cells.

Acknowledgments

We greatly acknowledge the financial support received from U.S. Department of Energy, Office of Sciences (BER) awarded to ASB (DE-FG02-05ER64055) and CRG (DE-FG02-05ER64054). CRG and ASB acknowledge the financial support received from NIH/NCI (5P01CA49062-16). The charged particle irradiations were undertaken at the Radiological Research Accelerator Facility (RARAF) of our center supported by NIH P41 research grant awarded to Dr. David Brenner, CUMC, NY, USA.

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Fig.1.

Signal Transduction Pathway array (Super Array, Frederick, MD, USA) analysis of gene expression in directly targeted and bystander cells with their corresponding sham treated

control cells. Total RNA was isolated from irradiated and sham irradiated (lower Mylar surface) and bystander and sham treated control (upper Mylar surface) cells 3 h after α -particles irradiation. Expression of genes was normalized to Actin B and GAPD. Genes that showed more than 2-fold increase over sham treated control were considered to be up regulated genes (Red circles). Genes that showed below 1 fold were considered to be down regulated (blue circles).

Fig.2.

Analysis of PKC ϵ expression by quantitative real time PCR in irradiated and bystander cells. The complementary DNA was synthesized from the total RNA isolated 3 h after irradiation with 5 Gy of α - particles (for details see materials and methods). Data were pooled from 2 independent biological experiments. Error bars represent $\pm$ SD.

Fig.3.

Effect of PKC ϵ inhibitor Ro 31-8220 on PKC ϵ expression in NHDF cells. Exponentially growing NHDF cells were treated with different concentrations (1-10 μ M) of Ro 31-8220 for 1.5h prior to γ -rays radiation. Treated and mock-treated cells were irradiated with 5Gy γ -rays radiation and the total cellular proteins were extracted 1h after radiation treatment. The optimum concentration required for complete inhibition of PKC ϵ protein level was determined by western blot analysis. The values for the band intensity of PKC ϵ are shown on the top of each lane.

Figure 4.

Inhibitory effect of Ro 31-8220 on micronuclei induction in directly targeted and bystander cells. Cells were treated with or without 1 μ M Ro 31-8220 and incubated for 1.5h. The ICM collected after 1 h from the sham irradiated and 5 Gy of α -particles treated cells (donor) were transferred to the corresponding recipient cells (bystander) and incubated for an additional 1h. Cells were subsequently trypsinized and reseeded for MN analysis. Cells were fixed 48 h later, stained with DAPI and scored for MN. The MN frequencies observed in directly targeted (a) and bystander cells (b) were shown in the form of histograms. At least 500 cells were scored for each treatment. Error bars represent $\pm$ SD. Representative pictures of binucleated cells with MN in directly targeted (c) and bystander (d) cells are shown.

Fig.5.

Transfection of PKCε specific siRNA efficiently reduces the PKCε expression in NHDF cells. NHDF cells in exponential growth phase were transfected with PKCε specific SiRNA using Lipofectamine RNAiMax (Invitrogen, USA). Total cellular proteins were isolated 72 h after transfection and the level of inhibition of PKCε expression was monitored by western blot analysis using PKCε specific antibody (Santa Cruz Biotechnology, CA, USA).

1. Recipient: control SiRNA; Donor: control SiRNA

2. Recipient: control SiRNA; Donor: PKCε SiRNA

3. Recipient: PKCε SiRNA; Donor: PKCε SiRNA

4. Recipient: PKCε SiRNA; Donor: control SiRNA

Fig.6.

Inhibition of PKCε expression by SiRNA reduces MN frequency in both directly targeted and bystander cells. Cells 48 h after transfection were trypsinized and seeded on 60mm Petri-dishes and 2-well chamber slides and incubated for an additional 8 h. The ICM collected after 1.5 h from the sham irradiated and irradiated transfected cells (donor in 60mm Petri-dishes) were transferred to the recipient cells (bystander cells in 2-well glass chamber slide) and incubated for an additional 72 h. Data were pooled from 5 independent experiments. Error bars represent $\pm$ SD.