**In Vivo** Modulation of Signaling Factors Involved in Cell Survival

Anirban KUMAR MITRA and Malini KRISHNA*

PKC/NF-κB/ERK/Radiation and fibrosarcoma.

*In vivo* expression of cell survival factors protein kinase C (PKC), nuclear factor κB (NFκB), and extracellular signal-regulated kinase (Erk), which may contribute to the development of radioreistance following radiotherapy, was looked for. Their modulation with natural compounds (curcumin, rutin or nicotinamide) was attempted in mice bearing a serially transplanted fibrosarcoma. Expression of protein kinase C was isoform specific. No translocation of any of the isozymes was noticed following γ-irradiation as has been reported elsewhere. None of the isoforms could be significantly inhibited by the modulators. However, significant inhibition of radiation-induced ERK and NFκB was observed with both curcumin and nicotinamide. Therefore we conclude that use of inhibitors of MAP kinases or NFκB may be a more promising strategy to enhance tumour cell killing or to prevent the development of radioreistance during radiotherapy.

**INTRODUCTION**

Ionising radiation is known to activate existing cellular signalling pathways, prominent among them being the cytoprotective and cytotoxic pathways. Activation of the survival pathways could confer radioreistance on the tumour cells or inhibition of these at the time of radiotherapy could push the cells into apoptosis.

Among the cytoprotective pathways that are activated following γ irradiation and which may confer radioreistance on the cell are protein kinase C (PKC), mitogen activated protein (MAP) kinase and the nuclear factor-κB (NFκB) pathways. PKC is known to exist in eleven different isoforms whose cofactor requirements are different. Activation of PKC has been implicated in the development of radioreistance, where it is reported to inhibit the ceramide pathway leading to inhibition of apoptosis and cell survival.

Growth factor-induced MAP kinase signaling has been proposed to regulate both proliferation and differentiation. Stress induced activation of MAP kinase, as happens in γ irradiation, plays a cytoprotective role by the activation of DNA repair genes.

The activation of NFkB by ionising radiation has been found to protect cells from apoptosis and its inhibition enhanced radiation-induced apoptosis. Therefore, strategies that inhibit the activation or nuclear translocation may prove beneficial in the radiation therapy of tumours.

Many synthetic drugs, at present under clinical trials target protein kinase C and MAP kinases. In recent years, the focus has shifted to natural compounds like polyphenols and other natural derivatives. We had earlier observed an inhibition of protein kinase C activity by curcumin, rutin and nicotinamide. The present study extends the work to modulation by these compounds in tumours, *in vivo*.

**MATERIALS AND METHODS**

*Animals*

Animals belonging to a conventional inbred colony of Swiss mice. Male mice (8 weeks), weighing 20–25 g and maintained on a standard laboratory diet, with water *ad libitum* were used. Tumour bearing animals were randomly distributed into four groups (a) control animals, fed the normal laboratory diet and those receiving (b) 1% (w/w) curcumin (c) 1% (w/w) nicotinamide or (d) 1% (w/w) rutin in the diet for a period of 9 days and were irradiated on the tenth day. All experiments were conducted with strict adherence to the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

*Tumour*

A serially transplanted mouse fibrosarcoma developed by subcutaneous injection of 6,12-dimethyl benzo [1,2-b,5,4-6’] dithionaphene into swiss mice was used as the test system. Tumours were grown on the gastrocnemius muscles in the left hind leg of eight-week old male mice.

---

*Corresponding author: Phone: +91-22-2559 3868, Fax: +91-22-2550 5151, E-mail: malini@magnum.barc.ernet.in*  
Radiation Biology and Health Sciences Division Bhabha Atomic Research Centre Trombay, Mumbai - 400 085, India.
Irradiation and sample preparation

Unanaesthetized animals were restrained in specially designed, well ventilated, acrylic boxes and the leg was locally irradiated with a single dose of 3 Gy of $^{60}$Co $\gamma$-radiation at a dose rate of 0.49 Gy/min using a $^{60}$Co Junior Theratron unit. The rest of the animal body was shielded with lead jigs designed for the purpose. Three mice from each group a, b, c or d were irradiated and three were sham irradiated. These were humanely sacrificed by cervical dislocation, 30 min post-irradiation. Radial sections of the tumours were taken and a single cell suspension was made in phosphate buffered saline. The cells were washed and suspended in 200 $\mu$L of cold lysis buffer (10 mM HEPES, 0.25 M sucrose, 5 mM EDTA, 5mM EGTA, 2 mM PMSF, 1 mM leupeptin, 10 $\mu$g ml$^{-1}$ aprotinin and 10 $\mu$g ml$^{-1}$ pepstatin; pH 7.5) and ultrasonicated using a mini sonication probe, with 10 bursts at 50% output control and 50% duty cycle. The lysate was quickly frozen in liquid $\text{N}_2$ and stored at $-70^\circ$C.

For the cytosolic fraction, the whole lysate obtained as above was kept on ice for 15 min and then centrifuged at 100 000 $\times$ g for 1 h at 4$^\circ$C in an ultracentrifuge. The supernatant (cytosolic fraction) was quickly frozen in liquid $\text{N}_2$ and stored at $-70^\circ$C. The remaining pellet was solubilized in the same cell lysis buffer containing 1% Triton X-100 and allowed to stand on ice for 30 min. The lysate was then centrifuged at 100 000 $\times$ g for 1 h at 4$^\circ$C in an ultracentrifuge. The supernatant of this constituted the particulate fraction was quickly frozen in liquid $\text{N}_2$ and stored at $-70^\circ$C.

Western Blotting

Protein was estimated using Lowry’s method. The lysates (125 $\mu$g protein for PKC, 200 $\mu$g for NF$\kappa$B and p44/42 MAP kinase) were run on 8% SDS polyacrylamide gel followed by transfer to nitrocellulose membrane (Amer sham, USA). Membranes were probed with anti PKC $\alpha$, PKC $\delta$, PKC $\zeta$ (Roche Molecular Biochemicals, Germany) and PKC $\beta$ (Transduction Laboratories, USA) at a dilution of 1:500 while p44/42 MAP kinase (Cell Signaling Technology, USA) and NF$\kappa$B (Transduction Laboratories, USA) were used at a dilution of 1:1000.

Fig. 1. Modulation of radiation-induced expression of PKC$\alpha$ by curcumin, nicotinamide and rutin. Tumour bearing mice were fed with curcumin (CUR.), nicotinamide (NIC.) or rutin (RUT.) for nine days prior to irradiation with 3 Gy of $^{60}$Co $\gamma$ rays. Control animals (CONT.) were fed with standard laboratory diet. The mice were sacrificed 30 minutes post irradiation and tumour lysate was obtained as described in materials and methods. The cytosolic (C) and particulate (P) PKC fractions were separated on 8% SDS polyacrylamide gel and hybridised with the antibody mentioned in materials and methods. Irradiation is indicated as RAD. Each data point ($\pm$ S.E.) represents an average of three independent experiments. Key: # - p<0.01 as compared to irradiated particulate fraction. * - p<0.05 as compared to irradiated particulate fraction.

Fig. 2. Modulation of radiation-induced expression of PKC$\beta$ by curcumin, nicotinamide and rutin. Tumour bearing mice were fed with curcumin (CUR.), nicotinamide (NIC.) or rutin (RUT.) for nine days prior to irradiation with 3 Gy of $^{60}$Co $\gamma$ rays. Control animals (CONT.) were fed with standard laboratory diet. The mice were sacrificed 30 minutes post irradiation and tumour lysate was obtained as described in materials and methods. The cytosolic (C) and particulate (P) PKC fractions were separated on 8% SDS polyacrylamide gel and hybridised with the antibody mentioned in materials and methods. Irradiation is indicated as RAD. Each data point ($\pm$ S.E.) represents an average of three independent experiments. Key: # - p<0.01 as compared to irradiated particulate fraction. * - p<0.05 as compared to irradiated particulate fraction.
membranes were then probed with horseradish peroxidase conjugated secondary antibody against mouse/rabbit (Roche Molecular Biochemicals, Germany) at a dilution of 1:2000 and developed using BM Chemiluminescence Western Blotting Kit (Roche Molecular Biochemicals, Germany). Densitometry was done using Shimadzu CS 9000 Dual wavelength flying spot scanner. Statistical analysis was done by ANOVA.

RESULTS AND DISCUSSION

The protein kinase C isoforms showed varied expression in the irradiated animals. It is noteworthy that no translocation of any of the isoforms was observed followingγ-irradiation in the fibrosarcoma cells. There was an increase in expression in both the cytosolic and the particulate fractions of the isoforms α and β (Fig. 1 A and B; lanes 1–4). However, PKCδ showed an increase in the particulate with no concomitant decrease in the cytosol and PKCζ showed a decrease in the particulate fraction following irradiation, thus different isoforms reacted differently to radiation (Fig. 2 A and B).

Amongst the PKC isozymes, none showed a significant inhibition with any of the modulators except for PKCα with nicotinamide (Fig. 1A). The radiation-induced expression was marginally inhibited by the endobiotic. Rutin increased the particulate PKC expression of α, β, δ and ζ (Fig. 1 and 2). Although curcumin has been reported to inhibit phorbol ester induced expression of c jun, c fos and c myc protooncogenes,[12] in the present study, we did not find any inhibition of expression of any of the PKC isoforms by curcumin. Inhibition of PKC isoforms by curcumin may be an in vitro phenomenon only, as has been observed in an earlier study.[11] However, no inhibition of PKC could be seen in the present study where curcumin was fed to the animals bearing fibrosarcoma.

There was significant inhibition of radiation-activated p44/42 MAP kinase by curcumin (Fig. 3B). Although curcumin alone was found to increase the expression of p44/42 MAP kinase, curcumin administration to irradiated animals...
brought the levels down to the control. Unlike curcumin, nicotinamide alone did not have any effect on the p44/42 MAP kinase but could also bring down the levels of radiation-activated kinase. Rutin was marginally effective.

Treatment with curcumin alone was found to raise the NFκB expression, but could bring down the radiation induced levels (Fig. 3A). Although nicotinamide itself was effective in inhibiting NFκB expression, it was only marginally effective in inhibiting radiation induced NFκB expression. Here again rutin was the least effective.

The noteworthy finding of this study is the fact that the inhibition of radiation-induced NFκB expression by curcumin is not via inhibition of PKC or the isozymes tested, since radiation-induced PKC expression itself was not significantly inhibited by curcumin. PKC is known to phosphorylate IκB and lead to the activation of NFκB. Feeding of curcumin alone was found to increase the NFκB levels significantly. This may be due to the fact that curcumin, by virtue of its hydrophobicity, is predestined to locate in the membrane and may alter its properties. NFκB, being a highly redox sensitive factor, responds to the changes in the membrane by inducing the expression of NFκB. This may be apoptosis independent and non-specific signaling. Some potent anticancer drugs, like taxol, are known to be strong activators of NFκB where it was speculated that activation of NFκB could lead to the induction of immunoregulatory and cytotoxic cytokines, thereby contributing to the antitumour effects of taxol. In the present study, although curcumin increases the expression of NFκB in unirradiated animals, it is noteworthy that it decreases the NFκB and ERK expression in irradiated animals both of which have been implicated in enhanced cell survival and radioresistance. It appears from this study that there are two mechanisms that are operative when curcumin is administered to animals, one that is operative when curcumin is fed alone and which may be non-specific signaling, and the second, which is triggered after irradiation of the curcumin fed animals. Curcumin, as an inhibitor of radiation-induced MAP kinase and NFκB may prove to be an excellent modulator in enhancing the efficacy of tumour cell killing in cancer radiotherapy. In conclusion, it might be stated that the results presented are preliminary and the detailed mechanism of modulation by curcumin is yet to be elucidated. Other potential mechanisms of modulation of radiation-induced NFκB expression by curcumin include changes in the level of IκBα, a component of NFκB heterodimer, which may be, due to its redox-sensitivity, altered by changes in the radiation-induced redox state of the membrane. Although curcumin increases the expression of NFκB, this may be apoptosis independent and non-specific signaling. Some potent anticancer drugs, like taxol, are known to be strong activators of NFκB where it was speculated that activation of NFκB could lead to the induction of immunoregulatory and cytotoxic cytokines, thereby contributing to the antitumour effects of taxol. In the present study, although curcumin increases the expression of NFκB in unirradiated animals, it is noteworthy that it decreases the NFκB and ERK expression in irradiated animals both of which have been implicated in enhanced cell survival and radioresistance. It appears from this study that there are two mechanisms that are operative when curcumin is administered to animals, one that is operative when curcumin is fed alone and which may be non-specific signaling, and the second, which is triggered after irradiation of the curcumin fed animals. Curcumin, as an inhibitor of radiation-induced MAP kinase and NFκB may prove to be an excellent modulator in enhancing the efficacy of tumour cell killing in cancer radiotherapy. In conclusion, it might be stated that the results presented are preliminary and the detailed mechanism of modulation by curcumin is yet to be elucidated.
cumin and nicotinamide would be worth investigating.

REFERENCES


Received on November 20, 2003
1st Revision on July 20, 2004
2nd Revision on August 11, 2004
Accepted on August 26, 2004