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Fractionated and Acute Irradiation Induced Signaling in a Murine Tumor

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Abstract The effect of fractionated doses of Co^{60} γ-irradiation (2 Gy per fraction over 5 days), as is delivered in cancer radiotherapy, was compared with acute doses of 10 and 2 Gy, in a serially transplanted mouse fibrosarcoma grown in Swiss mice. The aspects that were studied included the three major mitogen-activated protein (MAP) kinases, namely p44 MAP kinase, p38 MAP kinase, and stress-activated protein (SAP) kinase, which are known to be involved in determining the cell fate following exposure to ionizing radiation. The response of dual specificity phosphatase PAC1 which is involved in the dephosphorylation of MAP kinases was also looked at. There were significant differences in the response to different dose regimens for all the factors studied. Fractionated irradiation elicited an adaptive response with a sustained activation over 7 days of prosurvival p44 MAP kinase which was balanced by the increased activation of proapoptotic p54 SAP kinase up to 1 day post-irradiation, whereas, phosphorylated p38 MAP kinase showed a decrease at most time points. PAC1 was induced following fractionated irradiation and may be acting as a feed back regulator of p44 MAP kinase. The activation of SAP kinase after fractionated irradiation may be a stress response, whereas, constitutively activated p44 MAP kinase may play an important role in the induction of radioresistance during fractionated radiotherapy of cancer and may serve as a promising target for specific inhibitors to enhance the efficacy of radiotherapy. J. Cell. Biochem. 101: 745–752, 2007.

Key words: fractionated irradiation; p44 MAP kinase; p38 MAP kinase; p54 SAP kinase and PAC1

Cellular response to ionizing radiation is a complex phenomenon where the dose, dose rate and fractionation play an equally important role in deciding the fate of the cell. Chronic exposure of cells to ionizing radiation induces an adaptive response that results in the increased tolerance to subsequent cytotoxicity caused by the same [Russell et al., 1995; Dahlberg et al., 1999; Pearce et al., 2001]. Evidence from a number of studies has indicated that a potential cause of radiation treatment failure may be that multifraction irradiation selects a population of radiation resistant cells from which regrowth of tumor progresses [Weichselbaum et al., 1988, Joiner, 1994; Henness et al., 2002]. Several reports demonstrate the isolation of clones of cells from human tumors that are distinctly radioresistant compared to other clones of the same tumor [Leith et al., 1982; Weichselbaum et al., 1988; Yang et al., 1990]. An understanding of how resistance is promoted at the molecular level can form the basis for new treatment strategies.

Extensive work has been done and published on the response of the cell to single doses of radiation, which lead to lesions in DNA and other cellular macromolecules [Dent et al., 2003; Li et al., 2006; Quick and Gewirtz, 2006]. Among the latter are the signaling proteins that act to signal the damage as well as to regulate processes such as cell-cycle progression and DNA repair. These pathways are also intricately linked with the intrinsic radioresistance of the cell and are being exploited as targets to enhance tumor radiosensitivity [Mackay and Twelves, 2003; Jameel et al., 2004; Belka et al., 2004; Osaki et al., 2004]. The targets have been chosen based on their activation following a single dose of radiation. However, more information about the response of these signaling factors at clinically relevant doses following a

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fractionated regimen is needed to understand their role, if any, in the development of a radioresistant phenotype. Mitogen-activated protein (MAP) kinases are known to play a major role in cellular radiation responses [Dent et al., 2003]. The three major MAP kinases, p44/42 MAP kinase, stress-activated protein (SAP) kinase, and p38 MAP kinase lie at the crossroads of many pathways and control complex processes like proliferation, differentiation, death, etc. and are potential targets for pharmacological intervention and specific inhibitors have been identified [English and Cobb, 2002]. There is evidence that inhibition of p44 MAP kinase leads to increased susceptibility of cells to radiation [Watanabe et al., 2004; Kobayashi et al., 2005]. The present study was therefore conducted to unravel how the MAP kinases respond to different irradiation regimens with an aim to identify markers of radioresistance which may serve as future targets for modulation to enhance the efficacy of radiotherapy. Additionally, the knowledge of the time of activation of the target signaling molecules and in vivo biological half life of inhibitors will help in determining the time of administration of the drug and maximizing its efficacy.

Moreover, most of the data on the radiobiological parameters involved in fractionated irradiation are derived from established cell lines and rarely does the clinical response of the patients to radiotherapy correlate with in vitro biological parameters [Weichselbaum et al., 1986]. This is because in vivo tumors contain a large fraction of quiescent or very slowly proliferating cells and hence respond very differently as compared to in vitro [Dahlberg et al., 1999; Sugie et al., 2006]. The present study was therefore deliberately restricted to the in vivo condition and a fibrosarcoma that grows in the gastrocnemius muscle of the mice was the system of choice.

MATERIALS AND METHODS

Mice

Male Swiss mice (6–8 weeks old), weighing 20–24 g, were maintained on a standard laboratory diet with water ad libitum. They were reared in air-conditioned ($24 \pm 2^{\circ}C$) rooms with a 12 h dark and light schedule. They were housed in sterilized polypropylene cages (4–5 mice/cage) with sterilized paddy husk. Mice used in the present study were a part of conventional inbred colony of Swiss mice maintained at the animal house facility of Bhabha Atomic Research Centre.

Tumor

A serially transplanted fibrosarcoma developed by subcutaneous injection of 6,12-dimethylbenzo[1,2-b,5,4–6′]dithionapthene into male Swiss mice was used as the test system [Waravdekar and Ranadive, 1957]. For experiments, tumors were grown in the gastrocnemius muscles of left hind leg of 8 weeks-old male Swiss mice weighing 20–24 g, by injecting intramuscularly 0.5×10^6 viable tumor cells suspended in sterile phosphate-buffered saline (PBS) in a volume of 0.2 ml using 26-guage needle. Tumor bearing animals were used for experiments when the tumors reached a mean volume of 150 ± 50 mm^3.

Irradiation

Unanesthetized animals were restrained in specially designed well-ventilated acrylic boxes for local irradiation of the leg. The tumors were locally irradiated at a dose rate of 0.51 Gy/min using a ^{60}Co Junior Theratron unit (Atomic Energy of Canada Ltd.) while the rest of the animal body was shielded by lead jigs designed for the purpose. Tumor bearing mice were divided into four groups, each consisting of 18 animals (sham-irradiated group had 3 animals). While (a) controls were sham irradiated, the rest were subjected to (b) 2 Gy of ^{60}Co γ-irradiation daily over a period of 5 days (fractionated irradiation) (c) 10 Gy acute dose (which is the sum total of all the fractions, i.e., 2 Gy $\times$ 5), or (d) 2 Gy acute dose (single dose that was delivered in each fraction) of ^{60}Co γ-irradiation. The 2 Gy dose was included to compare the effect of a single fraction that was delivered with the effect of the total fractionated regimen and the 10 Gy acute dose, which is the sum total of all the fractions delivered.

Immunoblotting

Radial sections of the tumors were taken and a single-cell suspension was made in ice cold PBS by mechanical disruption using scissors. The cells were subsequently washed with ice cold PBS at 2,000 rpm for 5 min and lysed in boiling lysis buffer (50 mM Tris-Cl, pH 6.8; 2% SDS and 20% glycerol without Bromophenol blue). Protein estimation was done by BCA

method using protein estimation kit (Bangalore Genei, India) and separated on 8% SDS–PAGE followed by transfer to nitrocellulose membrane and probing with specific antibody. Among the antibodies used, anti-p44/42 MAP kinase, anti-phospho SAP kinase and anti-phospho p38 MAP kinase were from Cells Signaling Technology (USA). Anti-phospho p44/42 MAP kinase was from Calbiochem (USA). Anti-SAP kinase, anti-p38 MAP kinase, and anti-actin antibodies were from Sigma (USA). Bands were detected using secondary antibodies and reagents from Lumi-Light plus Western blotting kit (Roche, Germany) and quantification was done using Gelquant (version 2.7) software. The data were analyzed using one-way ANOVA.

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) constituted by the Animal Welfare Division of Government of India on use of animals in scientific research.

RESULTS

To determine the temporal response of MAP kinases the animals were sacrificed at various time periods (1 h, 3 h, 7 h, 1 day, 3 days, and 7 days) post-irradiation. In the fractionated group also, the animals were sacrificed at the above time periods but after they had received the last fraction of the dose, that is, on the fifth day.

The 10 Gy acute dose could be followed up to 3 days only because the animals did not survive beyond that time. In the fractionated group p44 MAP kinase showed an increase in phosphorylation at 1 h which peaked at 3 h (Fig. 1A, Lanes 2–3 and Fig. 2, Lanes 2–3). There was a subsequent decline in the phosphorylated p44 MAP kinase levels at 7 h (Fig. 1A and Fig. 2, Lane 4) which, however, increased to peak again at 1 day (Fig. 1A and Fig. 2, Lane 5) and 7 days (Fig. 1A and Fig. 2, Lane 7). The response of p44 MAP kinase, therefore, exhibits an overall increase in phosphorylation with intermittent peaks of activity. This activation is sustained right up to the seventh day after the last dose. With the 10 Gy acute dose, there was an initial decrease in the phosphorylation of p44 MAP kinase at 1 h (Fig. 1A and Fig. 2, Lane 8) as compared to the sham-irradiated control (Lane 1), thereafter a transient increase was observed which is maximum at 7 h post-irradiation (Lane 10). The levels were below control at all later time points. On acute irradiation with 2 Gy, phosphorylation of p44 MAP kinase decreased below control levels at 1 h post-irradiation (Fig. 1A, Lane 2 and Fig. 2, Lane 13). However, it increased thereafter at 3 and 7 h post-irradiation (Fig. 1A, Lanes 3–4 and Fig. 2, Lanes 14 and 15) again going down below control levels beyond 7 h up to 3 days. A strong peak reappeared at 7 days post-irradiation (Fig. 1A, Lane 7 and Fig. 2, Lane 18).

Phosphorylated p38 MAP kinase levels decreased below control at 1 and 3 h after fractionated irradiation (Fig. 1B, Lanes 1–3 and Fig. 3, Lanes 1–3). Thereafter, it remained low but for partial recoveries at 7 h and 7 days post-irradiation (Fig. 1B and Fig. 3, Lanes 4 and 7, respectively). Following an acute dose of 10 Gy, p38 MAP kinase decreases strongly at 1 h (Fig. 1B and Fig. 3, Lane 8) as compared to sham-irradiated control (Lane 1). Thereafter there was a slight resurgence at 3–7 h (Fig. 1B and Fig. 3, Lanes 9 and 10) following which it fell back to very low levels till 3 days (Fig. 1B and Fig. 3, Lanes 11 and 12).

For 2 Gy acute dose, there was no change in phosphorylated p38 MAP kinase at 1 h (Fig. 1B, Lane 2 and Fig. 3, Lane 13) with a subsequent increase at 3 h (Fig. 1B, Lanes 3 and Fig. 3, Lanes 14), thereafter it reduced below control levels (Fig. 1B, Lanes 4–7 and Fig. 3, Lanes 15–18).

Fractionated irradiation led to a continual increase in the phosphorylated p54 SAP kinase levels, which is a stress-activated kinase, at 1 h till 1 day (Fig. 1C, Lanes 1–5 and Fig. 4, Lanes 1–5). Subsequently, it disappeared at all later time periods till 7 days (Fig. 1C and Fig. 4, Lanes 6 and 7). With an acute dose of 10 Gy, the response was biphasic, with a steady increase till 7 h post-irradiation (Fig. 1C and Fig. 4, Lanes 8–10) followed by a decline at 1 day (Lane 11) and again a revival at 3 days (Lane 12) post-irradiation. For 2 Gy acute dose, a very rapid increase in the phosphorylated p54 SAP kinase was observed at 1 h (Fig. 1C, Lane 2 and Fig. 4, Lane 13) with a gradual decrease to control levels by 1 day (Fig. 1C, Lanes 3–5 and Fig. 4, Lanes 14–16). Although there was some similarity in the response of phosphorylated SAP kinase to fractionated and 10 Gy acute irradiation, the response to 2 Gy acute was totally different. Therefore the magnitude and

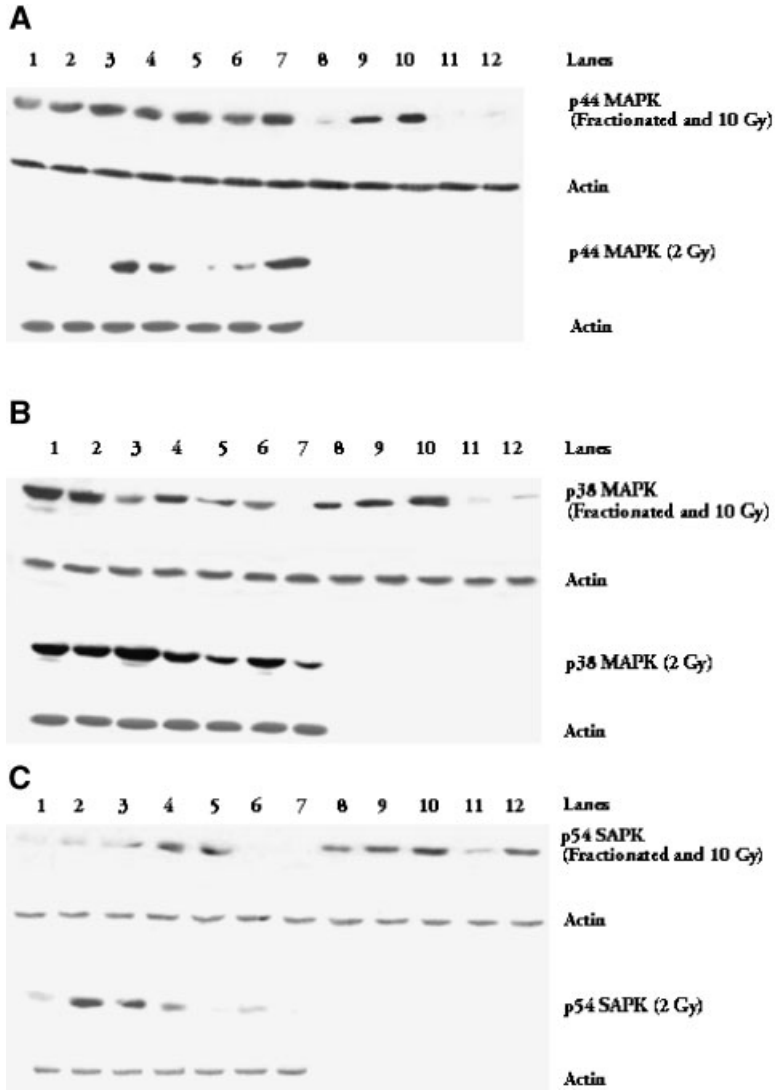

Fig. 1. Effect of fractionated and acute irradiation of mouse fibrosarcoma on the temporal activation of MAP kinases. Tumor bearing mice were irradiated as mentioned in the text, mice were sacrificed at different time periods, lysates prepared and separated on 8% SDS–PAGE and probed with specific antibody. (**A**) Immunoblot of phosphorylated p44 MAP kinase following fractionated and 10 Gy acute dose (**Lanes 1–12**) and immunoblot of p44 MAP kinase following 2 Gy acute dose (Lanes 1–7), (**B**) Immunoblot of phosphorylated p38 MAP kinase following fractionated and 10 Gy acute dose (Lanes 1–12) and immunoblot of p38 MAP kinase following 2 Gy acute dose (Lanes 1–7), and (**C**) Immunoblot of phosphorylated p54 SAP kinase following fractionated and 10 Gy acute dose (Lanes 1–12) and immunoblot of p54 SAP kinase following 2 Gy acute dose (Lanes 1–7). Key: Lane 1: Sham-irradiated control, Lanes 2 and 8: 1 h post-irradiation, Lanes 3 and 9: 3 h post-irradiation, Lanes 4 and 10: 7 h post-irradiation, Lanes 5 and 11: 1 day post-irradiation, Lanes 6 and 12: 3 days post-irradiation, Lane 7: 7 days post-irradiation. In fractionated and 10 Gy: Lanes 2–7: Fractionated irradiation, Lanes 8–12: 10 Gy acute irradiation.

duration of the stress elicited a differential response in the case of p54 SAP kinase.

The 3 h point was the time at which p44 MAP kinase showed its first peak for both fractionated and acute irradiation. This time point was chosen to study the induction of phosphatase of activated cells 1 (PAC1) which specifically dephosphorylates p44/42 MAP kinase and is itself induced by activated p44/42 MAP kinase [Ward et al., 1994; Yin et al., 2003; Zhang et al., 2005]. Fractionated irradiation lead to a significant increase in PAC1 at 3 h post-irradiation (Fig. 5A,B, Lane 2) while there was no change in case of 10 and 2 Gy acute doses (Lanes 3 and 4).

DISCUSSION

Following fractionated irradiation p44 MAP kinase showed a biphasic response and the

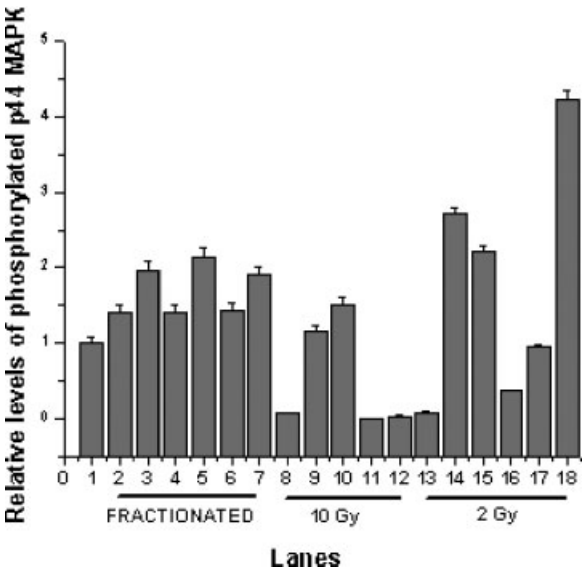

Fig. 2. Effect of fractionated and acute irradiation of mouse fibrosarcoma on the temporal activation of p44 MAP kinase. The Western blots were quantified using Gelquant (version 2.7) software and plotted. Data represent mean ± SE of three independent experiments. Key: **Lane 1**: Sham-irradiated control, **Lanes 2, 8**, and **13**: 1 h post-irradiation, **Lanes 3, 9**, and **14**: 3 h post-irradiation, **Lanes 4, 10**, and **15**: 7 h post-irradiation, **Lanes 5, 11**, and **16**: 1 day post-irradiation, **Lanes 6, 12**, and **17**: 3 days post-irradiation, **Lanes 7** and **18**: 7 days post-irradiation. Lanes 2–7: fractionated irradiation, Lanes 8–12: 10 Gy acute irradiation, Lanes 13–18: 2 Gy acute irradiation. $P < 0.01$ compared to control for all lanes except for Lane 9.

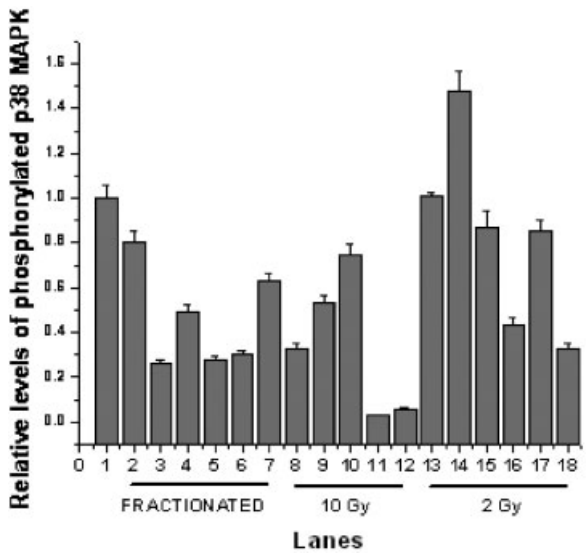

Fig. 3. Effect of fractionated and acute irradiation of mouse fibrosarcoma on the temporal activation of p38 MAP kinase. The Western blots were quantified using Gelquant (version 2.7) software and plotted. Data represent mean ± SE of three independent experiments. Key: **Lane 1**: Sham-irradiated control, **Lanes 2, 8**, and **13**: 1 h post-irradiation, **Lanes 3, 9**, and **14**: 3 h post-irradiation, **Lanes 4, 10**, and **15**: 7 h post-irradiation, **Lanes 5, 11**, and **16**: 1 day post-irradiation, **Lanes 6, 12**, and **17**: 3 days post-irradiation, **Lanes 7** and **18**: 7 days post-irradiation. Lanes 2–7: fractionated irradiation, Lanes 8–12: 10 Gy acute irradiation, Lanes 13–18: 2 Gy acute irradiation. $P < 0.01$ compared to control for Lanes 3–12, 14, 16, and 18. $P < 0.05$ compared to control for Lanes 2 and 17.

activation was sustained right up to the last time point studied, that is, 7 days post-irradiation and may be even beyond that (Fig. 2, Lanes 1–7). Since p44 MAP kinase is predominantly a prosurvival factor, this is a clear survival response elicited by repeated doses of γ-irradiation. In case 10 Gy acute dose, which is the sum total of all the fractions given (2 Gy per day over 5 days), the cells elicited a response contrary to that of fractionated and appeared to be an inhibition of p44 MAP kinase. This may push the balance towards cell death. The response to 2 Gy acute dose which is equal to a single dose of the fractionated regimen was similar to that observed with fractionated but was not sustained at all the time points as with the fractionated regimen. The common features of the response to acute doses were an initial decrease in the phosphorylation at 1 h with a subsequent transient increase which was less in magnitude in case of the higher dose. Repeated irradiations, as in case of the fractionated regimen, obliterated the initial decrease and lead to a sustained activation of p44 MAP kinase and in turn a possible prosurvival response. These facts, when put together, implicate p44 MAP kinase as a possible mediator of induced radioresistance in the tumor cells. Whether and how this sustained activation of p44 MAP kinase contributes to the development of radioresistance needs to be investigated.

A crucial role in mediating responses to cellular stress is played by p38 MAP kinase [Dent et al., 2003]. The kinase has been shown to be involved in both death and survival signaling with a lot depending upon the cell type [Juretic et al., 2001; Liu, 2001]. The fibrosarcoma showed a high basal activity of p38 MAP kinase. The ability of ionizing radiation to activate p38 MAP kinase has been reported to be either weak or nonexistent [Dent et al., 2003]. However, p38 MAP kinase is known to play a role in radiation-induced apoptosis [Kumar et al., 2004; Choi et al., 2006]. Therefore, the downregulation of p38 MAP kinase observed may also lead to a

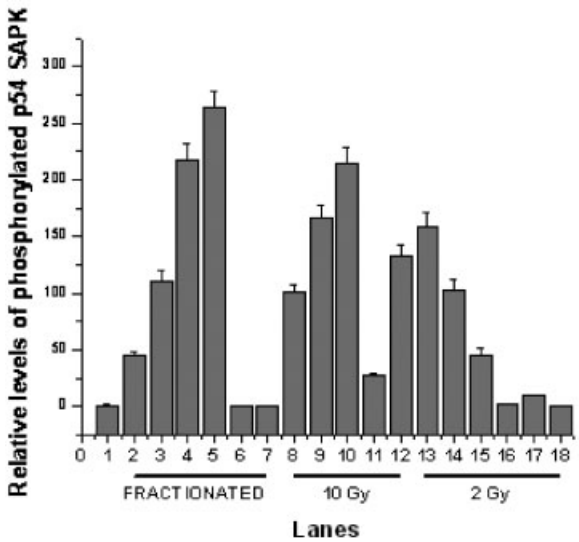

Fig. 4. Effect of fractionated and acute irradiation of mouse fibrosarcoma on the temporal activation of p54 SAP kinase. The Western blots were quantified using Gelquant (version 2.7) software and plotted. Data represent mean ± SE of three independent experiments. Key: **Lane 1**: Sham-irradiated control, **Lanes 2, 8**, and **13**: 1 h post-irradiation, **Lanes 3, 9**, and **14**: 3 h post-irradiation, **Lanes 4, 10**, and **15**: 7 h post-irradiation, **Lanes 5, 11**, and **16**: 1 day post-irradiation, **Lanes 6, 12**, and **17**: 3 days post-irradiation, **Lanes 7** and **18**: 7 days post-irradiation. Lanes 2–7: fractionated irradiation, Lanes 8–12: 10 Gy acute irradiation, Lanes 13–18: 2 Gy acute irradiation. $P < 0.01$ compared to control for all lanes except for Lane 6, 7, 16, and 18.

decrease in apoptosis and indirectly play a role in enhanced survival and radioresistance of tumor cells.

MAP kinase pathways are known to regulate the fate of the cell following exposure to ionizing radiation wherein the prosurvival p44 MAP kinase acts antagonistically to the proapoptotic SAP kinase and the balance between the two determines the cells destiny [Dent et al., 2003]. The response of p54 SAP kinase, which is activated by various stresses, to fractionated irradiation was a steady increase in phosphorylation right up to 1 day post-irradiation (Fig. 4). This is unlike the much shorter periods of activation for both 10 and 2 Gy acute doses. For 10 Gy acute dose there is a reactivation at 3 days post-irradiation which coincides with a decrease in p44 MAP kinase at the same time point. Therefore, at the higher acute dose the cell is definitely pushed towards a death response due to the equilibrium shifting towards SAP kinase. However, in fractionated irradiation, although there is a much stronger response of p54 SAP kinase as compared to acute, the sustained activation of p44 MAP kinase may drive the cells towards survival once the SAP kinase levels have come back to normal after 1 day post-irradiation.

The activation of PAC1, an inducible dual specificity phosphatase, is induced by activated p44/42 MAP kinase and requires the binding of the same in order to be activated [Tamura et al., 2002; Zhang et al., 2005]. It is known to specifically dephosphorylate and inactivate p44/42 MAP kinase, acting as a negative feedback regulator [Ward et al., 1994; Tamura et al., 2002]. Fractionated irradiation increased the levels of PAC1 at 3 h post-irradiation while it is unaffected by the acute doses (Fig. 5). This indicated that the sustained and immediate activation of p44 MAP kinase by fractionated irradiation lead to the induction of PAC1 which,

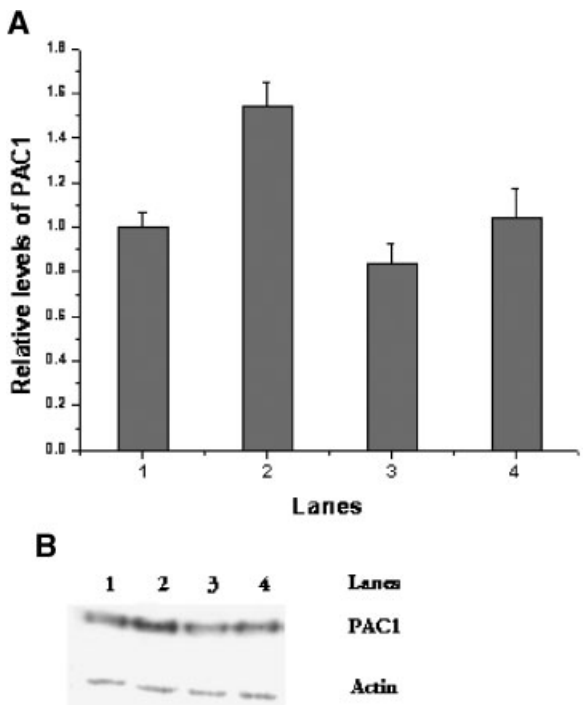

Fig. 5. Effect of fractionated and acute irradiation of mouse fibrosarcoma on PAC1. Tumor bearing mice were irradiated as mentioned in the text, mice were sacrificed at different time periods, lysates prepared and separated on 8% SDS–PAGE and probed with specific antibody. The Western blots were quantified using Gelquant (version 2.7) software and plotted. (**A**) Plot and (**B**) Western blots showing protein levels of PAC1. Data represent mean ± SE of three independent experiments. Key: **Lane 1**: Sham-irradiated control, **Lane 2**: fractionated irradiation (2 Gy × 5 days), **Lane 3**: 10 Gy acute dose, **Lane 4**: 2 Gy acute dose. $P < 0.01$ compared to control for Lane 2.

in turn, acted as a feedback regulator to regulate the extent of activation of the former. Therefore, the degree of activation of p44 MAP kinase in case of fractionated irradiation is not as much as in 2 Gy acute dose (Fig. 2).

The study clearly indicates that there are significant differences in response to fractionated and acute irradiation of the mouse fibrosarcoma in vivo. Repeated doses of ionizing radiation lead to an adaptive response in the cells wherein a sustained activation of the prosurvival p44 MAP kinase was observed which was balanced up to 1 day post-irradiation by the activation of the proapoptotic SAP kinase. Thereafter the direction of the equilibrium shifted towards p44 MAP kinase which, however, was tightly regulated by the dual specificity phosphatase PAC1. The third MAP kinase p38 appeared to be inhibited by fractionated irradiation.

These studies point towards the fact that assumptions based on a single dose of irradiation could be erroneous in case of testing of specific modulators of MAP kinases with the view of improving the efficacy of radiotherapy. The expression pattern varies both with the radiation dose as well as the fractionation of the dose. The constitutively activated p44 MAP kinase may play an important role either by itself or by inducing other factors in conferring radioresistance to the tumor cells following repeated irradiation as occurs in fractionated radiotherapy and may serve as a promising target for specific inhibitors to increase the effectiveness of subsequent doses delivered in radiotherapy of cancer. Alternatively, any means of sustained upregulation of p54 SAP kinase and PAC1 may be a possible approach.

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