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Role of nuclear factor-κB and P53 in radioadaptive response in Chang live cells

Dexiao Yuan, Yan Pan, Jianghong Zhang, Chunlin Shao*

Institute of Radiation Medicine, Fudan University, Shanghai 200032, China

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ABSTRACT

Understanding the mechanism governing radioadaptive response (RAR) has important implication for cancer risk assessment of a low-dose radiation (LDR). However the related knowledge especially the key gene of RAR is still limited. In this study, Chang liver cells were irradiated with a priming dose of 0.016 Gy, 0.08 Gy, or 0.16 Gy of γ-rays, and with 4 h interval, they were irradiated again with a challenging dose of 2 Gy or 3 Gy. It was found that only 0.08 Gy, but not 0.016 Gy or 0.16 Gy, induced RAR of micronuclei induction to the challenging irradiation. This RAR could be slightly reduced by pifithrin-α, an inhibitor of P53, however it was completely suppressed by BAY11-7082, an inhibitor of nuclear factor-κB (NF-κB). Further assays using western blotting and luciferase reporter gene found that nuclear NF-κB and its activity could be triggered by the priming irradiation of 0.08 Gy so that the expressions of them in the primed cells were higher than those in the cells exposed to the challenging dose alone. In contrast, LDR neither influenced the expressions of both P53 and phospho-P53 (ser15) nor enhanced P53 activity; the expression of phospho-P53 and the activity of P53 in the primed cells were lower than that in the non-primly challenged cells. Our results demonstrate that the induction of RAR relays on an optimum priming irradiation dose and it is NF-κB rather than P53 that contributes to RAR.

1. Introduction

Currently, the biological effects of a low-dose radiation (LDR) are commonly estimated by using the theory of linear-non-threshed model and extrapolating the data obtained from high-dose exposure. However, the validity of this dose–response model is controversial because evidence accumulated over past decade has indicated that cells respond to LDR differently from that they do to high-dose radiation [1]. One phenomenon is radioadaptive response (RAR), a biological defense reaction of which a LDR induces cellular resistance to the genotoxic effects of subsequent challenging irradiation. The first report of RAR is that the incorporation of low concentration of radioactive thymidine reduced the chromosomal aberration due to subsequent high-dose irradiation [2]. Since then, RAR has been observed by many laboratories in a variety of systems *in vivo* and *in vitro* [3–8]. It is known that RAR has high degree of inter- and intra-individual variability so that even a similar experimental protocol may yield different responses, which may because of various factors such as cell type [9], genetic variation [10], dose rate [11], and the endpoint used for radiation response assay [12].

It is well known that cancerous cells often bear mutated p53 that leaves the cell with an unbalanced pro-survival signal and favors both the occurrence of mutation and the development of radioresistance. A better understanding of the role of p53 in RAR may provide deep insights for understanding the phenomenon of tumor radioresistance. The elevated level of NF-κB activity in certain cancers has been linked with tumor resistance to radiation and chemotherapy [13,14]. For instance, it has been found that the fractional irradiation enhanced NF-κB activation can be associated with an enhanced clonogenic survival of breast cancer cells [15]. Blocking NF-κB activation increased radiation-induced apoptotic response and decreased clonogenic survival of several human cancer cell lines [16–18]. Obviously, NF-κB plays a key role in tumor radioresistance.

Up to now, the molecular mechanism of RAR is still not well known. The characteristics of RAR include surviving better and less induction of apoptosis. These two endpoints are closely associated with NF-κB and P53, respectively, the activation of NF-κB can enhance cell survival [13] and the activation of P53 results in the progression of apoptosis [19]. In this study, the roles of NF-κB and P53 in the induction of RAR were investigated.

2. Materials and methods

2.1. Cell culture and irradiation

Chang liver cells (Shanghai Cell Bank), immortalized but non-tumorigenic human cells, were maintained in DMEM medium (Gibco, Hangzhou) supplied with

Abbreviations: RAR, radioadaptive response; LDR, low-dose radiation; NF-κB, nuclear factor-κB; PFT-α, pifithrin-α; BAY, BAY 11-7082; MN, micronuclei.

* Corresponding author. Tel.: +86 21 64048677; fax: +86 21 64048677.
E-mail address: clshao@shmu.edu.cn (C. Shao).

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10% fetal bovine serum (Gibco Invitrogen, CA), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin and cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C. The cells were propagated every other day and reseeded at 5×10^5 cells per T-25 flask.

One day before irradiation, 1.5×10^5 cells were seeded in a ϕ60 mm Petri dish. The cells were irradiated at room temperature with a priming dose of 0.016 Gy, 0.08 Gy, or 0.16 Gy (dose rate 0.16 Gy/min) of γ-rays that were generated by a ^{137}Cs gamma irradiator (Gammacell-40, MDS Nordian, Canada). With an interval time of 4 h that has been widely used for radioadaptive study [20], some of the cells were further irradiated with a challenging dose of 2 Gy or 3 Gy at a dose rate of 0.8 Gy/min for the investigation of RAR. For the non-primly challenged cells, the cells were just irradiated with a single dose of 2 Gy or 3 Gy. Sham-irradiated cells were used as control.

2.2. Chemical treatment

Pifithrin-α (PFT-α) is a reversible inhibitor of p53-mediated apoptosis and p53-dependent gene transcription such as the expressions of cyclin G, p21/waf1, bax, and mdm2. PFT-α can enhance cell survival after genotoxic stress such as UV irradiation and treatment with cytotoxic compounds [21]. BAY 11-7082 (BAY) is an irreversible inhibitor of IκB phosphorylation, which results in the inactivation of NF-κB [22]. In this study, exponentially growing cells were treated with 20 μM PFT-α (Sigma) or 3 μM BAY (Sigma) 1 h before priming irradiation to inhibit the activation of P53 or NF-κB, respectively. The drug was well washed away after priming IR or kept until challenging irradiation.

2.3. Cell growth assay

Radiation-induced cell growth suppression was evaluated by cell proliferation assay. 2.5×10^5 cells per flask were plated 1 day before irradiation, and the cells were irradiated with different doses of γ-rays at room temperature. Two days after irradiation, cell number in each group was measured using a cell counter (Beckman counter Z2, USA) and the relative cell growth rate was calculated by normalizing the cell number to the control without irradiation.

2.4. Micronuclei and binucleation assay

As a biological endpoint of adaptive response, micronuclei (MN) were measured with the cytokinesis-block technique. Briefly, after final irradiation, cells were treated with 1.0 μg/ml cytochalasin-B (Sigma Co.) for 24 h followed by 0.075 M KCl hypotonic treatment for 1–3 min and then fixed *in situ* with methanol-acetic acid (9:1, v/v) for 30 min. Air-dried cells were stained with 0.01% acridine orange for 5 min. MN were scored in at least 1000 binucleated cells each sample under a fluorescence microscope. The MN yield, Y_{MN}, was calculated as the ratio of the number of MN to the scored number of binucleated cells. Since the MN formation is strongly dependent on the cell proliferation, the binucleation index, i.e. the percentage of binucleated cells in the population was also measured on the slides in 1000 cells per experiment and point.

2.5. Reporter gene transfection and luciferase activity assay

Cells in 80–90% confluent growing on a 35 mm dish were co-transfected with the firefly luciferase reporter of NF-κB or P53 containing a TA promoter (pNFκB-TA-luc, pp53-TA-luc, Beyotime Biotechnology, China) (1 μg) along with the Renilla luciferase reporter (Promega Co.) (0.1 μg) for 6 h using a lipofectamine reagent (Invitrogen) according to the protocols supplied by manufacturers. Some cells were further incubated with 20 μM PFT-α or 3 μM BAY for 1 h before the priming irradiation. Twenty-four hours after the final irradiation, the luciferase activity was measured in the cellular extracts using a dual luciferase reported gene assay kit (Beyotime Biotechnology, China). Briefly, the relative fluorescence light unit (RLU) at 560 nm of the mixture of 50 μl total cell lysate and 100 μl of the firefly luciferase assay reagent was measured by a multimode microplate readers (TECAN Infinite M200, Switzerland) for a total period of 10 s. Then 100 μl of Renilla luciferase assay reagent was added into the above mixture and its fluorescence at 465 nm was measured. The relative activity of reporter gene was calculated by dividing RLU at 560 nm into that at 465 nm.

2.6. Nuclear protein extract

Nuclear NF-κB protein was isolated by the method described before [23] with a few modifications. Briefly, 4 h after the final irradiation, cells were washed triply with ice-cold PBS in a 15-ml conical tube and resuspended in 5 pellet cell volumes (PCV) of nuclei wash buffer (10 mM HEPES, pH 7.9, 10 mM Tris.60 mM KCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF) with 0.25% NP40. The cells were pipetted up and down several times until the cell membrane in over 90% of cells were broken, then the nuclei were centrifuged for 1 min at 12,000 rpm and suspended in 5 PCV of ice-cold nuclei wash buffer, and re-centrifuged at 12,000 rpm for 1 min and resuspended in 1 PCV of nuclear extract buffer (250 mM Tris, pH 7.8, 60 mM KCl, 1 mM DTT, and 1 mM PMSF). The nuclei were lysed by three cycles of freeze–thaw and vortex, and then DNA and nuclear debris were removed by centrifugation. The concentration of obtained nuclear protein was determined using the BCA Protein Assay kit (Beyotime Biotechnology, China), and the nuclear extracts were aliquoted and stored at −80 °C until ready for further use.

2.7. Western blot assay

After denatured at 100 °C for 10 min, aliquots of protein (30 μg) were electrophoresed on 10% polyacrylamide gel using an electrophoresis cell tank (BIO-RAD Laboratories, Inc.). After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore Corporation). This membrane was blocked for 1 h with 5% skim milk in Tris-buffered saline/Tween 0.05% (TBST) and then incubated with a primary antibody of anti-P53 antibody (1:1000, Beyotime Biotechnology, China), anti-phospho-P53 (ser15) (1:1000, Cell signaling, USA), anti-NF-κB (1:1000, Santa Cruz Biotechnology, Inc.), and anti-tubulin (Santa Cruz Biotechnology, Inc.). After 2–3 h at room temperature, the membrane was triply washed with TBST at room temperature for 10 min and treated with a peroxidase-conjugated secondary antibody (1:10,000, Beyotime Biotechnology, China) for 1 h. After several washes, proteins in the membrane were detected by the enhanced chemiluminescence system (ECL advance, Amersham Biosciences) and the protein image was recorded and analyzed by the BIO-RAD ChemiDoc XRS system.

2.8. Statistical analysis

Data were resulted from at least three independent experiments and three to four replicates in each experimental point were counted for MN determination. All data are presented as means ± SE. Significance was assessed using a two-tailed Student's *t*-test. The criterion for significance [α] was set at 0.05, thus, data with a $P < 0.05$ was considered as significantly different.

3. Results

3.1. Effect of radiation on cell growth and MN formation

Fig. 1 illustrates that, when the cells were irradiated with a high dose of γ-rays, the cell growth and the binucleation index were reduced and the yield of MN was enhanced. But when the radiation dose was less than 0.16 Gy, the cell growth, the binucleation index, and the yield of MN of the irradiated cells had no significant difference to those of nonirradiated control. Accordingly, we chose 0.016 Gy, 0.08 Gy, and 0.16 Gy as priming doses and 2 Gy and 3 Gy as challenging doses for the following study on RAR.

3.2. Radiation-induced adaptive response of MN induction

To know whether an adaptive response can be induced in Chang liver cells, the cells were pretreated with a priming dose of 0.016 Gy, 0.08 Gy, or 0.16 Gy of γ-rays, after 4 h, they were subsequently irradiated with a challenging dose of 2.0 Gy or 3.0 Gy. It was found that the priming irradiation of 0.08 Gy clearly rendered the cells to be less susceptible to the challenging irradiation so that the yield of MN in the 0.08 Gy primed cells was 20.4% and 22.4% less than that in the cells irradiated with 2 Gy and 3 Gy alone, respectively (Fig. 2A). However, the priming doses of 0.016 Gy and 0.16 Gy did not significantly influence the induction of MN in the cells that were challenged with 2 Gy or 3 Gy, which indicates that the priming irradiation of 0.016 Gy and 0.16 Gy could not induce RAR in the Chang liver cells. This result suggests that an optimum dose of the priming irradiation is necessary for the induction of RAR.

To know whether the variation of the yield of MN is due to any change of cell proliferation after the priming irradiation, we analyzed the binucleation index of the cells as well. Fig. 2B shows that, although the binucleation index decreased along with challenge dose, it had similar values among primed cells and non-primed cells and hence was not influenced by the priming irradiation. Moreover, we found that, when the cells were treated with PFT-α and BAY, the cell proliferation was also not influenced by the priming irradiation (data not shown).

3.3. P53 is not necessary for the induction of RAR

To investigate whether P53 is involved in the induction of RAR, we examined the effect of P53 inhibitor on the MN formation and

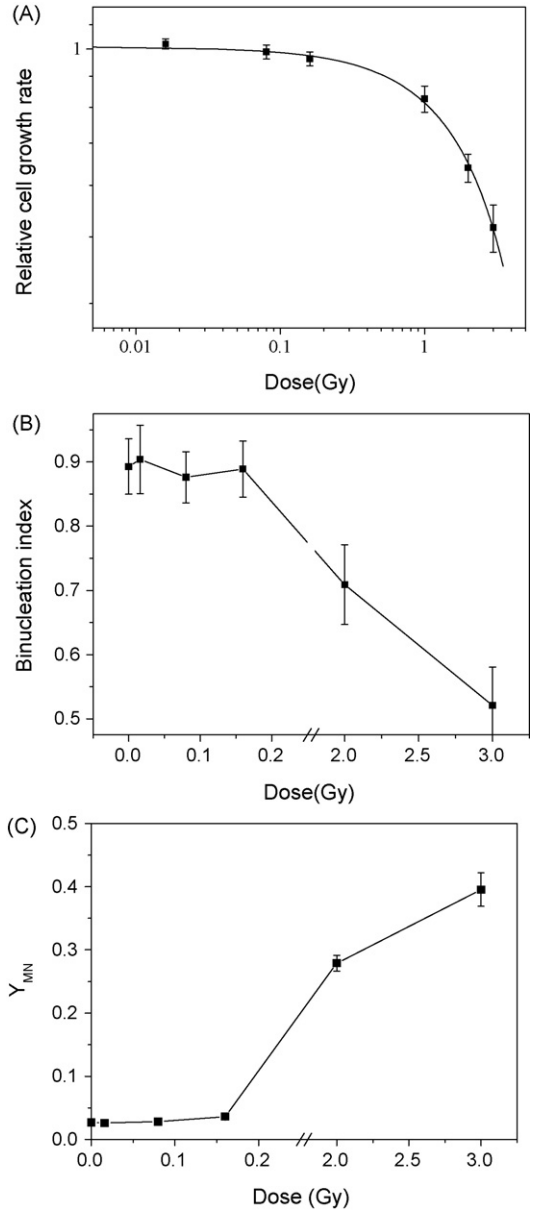

Fig. 1. Dose responses of the relative cell growth rate (plot A), binucleation index (plot B) and micronuclei formation (plot C) of Chang liver cells irradiated with γ-rays. Cell growth rate was normalized to sham-irradiated control.

P53 activation. Fig. 3A illustrates that the treatment with PFT-α decreased the yield of MN in the cells irradiated with 2 Gy or 3 Gy, indicating that p53 is required for the sensitivity of radiation. Moreover, under the situation of PFT-α treatment, the priming irradiation of 0.08 Gy could still suppress the MN induction in the challenged cells although this reduction was less than that without PFT-α treatment. Accordingly, the induction of RAR could not be inhibited by PFT-α.

The result of P53-controlled luciferase reporter assay shows that the P53 function in Chang liver cells was normal. Following to a high-dose irradiation, the cellular P53 activity could be increased but it was remarkably suppressed by the treatment of cells with PFT-α (Fig. 3B). The priming irradiation of 0.08 Gy itself did not change the P53 activity. The P53 activity in the primed cells that exposed with both priming and challenging doses was lower than that in the cells exposed to challenging irradiation alone. These results suggest P53 plays an essential role in the cellular radiosensitivity but is not required for the induction of RAR in Chang liver cells.

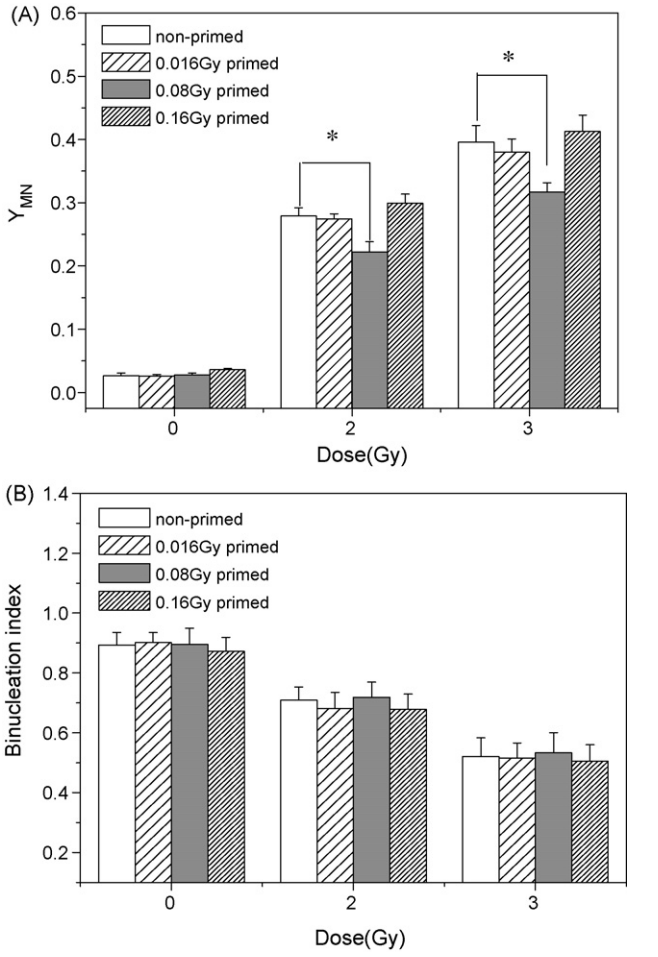

Fig. 2. Micronuclei formation (plot A) and binucleation index (plot B) of Chang liver cells. The cells were irradiated with a priming dose of 0.016 Gy, 0.08 Gy, and 0.16 Gy and further challenged with 2 Gy and 3 Gy. The interval between priming irradiation and challenging irradiation was 4 h. *$P < 0.05$ between primed group and non-primed group.

3.4. NF-κB regulates the induction of RAR

In contrast to the effect of P53 inhibitor, treatment of cells with NF-κB inhibitor of BAY increased MN formation significantly (Fig. 4A). Under this treatment, the priming irradiation of 0.08 Gy could not induce cellular adaptive response but contrarily increased DNA damage when the cells were further challenged with 2 Gy or 3 Gy of γ-rays. Meanwhile, the result of NF-κB-controlled luciferase reporter assay discloses that, after the priming irradiation of 0.08 Gy, the activity of NF-κB was significantly increased (Fig. 4B). In addition, the activity of NF-κB in the primed cells was also higher than that in the non-primed cells. But the activity of this protein could be remarkably suppressed by the NF-κB inhibitor of BAY. Therefore, LDR can trigger cellular resistance to subsequent cytotoxic effect of ionizing radiation through a NF-κB related Pathway.

3.5. Expression of P53 and nuclear NF-κB

To get further evidence supporting the above findings about roles of P53 and NF-κB in RAR, we measured the protein expressions of P53, phospho-P53, and NF-κB in the Chang liver cells under various irradiation conditions by Western blotting. The representative protein expressions were shown in Fig. 5. According to the gray values of protein bands, the semi-quantitative results of the

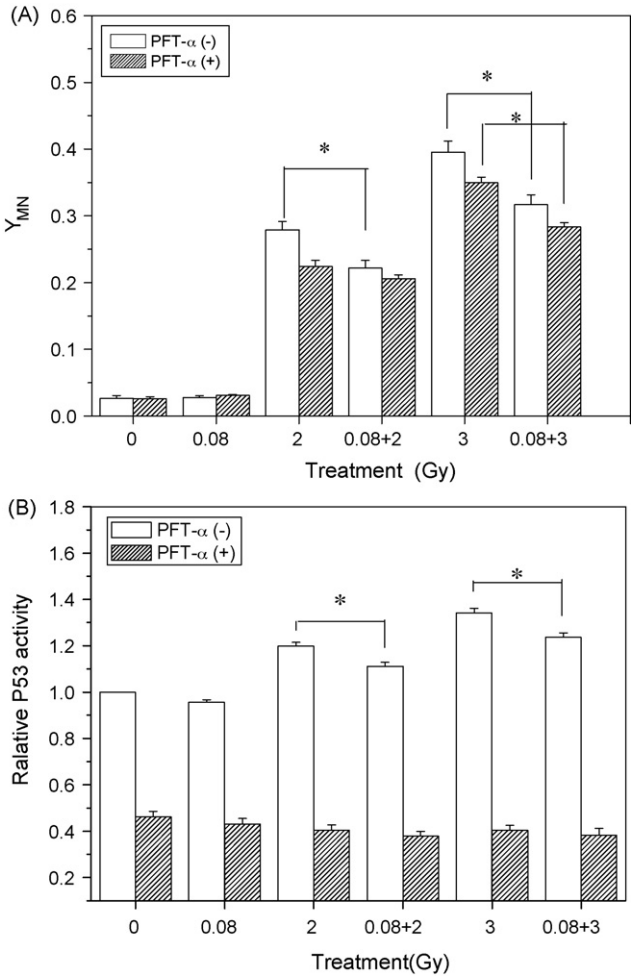

Fig. 3. Influence of PFT-α on the micronuclei formation (plot A) and P53 activity (plot B) of Chang liver cells. Cells were primed with 0.08 Gy of γ-rays and then challenged with 2 Gy or 3 Gy of γ-rays. The interval between two irradiations was 4 h. $^{*}P < 0.05$ between primed group and non-primed group.

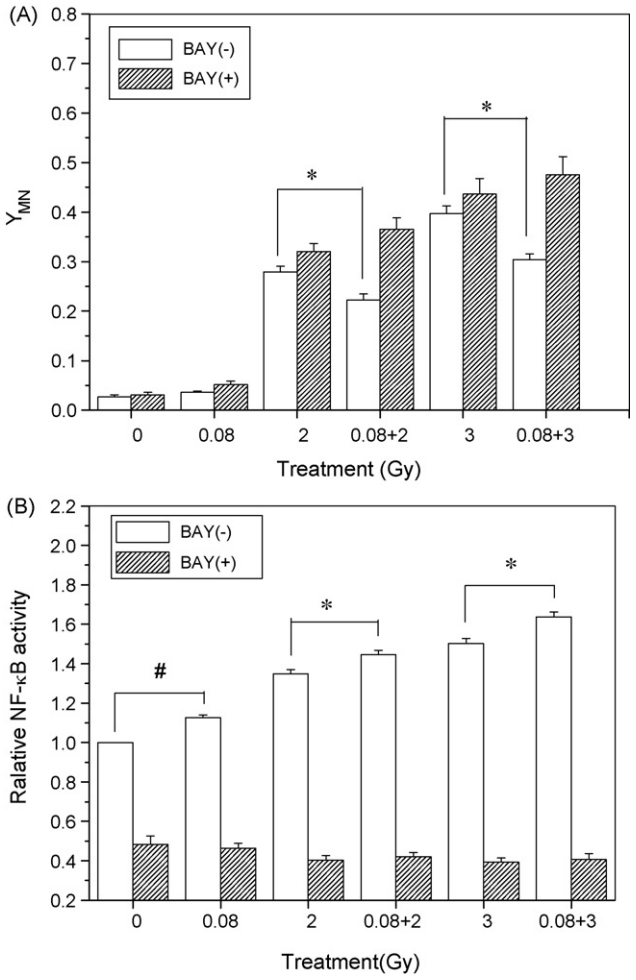

Fig. 4. Influence of BAY 11-7082 on the micronuclei formation (plot A) and NF-κB activity (plot B) of Chang liver cells. Cells were primed with 0.08 Gy of γ-rays and then challenged with 2 Gy or 3 Gy of γ-rays. The interval between two irradiations was 4 h. $^{\#}P < 0.05$ compared with sham-irradiated cells. $^{*}P < 0.05$ between primed cells and non-primed cells.

protein expressions were calculated and listed in Table 1 where each protein expression level was first normalized to tubulin and then compared to its sham-irradiated control. It is seen that the expression of P53 was almost not influenced by 0.08 Gy irradiation but could be enhanced by a high-dose irradiation. Under the challenge irradiation, the P53 expression in the primed cells was slightly lower than that in the non-primed cells. In addition, the expression of phosphor-P53 could be more obviously up-regulated by a high-dose irradiation. With an irradiation of 2 Gy and 3 Gy, this expression approached to about 38-folds and 50-folds of non-irradiated control, respectively. However, a priming irradiation of 0.08 Gy decreased the expression of phospho-P53 in these challenged cells. The expression of phospho-P53 in the cells exposed with both priming and challenging doses was lower than that irradiated with a challenging dose alone. With respect to the nuclear NF-κB, we found that its level in nucleus could be aroused up by the priming irradiation of 0.08 Gy so that the expression of nuclear NF-κB in the primed cells was obviously higher than that in the non-primed cells which only received a challenging dose of 2 Gy or 3 Gy.

4. Discussion

It is found here that adaptive response of MN induction could be induced by 0.08 Gy but was not observed in the cells primed with 0.016 Gy and 0.16 Gy, which is consistent with a previous report that the priming doses less than 5 mGy or greater than about 0.2 Gy yield very little if any adaptation [24]. It was believed that the gene expression resulted from a suitable priming irradiation could serve as triggering signals for the adaptation of human lymphocytes against ionizing radiation [25,26]. But if the priming dose is too low, the gene expression profile will not induce any cellular resistance to further high-dose challenging irradiation. On the other hand, radiation can stimulate both pro- and anti- survival responses [27,28].

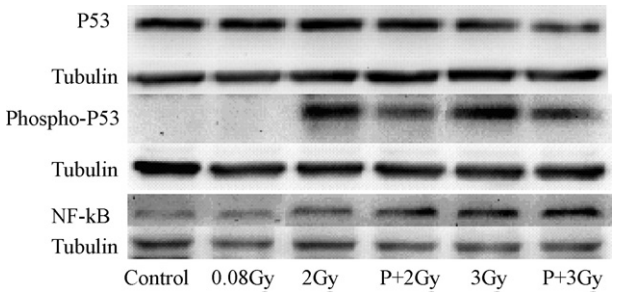

Fig. 5. A representative result of the western blotting assay for the expressions of P53, phospho-P53 (ser15), nuclear NF-κB and tubulin. Proteins were extracted 4 h after the final irradiation under various irradiation conditions.

Table 1
Relative amounts of P53, phospho-P53, and nuclear NF-κB under various irradiation conditions. The proteins were measured 4 h after the final irradiation. Each protein was normalized to tubulin first, then the ratio of each normalized value to its corresponding control was calculated.

	Groups				
	0.08 Gy	2 Gy	0.08 Gy + 2 Gy	3 Gy	0.08 Gy + 3 Gy
P53	1.06 ± 0.21	1.17 ± 0.16	1.13 ± 0.21	1.33 ± 0.19	1.24 ± 0.31
Phospho-P53	1.03 ± 0.25	38.67 ± 3.28	20.12 ± 4.12[b]	59.08 ± 5.21	26.91 ± 4.66[c]
NF-κB	1.34 ± 0.19[a]	4.27 ± 0.72	6.33 ± 1.13[b]	7.71 ± 1.21	10.87 ± 1.32[c]

[a] $P < 0.05$ compared with sham-irradiated group.
[b] $P < 0.05$ compared with 2 Gy irradiation group.
[c] $P < 0.05$ compared with 3 Gy irradiation group.

When the priming dose is too high, the pro-survival response may not sufficient to compensate the anti-survival response so that RAR cannot be induced as well. Our finding suggests that the induction of adaptive response requires an optimum priming dose which may vary in different cell lines.

The present experiments highlighted the role of P53 and NF-κB in the induction of RAR by focusing on three aspects: (i) the effect of the inhibitors of P53 and NF-κB on the induction of RAR, (ii) whether LDR activates P53, NF-κB, (iii) the difference of the expression and activity of P53 and NF-κB between the primed cells and the non-primed cells. It has long been observed that P53 function is closely related to cellular radiosensitivity. For instance, after irradiation, the frequency of chromosome aberration in p53-deficient cells was lower than that in p53 wild cells [29]. Our results show that the treatment of cells with PFT-α decreased the MN formation and slightly weakened the RAR, where the reduction of adaptive response can be due to the decreased radiosensitivity.

The P53-controlled luciferase reporter assay demonstrates that the Chang liver cells have a positive p53 function and that its activity can be enhanced by a high-dose irradiation. Compared to the expression of P53, the activation of P53 such as phosphorylation at serine 15 (Ser-15) is more critical for p53-mediated DNA damage responses [30]. Ser-15 is an evolutionarily conserved residue that can be phosphorylated by several related protein kinases of the ATM family including DNA-PK, ATR, and ATM itself [31–35]. Recent studies have shown that, following to DNA damage, the rapid phosphorylation of human P53 at Ser-15 requires ATM protein kinase [36], which suggests that Ser-15 phosphorylation is a marker of DNA damage. We found here that the expression of P53 and phosphor-P53 (at Ser-15) was not on parallel. The expression of P53 almost remained no difference between the primed cells and the non-primed cells, whereas the phosphor-P53 in the primed cells was extensively suppressed by the priming irradiation. A possible reason of this phenomenon is that the activity of P53 is mainly controlled through post-translational modification [37]. The finding that a RAR-inducible priming dose down-regulates the expression of phospho-P53 indicates that there is no inevitable linkage between P53 and RAR induction in Chang liver cells. Although the P53-independent RAR was also reported by other investigators [12,38], there are articles suggesting that the induction of RAR requires an intact P53 response [39–42]. One possible reason of this biphasic response may due to the difference of the experimental protocols used. For example, Rigaud et al. reported that a low-dose exposure could trigger a adaptive response of mutation induction but it could not affect cell survival following to a high-dose challenging irradiation [43].

With respect to the transcription factor of NF-κB, it exists mostly in the cytoplasm as an inactive form which binds with its family inhibitors including IκBa, IκBβ, IκBγ, IκBε, Bcl-3 and the precursor proteins p100 and p105. Under stress conditions, NF-κB can be unbound and moves into the nucleus to regulate the transcription of various protective genes [44–46]. We found here that the expression of nuclear NF-κB and its activity could be aroused by a low-dose irradiation so that they had higher levels in the adapted cells. In fact, accumulating evidence demonstrates that, following to irradiation and anticancer drug treatment, NF-κB has a pro-survival function [45,46]. Radiation-induced activation of NF-κB can also be involved in the cell cycle arrest which allows cell to repair damaged DNA properly [47]. In addition, the constitutive activation of NF-κB can prevent cancerous cells from apoptosis [48,49]. Liu et al found that a severe DNA damage and apoptotic death could be induced in the NF-κB knocked out cells when they were exposed to CSE, a DNA damage reagent [50]. Thus, up-regulation of NF-κB may offer a significant survival advantage leading to the decreased cell damage, and a pro-survival pathway initiated by NF-κB may be responsible for the induction of RAR. Inhibition of NF-κB allows cell to change its life/death balance towards apoptosis, increases radiosensitivity and the efficiency of anticancer treatment, and prevents primly irradiated cells from resistant to the challenging irradiation. Interestingly, with respect to the BAY treated cells, the MN frequency of 0.08 + 2 Gy group had a tendency of being higher than that of 2 Gy group, which indicates that once the cellular radioprotection function is inhibited, a priming low-dose irradiation and subsequent challenging irradiation will have a complex effect on cellular damage. Based on the present results and the above mentioned reports, we summarize a novel possible RAR model centered on NF-κB activation: a primary low-dose irradiation causes a redox imbalance and triggers the activation of NF-κB that further enhances the functions of a series of defense systems including DNA repair, cell cycle regulation, and antioxidant defense so that the primly irradiated cells acquire resistance to the subsequent high-dose challenging irradiation.

In conclusion, a deeper understanding of the molecular mechanism of RAR may lead to the improvement of the risk assessment for LDR and then opens up new approaches of cell protection. A better understanding of RAR in tumor cells may be helpful for preventing tumors from radioresistance and thus has potential implications in increasing the efficiency of anticancer treatment.

Conflict of interest

The authors declare that there are no conflicts of interests.

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