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Unirradiated cells rescue cells exposed to ionizing radiation: Activation of NF-κB pathway in irradiated cells

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ABSTRACT

We studied the involvement of NF-κB pathway activation in the rescue effect in HeLa and NIH/3T3 cells irradiated by α particles. Firstly, upon irradiation by 5 cGy of α particles, for both cell lines, the numbers of 53BP1 foci/cell at 12 h post-irradiation were significantly smaller when only 2.5% of the cell population was irradiated as compared to 100% irradiation, which demonstrated the rescue effect. Secondly, we studied the effect of NF-κB on the rescue effect through the use of the NF-κB activation inhibitor BAY-11-7082. Novel experimental setup and procedures were designed to prepare the medium (CM) which had conditioned the bystander cells previously partnered with irradiated cells, to ensure physical separation between rescue and bystander signals. BAY-11-7082 itself did not inflict DNA damages in the cells or have effects on activation of the NF-κB response pathway in the irradiated cells through direct irradiation. The rescue effect was induced in both cell lines by the CM, which was abrogated if BAY-11-7082 was added to the CM. Thirdly, we studied the effect of NF-κB on the rescue effect through staining for phosphorylated NF-κB (p-NF-κB) expression using the anti-NF-κB p65 (phospho S536) antibody. When the fraction of irradiated cells dropped from 100% to 2.5%, the p-NF-κB expression in the cell nuclei of irradiated NIH/3T3 cells increased significantly, while that in the cell nuclei of irradiated HeLa cells also increased although not significantly. Moreover, the p-NF-κB expression in the cell nuclei of irradiated HeLa cells and NIH/3T3 cells treated with CM also increased significantly.

1. Introduction

One of the most interesting findings in radiobiology in the past decades was the discovery of radiation-induced bystander effect (RIBE). RIBE between cells refers to the phenomenon that unirradiated cells, after receiving signals from irradiated cells, respond as if they have themselves been irradiated. RIBE was first demonstrated in in vitro studies by Nagasawa and Little [1] who used the frequency of sister chromatid exchanges as the biological end point. There are many excellent reviews on RIBE [2–7]. There are two widely accepted models for RIBE, which involve (1) gap junction intercellular communication (GJIC) in the presence of cell–cell contact, and (2) communication of soluble signal molecules released by the irradiated cells into the medium conditioning the non-irradiated cells. Suggested soluble molecules involved in RIBE include tumor necrosis factor-α (TNF-α) [8], transforming growth factor-β1 (TGF-β1) [9], interleukin-6 (IL-6) [10], interleukin-8 (IL-8) [11], nitric oxide [12–14] and reactive oxygen species (ROS) [15].

Recently, our group found the "rescue effect" which was closely related to RIBE [16]. Briefly, after co-culturing with unirradiated bystander cells, the numbers of p53-binding protein 1 (53BP1) foci and micronucleus (MN) formation, and the extent of apoptosis in the irradiated cells were found to be decreased when compared to those in the irradiated cells that were not co-cultured with the bystander cells. The effect was detected in human primary fibroblast (NHLF) and human cervical cancer cells (HeLa). In particular, unirradiated normal cells were able to rescue the irradiated cancer cells, which might have a significant impact on the efficiency of radiation therapy.

Subsequently, Widel et al. [17] confirmed the rescue effect through demonstrating that the frequencies of MN formation and apoptosis in irradiated human melanoma (Me45) cells having co-cultured with normal human dermal fibroblasts (NHDF) cells were significantly lower than the corresponding values observed in those

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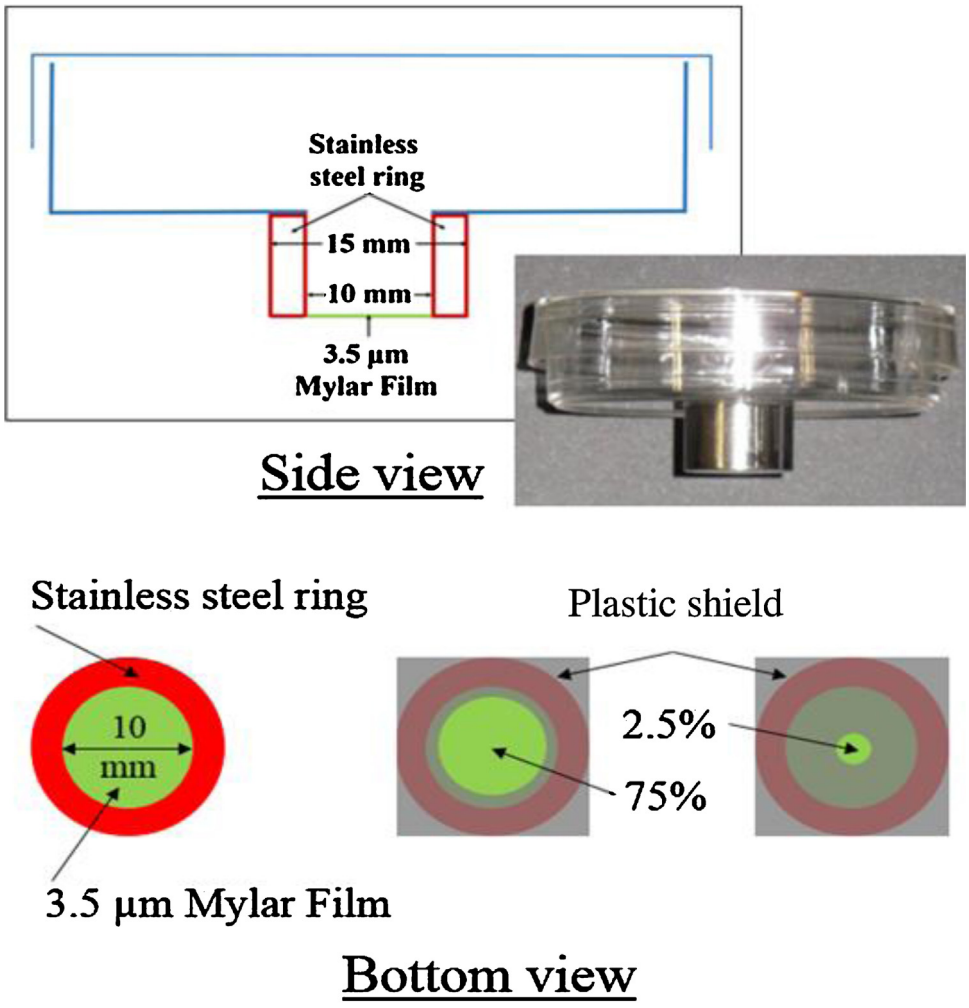

Fig. 1. Side view and bottom view of the variable-aperture (VA) dish. The VA dish was a tissue culture dish with a 60 mm diameter, with a hole of 10 mm diameter at the center and aligned to an attached stainless-steel ring with an inner diameter of 10 mm. The bottom of the ring was covered by a Mylar film, and had an option to be covered by a plastic shield with a thickness of ~0.25 mm and an aperture which could cover 25% or 97.5% of the hole area.

cells cultured alone. The study also showed that the rescue effect was associated with a significant lowering in the ROS level in the irradiated cells. Interestingly, however, they found that non-irradiated Me45 cells did not provide the rescue effect. Pereira et al. [18] also claimed that rescue effect was present between embryonic zebrafish (ZF4) cells. ZF4 cells which were first exposed to gamma rays for 4 h, then partnered with non-irradiated bystander ZF4 cells for 1 h, and finally irradiated again with gamma rays for another 20 h, developed significantly fewer γ-H2AX foci than those ZF4 cells which were irradiated for 24 h from the beginning. It is remarked here that the 1-h gap might have helped the cells develop some kind of adaptation, so the fewer γ-H2AX foci could have been a combined effect of rescue effect and radioadaptation. Desai et al. [19] also confirmed the rescue effect between proton-irradiated lung cancer (A549) cells and normal (WI38) cells. Through using γ-H2AX foci as the biological endpoint, the authors revealed that the irradiated A549 cells did not affect bystander WI38 cells, but the bystander WI38 cells exerted a rescue effect on the irradiated A549 cells.

Despite confirmations of the rescue effect, studies on the underlying mechanisms are scarce. He et al. [20] proposed that the rescue effect between co-cultured irradiated human macrophage U937 cells and bystander unirradiated HL-7702 hepatocyte cells was mediated by the second messenger cyclic adenosine monophosphate (cAMP). It is understood that cAMP can activate the cAMP-dependent protein kinase (or protein kinase A, PKA), so it is pertinent to identify the involvement of PKA in the rescue effect.

Most previous studies on the rescue effect employed human cells [16,17,19,20] so we included the HeLa cell line in the present study, which was also employed in our initial study on the rescue effect [16]. Since there was recent evidence that rescue effect was also observed in non-human cells, namely, the embryonic zebrafish fibroblast cells [18], we also included the mouse embryo fibroblast NIH/3T3 cells in the present study. We studied the rescue effect in these two cell lines exposed to α-particle irradiation, and explored the involvement of the nuclear factor-κB (NF-κB) response pathway. Many cellular stimuli could activate NF-κB, and expression of NF-κB target genes in general promoted cellular survival, despite a few exceptions where NF-κB contributed to cell death.

2. Materials and methods

2.1. Cell culture

Both the HeLa (ATCC® CCL-2™) and NIH/3T3 (ATCC® CRL-1658™) cells were obtained from American Type Culture Collection. Authentication of cell lines was frequently and regularly performed through checking the cellular morphology under the microscope since acquiring the cell lines. The cells were routinely cultured in DMEM (Gibco, 10569), supplemented with 10%

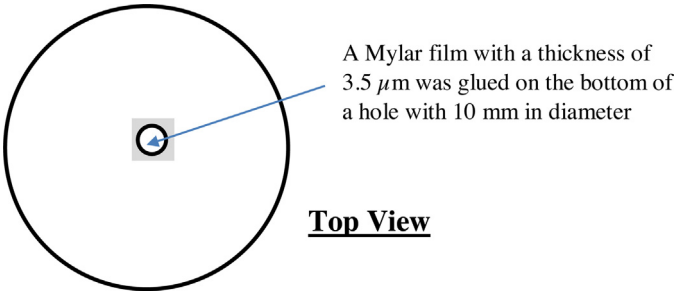

Fig. 2. Top view of the fixed-aperture (FA) dish. The FA dish was a tissue culture dish with a 100 mm diameter, with a hole of 10 mm diameter at the center and covered by a Mylar film glued to the bottom of the dish.

FBS (Gibco, 10270) and 1% penicillin-streptomycin solution (Gibco, 15140-122) and maintained at 37 °C in a humidified 5% CO_2 incubator.

2.2. Alpha-particle irradiation

In the present investigation, irradiation of the cells served three purposes, namely, (1) to prepare irradiated cells on which the effects of abundance of bystander cells would be studied, (2) to prepare irradiated cells to which the consequences from different treatments would be examined, and (3) to prepare the medium (CM) which had conditioned the bystander cells previously partnered with irradiated cells. The approach using CM was crucial for confirming the activation pathways in the irradiated cells responsible for the rescue effect by using activation inhibitors, due to physical separation between rescue and bystander signals, which avoided the question whether the activation inhibitors actually stopped the RIBE or the rescue effect.

A total of 4.5×10^4 cells (for HeLa cells) or 3×10^4 (NIH/3T3 cells) were plated on Mylar films and incubated overnight before α-particle irradiation. The cells were irradiated under ~80% confluence and were incubated in 5 mL culture medium. The Mylar films had a thickness of 3.5 μm, which allowed alpha particles to pass through and hit the cells without significant energy loss. All α-particle irradiations were made with a delivered dose of 5 cGy using an ^{241}Am irradiation source (diameter of active area = 12 mm, average alpha particle energy = 5.16 MeV, activity = 5.02 μCi, dose rate = 18 cGy/min). We had tried different alpha-particle doses including 5, 20 and 40 cGy in our preliminary experiments, and found that the rescue effects corresponding to 5 cGy were the most significant. The rescue effect was also found to diminish when higher doses of radiation were used. As such, we chose 5 cGy for our follow-up experiments to study the underlying mechanisms.

For (1), irradiation of the cells took place in a variable-aperture (VA) dish as shown in Fig. 1, which was a tissue culture dish with a 60 mm diameter, with a hole of 10 mm diameter at the center and aligned to an attached stainless-steel ring with an inner diameter of 10 mm. The bottom of the ring was covered by a Mylar film, and had an option to be covered by a plastic shield with a thickness of ~0.25 mm and an aperture which could cover 25% or 97.5% of the hole area. The plastic material with such a thickness stopped all α particles. Exponentially growing cells were plated onto the Mylar film which was glued on the bottom of the stainless steel ring with an inner diameter of 10 mm. Four irradiation strategies were employed, namely, 0% (sham-irradiated control), 2.5%, 75% and 100% (uniformly irradiated control) of a cell population were irradiated with α particles. Here, bystander signals and rescue signals co-existed in the medium.

For (2), irradiation of the cells took place in a fixed-aperture (FA) dish as shown in Fig. 2, which was a tissue culture dish with a diameter of 100 mm, and with a hole (diameter = 10 mm) at the center and covered by a Mylar film glued to the bottom of the dish.

For (3), irradiation of the cells also took place in a FA dish as described above. The experiments which made use of the CM were relatively more complex, and the scheme was described in Fig. 3. Since a number of dishes were used in the experiments involving the preparation of the CM, clear nomenclature of these dishes were necessary to avoid confusion. The FA dish with 15 mL medium hosting the irradiated cells to provide the bystander signals was referred to as the IR (irradiation) dish. Bystander cells plated on eight 18 × 18 mm cover glass slips were first partnered with the irradiated cells in the "IR dish". This partnering setup was modified from our previous protocol [42]. At the time of irradiation, both the cells on the Mylar film and the cover glass slips were under ~80% confluence. After co-culturing for 2 h, the cover glass slips were relocated into a new standard 100 mm tissue culture dish without a drilled hole and the Mylar film (referred to as the "CM dish") with 15 mL fresh medium. After another 2 h, the final step was to harvest the CM and to transfer it into new FA dishes (referred to as the "recipient dishes") for subsequent experiments.

2.3. 53BP1 immunofluorescent staining

In the present study, we used the number of 53BP1 foci as the biological endpoint to assess the magnitude of rescue effect in the cells (fixed at 12 h post-irradiation). 53BP1 is a protein which binds to the central DNA-binding domain of the tumor suppressor protein p53. Within a short time after irradiation, foci of 53BP1 protein appear in the irradiated cells, with the number of foci matching the number of DNA double-strand breaks (DSBs) [43], which leads to the common use of fluorescent detection of 53BP1 foci as a marker of DNA damages [44–48]. It is well established that 53BP1 foci appear shortly after irradiation, but they will remain for a period of time after irradiation. For example, Markova et al. [49] reported that 53BP1 foci appeared rapidly at the sites of DSBs after gamma irradiation, the number of which peaked at about 0.5 h post-irradiation and then decreased gradually. In fact, residual 53BP1 foci remained at 24 h post-irradiation depending on the dose of the radiation. Asaithamby et al. [50] studied the kinetics of 53BP1 formation and found that the foci did not completely disappear even after 8 h. Other studies also revealed that the time required for recovery of 53BP1 in cells could be larger than 12 h [16,43,51,52]. In fact, in our preliminary studies, we had examined the induction of 53BP1 foci at earlier time points, namely, 10 min, 0.5, 1, 2, and 5 h, in the cells after irradiation with an alpha-particle dose of 5 cGy. Our results showed that the numbers of 53BP1 foci per cell peaked between 0.5 and 1 h, and then decreased continuously up to 12 h, which was similar to the findings of other studies [49,50]. In addition, the ratios between the numbers of 53BP1 foci on the irradiated cells in the presence and absence of bystander cells were relatively small for time points of 5 h or less post-irradiation. At 12 h post-irradiation, the ratio was the largest, and the difference between the corresponding numbers of 53BP1 foci was also statistically most significant. As such, the 12 h time point was chosen for more detailed studies in the present work. In our first study on the rescue effect [16] by using the cell co-culture model and the number of 53BP1 foci as the biological endpoint, we also found that rescue effect was not significant at 30 min post-irradiation, but was significant at 24 h post-irradiation after 53BP1 positive foci in the cells had decreased.

At 12 h post-irradiation, the cells were fixed in 4% paraformaldehyde at RT, then washed three times with PBS, permeabilized in 0.5% Triton X-100 at 37 °C, and finally incubated with 1.5% goat serum blocking solution followed by primary anti-53BP1 antibody (Abcam, ab21083) at 37 °C. The cells were further washed with PBS three times, treated with secondary Alexa Fluor 488 goat anti-rabbit

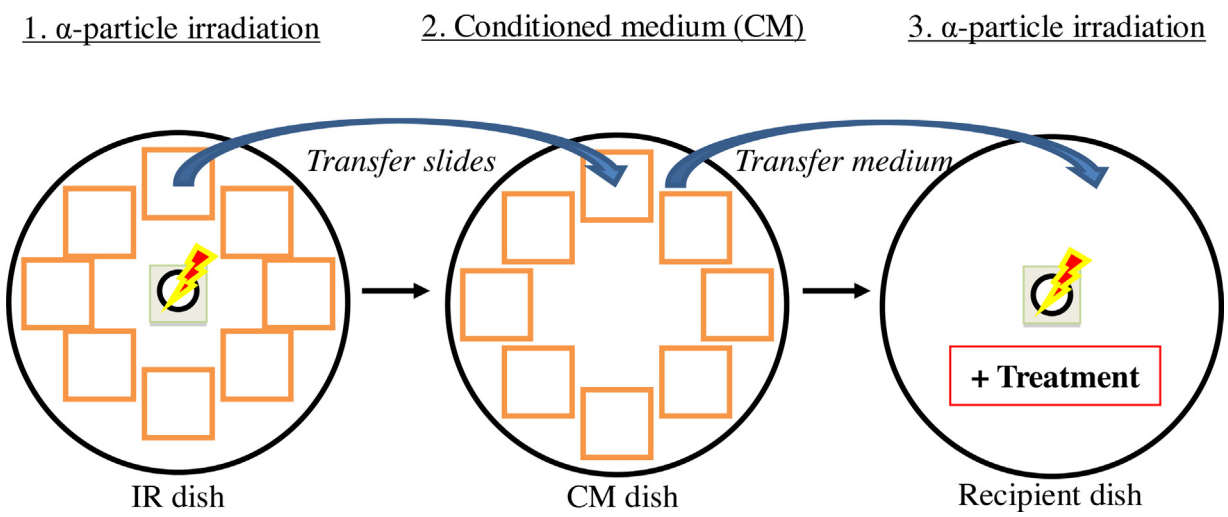

Fig. 3. Schematic representation of the procedures for preparing and transferring the conditioned medium (CM). The bystander cells plated on 18 × 18 mm cover glasses were first partnered with the irradiated cells in the "IR dish". After a specific time, the cover glasses were transferred into the "CM dish", from which the CM was harvested and transferred into "recipient dishes" for further experiments.

IgG antibody (Invitrogen, A11008), and counterstained with DAPI if necessary before the 53BP1 foci in the cell nuclei were scored under fluorescence microscopy.

2.4. Effect of NF-κB on the rescue effect

We studied the effect of NF-κB on the rescue effect using two different approaches, namely, through the use of (1) the NF-κB activation inhibitor BAY-11-7082, and (2) staining for phosphorylated NF-κB expression.

For the study with the NF-κB activation inhibitor, BAY-11-7082 (Cayman, 10010266) was employed. Phosphorylation of IκBα induced by TNF-α was blocked by BAY-11-7082 with the half maximal inhibitory concentration (IC50) of about 5–10 μM [53], while constitutive IκBα phosphorylation was not affected [53]. In the present work, all treatments with BAY-11-7082 referred to those with a final concentration of 5 μM (hereafter referred to as BAY5). The approach involving the preparation of CM was adopted. Unirradiated cells treated for 30 min with 15 mL of (1) fresh medium (FM) and (2) medium having conditioned unirradiated cells + BAY5 were assayed to provide references for assessing irradiated cells immediately treated after irradiation for 30 min with 15 mL of (3) FM, (4) FM + BAY5, (5) CM, and (6) CM + BAY5. After the different treatments for 30 min, the medium in the recipient dishes was replaced by FM for another 11.5 h (i.e., until 12 h post-irradiation) and the cells were then fixed for 53BP1 immuno-fluorescent staining.

The first objective here was to ensure that BAY5 itself did not inflict DNA damages in the cells by comparing the results from treatments (1) and (2). The second objective was to assess whether BAY5 affected the activation of the NF-κB response pathway in the irradiated cells induced by direct irradiation alone by comparing the results from treatments (3) and (4). The third objective was to confirm the presence of rescue effect induced by the CM, or otherwise, by comparing the results from treatments (3) and (5). If the rescue effect was present, the CM treatment would significantly reduce the number of 53BP1 foci/nucleus. Finally, a comparison of the results from treatments (5) and (6) would confirm or otherwise the importance of the NF-κB pathway in irradiated cells for the rescue effect.

For the study with the staining for phosphorylated NF-κB (p-NF-κB) expression, the anti-NF-κB p65 (phospho S536) antibody (Abcam, ab86299) was used. The VA dish as shown in Fig. 1 was employed. Different irradiation strategies were adopted, including delivery of zero α-particle dose (the control sample) and α-particle doses of 5 cGy in two irradiation patterns (100% and 2.5% of a cell population). At 1 h post-irradiation, the cells were fixed for staining for phosphorylated NF-κB expression. After staining, images were captured using SpotBasic (SPOT 4.7) under a fluorescent microscope (Nikon ECLIPSE 80i) with a magnification of 40×. The fluorescence (staining) intensities of p-NF-κB in the cell nuclei were quantified using the freely available ImageJ software. Briefly, the area for the cell nucleus was selected, and the measurements of area, integrated density and mean gray value of the selection were all recorded. The corresponding information for a (background) region next to the selected cell was also recorded for determining the Corrected Fluorescence (CF) of the selected cell nucleus, where

$$\mathrm{CF} = \text{Integrated density} - (\text{cell nucleus area} \times \text{mean background fluorescence})$$

At least 200 cells in each sample were analyzed. Finally, the average CF values of different treatment groups were further normalized using values obtained from non-treated controls. The objective here was to assess whether expression of p-NF-κB was significantly increased in irradiated cells when surrounded by a large number of bystander cells, when compared to the situation where the irradiated cells were not surrounded by bystander cells. To achieve this objective, different percentages (100% and 2.5%) of a cell population were irradiated. In particular, we compared the expression of p-NF-κB for a large number of bystander cells (2.5% of irradiated cells) and that for no bystander cells (100% of irradiated cells).

On the other hand, the p-NF-κB expressions in the nuclei of irradiated HeLa cells and NIH/3T3 cells treated with CM were also studied. The cells were irradiated with 5 cGy of alpha particles, treated with either FM or CM and fixed at 1 h post-irradiation. The p-NF-κB expressions in the cell nuclei of the irradiated cells treated with CM and FM were then compared.

2.5. Statistical analysis

All experiments were repeated at least three times, and the data were presented in terms of their means and SDs. The differences between the means for the treated and control groups were assessed through one-way ANOVA analysis and post-hoc Tukey HSD (honest significant difference) test. A p-value of 0.05 or smaller will be treated as demonstrating significant differences between the compared groups.

3. Results

3.1. Rescue effect in cells exposed to α-particle irradiation

Representative immuno-fluorescence 53BP1 staining results of HeLa and NIH/3T3 cells after irradiation in VA dishes (Fig. 1) are shown in Fig. 4. Fig. 5 shows the quantification of the number of 53BP1 foci per cell in the cell nuclei at 12 h post-irradiation for different cell lines (HeLa and NIH/3T3 cells) and for different irradiation patterns (100%, 75% and 2.5% of a cell population). One-way ANOVA analysis for data for HeLa cells gave a p value of 0.0023, and post-hoc Tukey HSD test gave p values of 0.0129, 0.0020, and 0.2140 for comparisons between 2.5% and 75%, between 2.5% and 100%, and between 75% and 100% irradiation patterns, respectively. Similarly, one-way ANOVA analysis for data for NIH/3T3 cells gave a p value of 0.0006, and post-hoc Tukey HSD test gave p values of 0.0052, 0.0010 and 0.0702 for comparison between 2.5% and 75%, between 2.5% and 100%, and between 75% and 100% irradiation patterns, respectively. For the case with 2.5% of the cells irradiated by 5 cGy of α particles, the numbers of 53BP1 foci/cell were found to be significantly reduced for both HeLa and NIH/3T3 cells as compared to the 100% irradiated cells, which demonstrated the rescue effect. For the case with 75% of the cells irradiated, the numbers of 53BP1 foci/cell were found to be reduced in both cells but not significantly as compared to the uniformly irradiated cells.

3.2. Effect of NF-κB on the rescue effect

We studied the effect of NF-κB on the rescue effect through two different approaches, namely, through the use of (1) the NF-κB activation inhibitor BAY-11-7082, and (2) staining for phosphorylated NF-κB (p-NF-κB) expression.

As regards the study using BAY5, irradiated cells cultured in FA dishes (Fig. 2) were assayed. Unirradiated cells treated for 30 min with 15 mL of (1) FM and (2) medium having conditioned unirradiated cells + BAY5 were assayed to provide references for assessing irradiated cells immediately treated after irradiation for 30 min with 15 mL of (3) FM, (4) FM + BAY5, (5) CM, and (6) CM + BAY5. After the different treatments for 30 min, the medium in the recipient dishes was replaced by FM for another 11.5 h (i.e., until 12 h post-irradiation) and the cells were fixed for the 53BP1 immuno-fluorescent staining. A total of 400–500 cells in each sample were analyzed. As a remark, in terms of the numbers of 53BP1 foci/nucleus, there were no significant differences between the non-irradiated cells and the sham-irradiated cells.

The results are shown in Fig. 6. For both HeLa and NIH/3T3 cells, there were no significant differences in the number of 53BP1 foci/cell between treatments (1) and (2), which ensured that BAY5 itself did not inflict DNA damages in the cells. Moreover, one-way ANOVA analysis for data for HeLa cells gave a p value of 0.0010, and post-hoc Tukey HSD test gave p values of 0.9000, 0.0490, 0.3995, 0.0150, 0.7800 and 0.0012 for comparisons between conditions (3) and (4), (3) and (5), (3) and (6), (4) and (5), (4) and (6), and (5) and (6), respectively. Similarly, one-way ANOVA analysis for data for NIH/3T3 cells gave a p value of 0.0002, and post-hoc Tukey HSD test gave p values of 0.9000, 0.0016, 0.9000, 0.0054, 0.7937 and 0.0010 for comparisons between conditions (3) and (4), (3) and (5), (3) and (6), (4) and (5), (4) and (6), and (5) and (6), respectively. There were no significant differences between treatments (3) and (4), which demonstrated that BAY5 had no effects on the NF-κB response pathway in the irradiated cells through direct irradiation. Treatment (5) produced significantly fewer 53BP1 foci/cell than treatment (3) for both cell lines, which confirmed the occurrence of rescue effect induced by the CM. Finally, treatment (6) produced significantly more 53BP1 foci/cell than treatment (5) for both cell lines. As shown above that BAY5 had no effects on the NF-κB response pathway in the irradiated cells through direct irradiation, the significant difference between treatments (5) and (6) proved the importance of activation of the NF-κB pathway in irradiated cells in the rescue effect.

As regards the study through immuno-staining for p-NF-κB expression, the anti-NF-κB p65 (phospho S536) antibody was used. Different fractions of the cell population, namely, 0% (Control), 100% and 2.5%, were irradiated with an α-particle dose of 5 cGy. The cells were fixed at 1 h post-irradiation for further immuno-fluorescence staining. Representative staining results of HeLa and NIH/3T3 cells in VA dishes after irradiation are shown in Fig. 7. The nuclei and cytoplasm of the cells were easily distinguishable from each other due to the large contrast between their p-NF-κB staining intensities. This facilitated quantification of p-NF-κB staining intensities in the cell nuclei as described in Section 2.4 above. Fig. 8 shows that when the fraction of irradiated cells dropped from 100% to 2.5%, the p-NF-κB expression in the nuclei of irradiated NIH/3T3 cells increased significantly, while that in the nuclei of irradiated HeLa cells also increased although not significantly.

On the other hand, Fig. 9 shows the relative p-NF-κB expression in the cell nuclei of HeLa cells and NIH/3T3 cells, after the cells were irradiated with 5 cGy of alpha particles, treated with either FM or CM and fixed at 1 h post-irradiation. The p-NF-κB expression in the nuclei of irradiated HeLa and NIH/3T3 cells treated with CM increased significantly when compared to those treated by FM.

We have also confirmed the findings by western-blotting analysis using the CM protocol. The nuclear fractions of cells were partitioned for the western blot analysis. The results are shown in Fig. 10. The p-NF-κB expression in the nuclei of irradiated HeLa and NIH/3T3 cells treated with CM increased by ~1.2 folds when compared to those treated by FM. These results agreed with those obtained in Fig. 9.

4. Discussion and conclusions

We studied the effects of abundance of bystander cells on the rescue effect. When the irradiated cells received an alpha-particle dose of 5 cGy, significant rescue effects were confirmed for a large ratio of (number N_U of unirradiated bystander cells)/(number N_I of irradiated cells), for both HeLa and NIH/3T3 cells. Our results showed that, when only 2.5% of the cells in a dish were irradiated with 5 cGy of α particles (or in other words 97.5% of the cells were unirradiated bystander cells, or $N_U/N_I = 39$), the numbers of 53BP1 foci/cell in the irradiated cells were significantly smaller than the corresponding numbers in the uniformly (100%) irradiated cells (or in other words, no unirradiated bystander cells). When the ratio N_U/N_I decreased, the rescue effects were also diminished. Our results showed that, when 75% of the cells in a dish were irradiated with 5 cGy of α particles (or in other words 25% of the cells were unirradiated bystander cells, $N_U/N_I = 0.33$), the number of 53BP1 foci/cell in the irradiated cells was still significantly smaller than the corresponding numbers in the uniformly irradiated cells for the case of NIH/3T3 cells, but not significantly smaller for the case of HeLa cells. These results showed that a significant induction of rescue effect critically depended on the ratio N_U/N_I. Moreover, our results revealed a non-linear relationship between the response (number of 53BP1 foci/cell) and the ratio N_U/N_I, showing a much smaller change in the response for large N_U/N_I, which could be due to signal amplification or receptor limitation.

The observations reported in the present paper were the first to prove the presence of a rescue signal in the CM, and reveal that the rescue effect (in terms of mitigating the 53BP1 foci) from the bystander cells was activated through the NF-κB pathway in the irradiated cells. These are also supported by our results showing increased expression of p-NF-κB in irradiated cells when

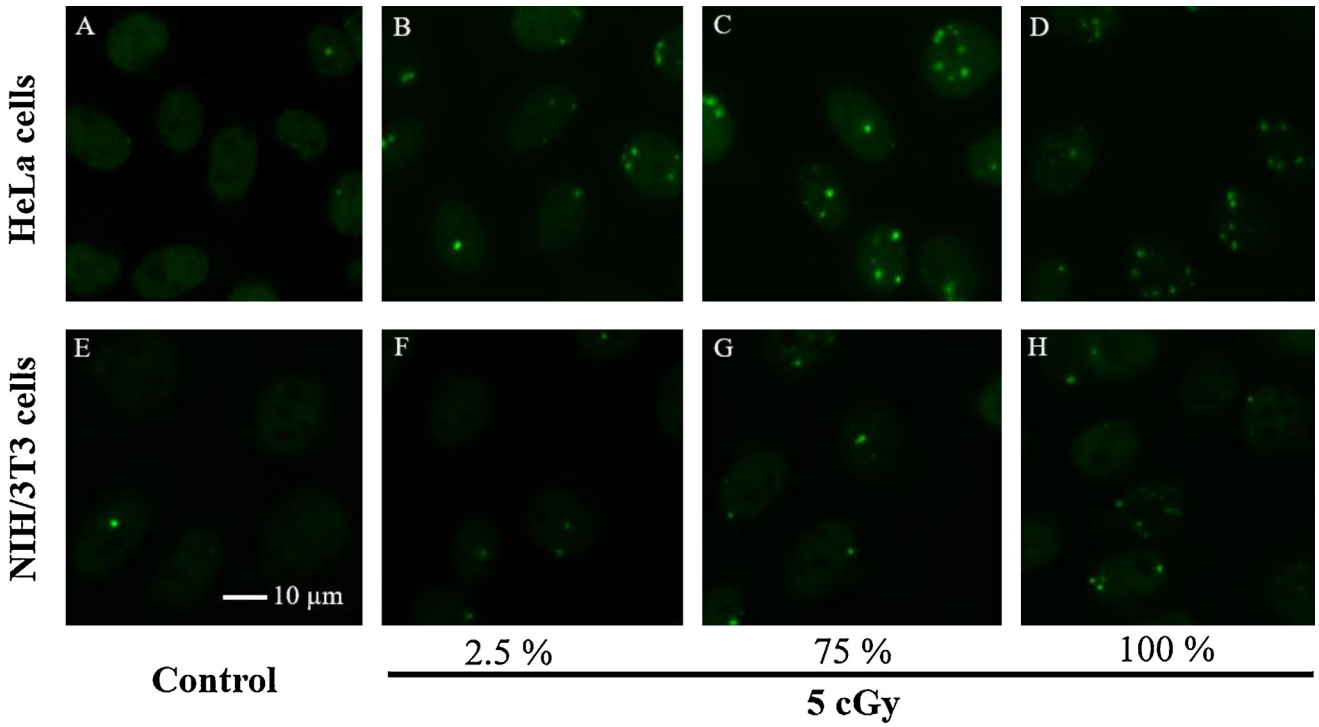

Fig. 4. Representative immunofluorescence 53BP1 staining results of HeLa cells (A–D) and NIH/3T3 cells (E–H) after irradiation in the VA dish. Different fractions of the cell population, namely, 0% (Control), 2.5%, 75% and 100%, were irradiated with an α-particle dose of 5 cGy. The cells were fixed at 12 h post-irradiation for further immuno-fluorescence staining.

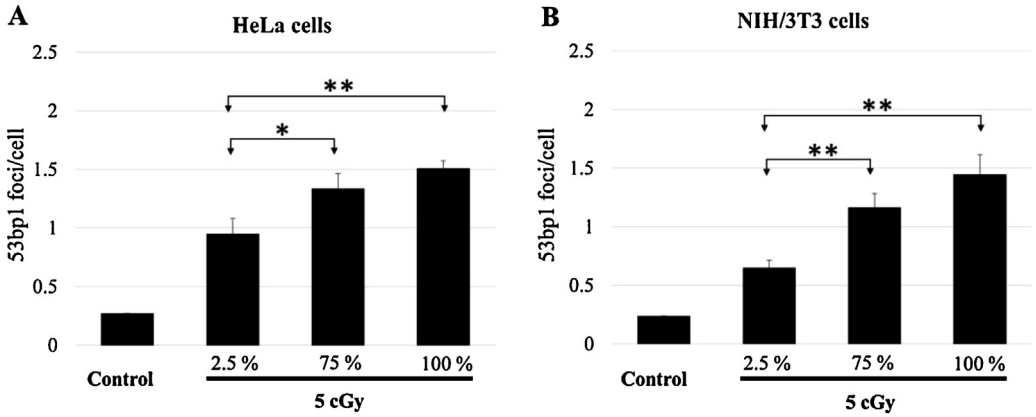

Fig. 5. Rescue effect induced by α-particle irradiation in (A) HeLa cells and (B) NIH/3T3 cells. Each figure shows the quantification of 53BP1 foci/cell in the cell nuclei at 12 h post-irradiation with zero α-particle dose and an α-particle dose of 5 cGy in three irradiation patterns (2.5%, 75% and 100% of a cell population). Experiments were repeated three times. $^{*}p < 0.05$, $^{**}p < 0.01$, and error bars represent ±SD.

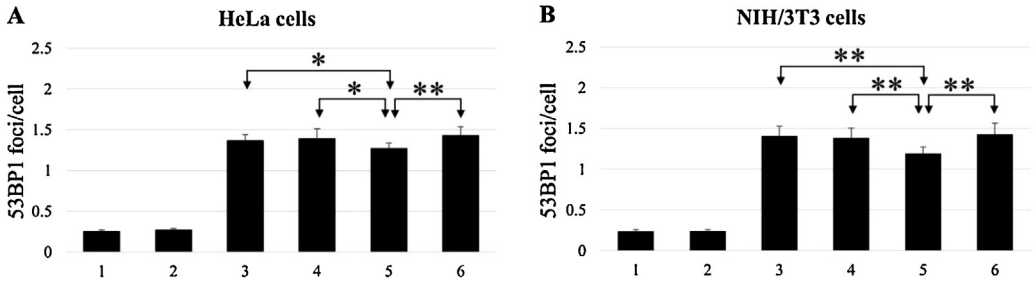

Fig. 6. Effects of different treatments on (A) HeLa cells and (B) NIH/3T3 cells in terms of number of 53BP1 foci/cell in nuclei at 12 h post-treatment. Experiments were repeated between 5 and 15 times. $^{*}p < 0.05$, $^{**}p < 0.01$, and bar represents ±SD. The approach involving the preparation of CM was adopted. Unirradiated cells treated for 30 min with 15 mL of (1) fresh medium (FM) and (2) medium having conditioned unirradiated cells + BAY5 were assayed to provide references for assessing irradiated cells immediately treated after irradiation for 30 min with 15 mL of (3) FM, (4) FM + BAY5, (5) CM, and (6) CM + BAY5. After the different treatments for 30 min, the medium in the recipient dishes was replaced by FM for a further 11.5 h (i.e., until 12 h post-irradiation) and fixed for the 53BP1 immuno-fluorescent staining.

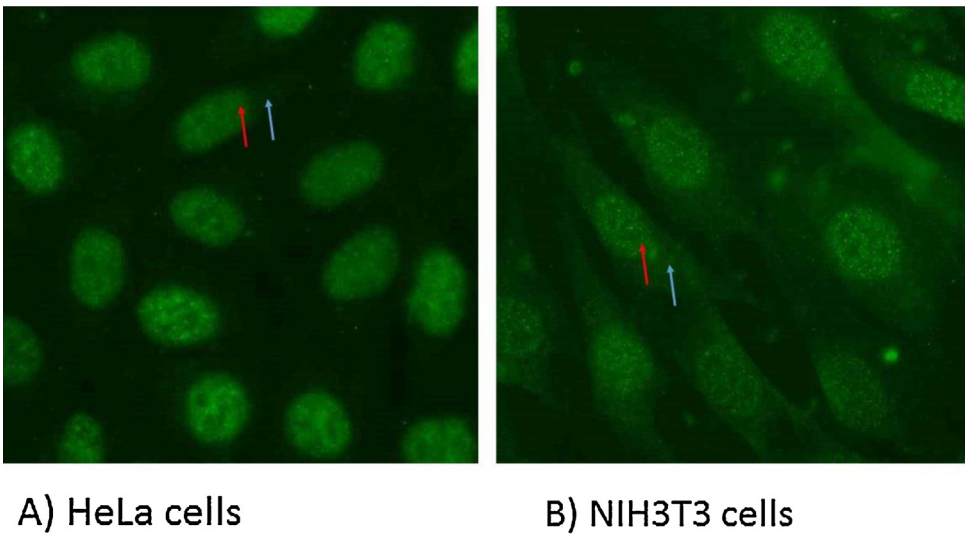

Fig. 7. Representative images from immunofluorescence staining with anti-NF-κB p65 (phosphor S536) antibody for (A) Hela cells and (B) NIH/3T3 cells after α-particle irradiation. The cells were fixed at 1 h post-irradiation for further immuno-fluorescence staining. Red arrows: cell nuclei; blue arrows: cytoplasm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

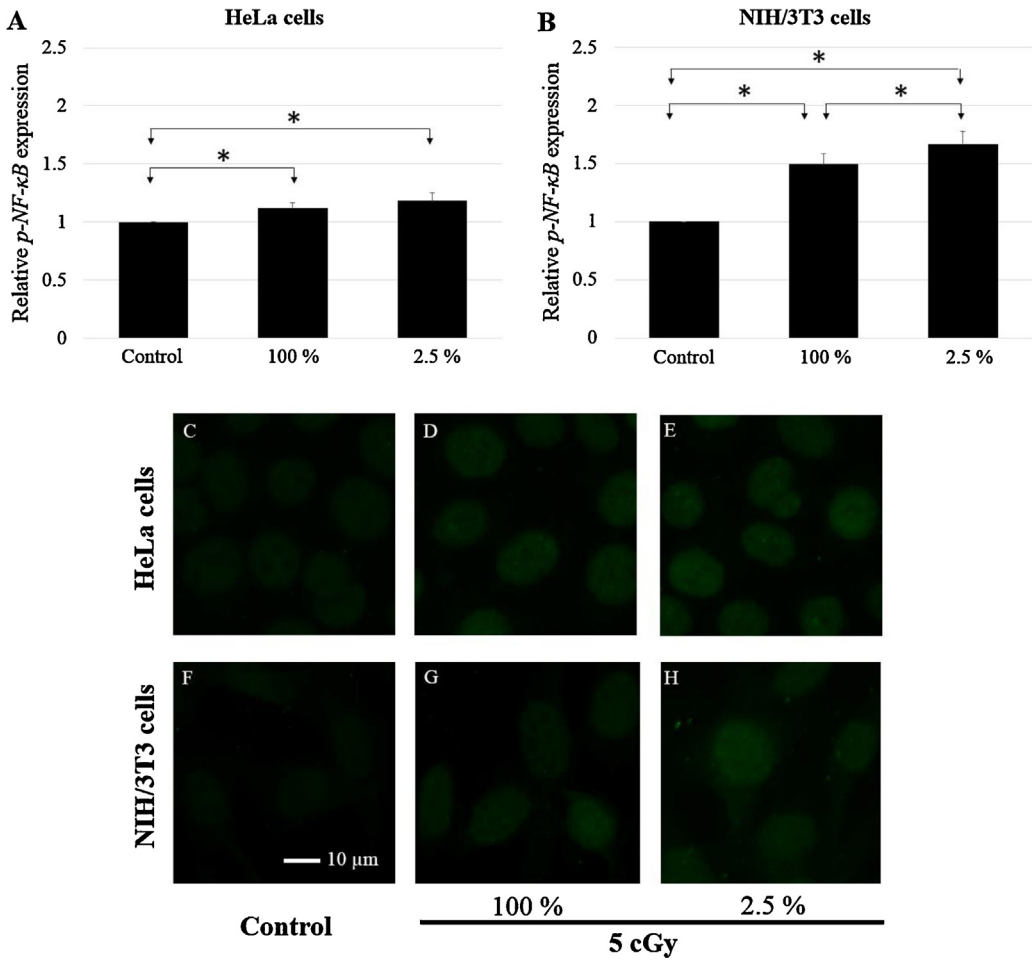

Fig. 8. Induction of phosphorylated NF-κB (p-NF-κB) expression in the cell nuclei of irradiated (A) HeLa cells and (B) NIH/3T3 cells, where the p-NF-κB expression results are presented relative to the values of the control samples. Representative images of p-NF-κB staining of cells are shown: (C)–(E) for HeLa cells; (F)–(H) for NIH/3T3 cells. Each figure shows the staining results for the control (zero α-particle dose) and for an α-particle doses of 5 cGy in two irradiation patterns (100% and 2.5% of a cell population). Experiments were repeated at least three times. $^{*}p < 0.05$, and bar represents ±SD.

surrounded by bystander cells or when treated with CM. The involvement of NF-κB response pathway was intriguing since TNF-α had been established as a soluble molecule involved in RIBE [8], which could activate the NF-κB pathway in both irradiated and

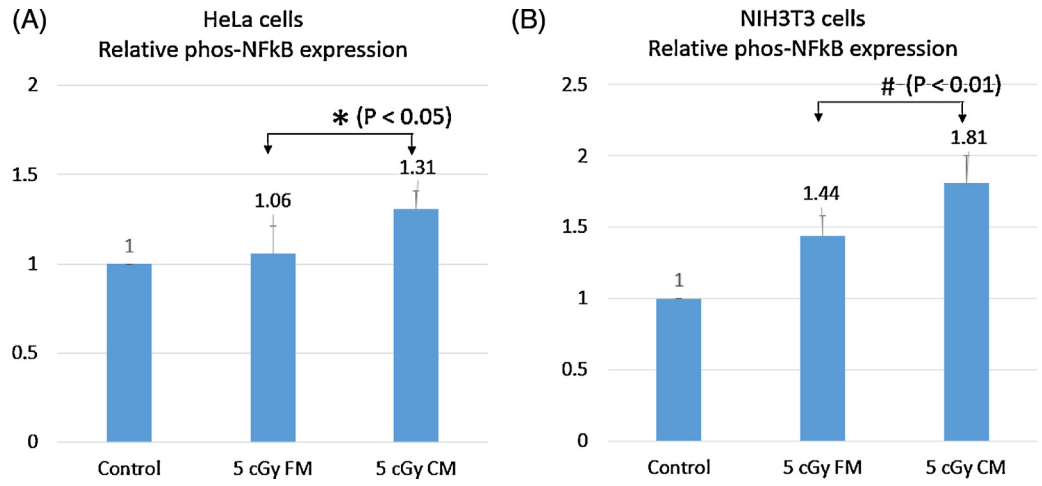

Fig. 9. Relative p-NF-κB expression in the nuclei of (A) HeLa cells and (B) NIH/3T3 cells, after the cells were irradiated with 5 cGy of alpha particles, treated with either FM or CM and fixed at 1 h post-irradiation. The p-NF-κB expression in the nuclei of irradiated HeLa cells and NIH/3T3 cells treated with CM increased significantly by 1.23 and 1.26 folds, respectively, when compared to those treated by FM.

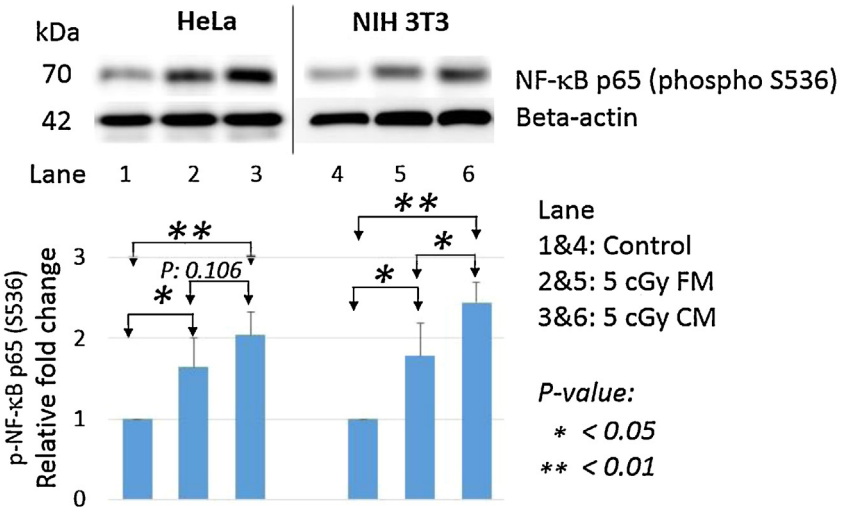

Fig. 10. Western blot analysis of NF-κB p65 (phospho S536) in nuclear extraction of Hela cells and NIH/3T3 cells after 5 cGy α-particle irradiation, and treated with either FM or CM and fixed at 1 h post-irradiation.

bystander cells. The failure for the irradiated cells to rescue themselves might be due to the presence of a threshold concentration of the rescue signal in activating the rescue response, below which no response will be triggered. Interestingly, such a threshold concentration (of bystander signal) is also present for the RIBE [21].

It has been reported in the literature that RIBEs involve the production of both inflammatory and anti-inflammatory cytokine and downstream signaling. TNF-α, which is commonly classified as a pro-inflammatory cytokine that can activate the NF-κB pathway, has been established as a soluble molecule involved in RIBE [8]. The present paper revealed that the rescue effect from bystander cells was in fact activated through the NF-κB pathway in the irradiated cells. However, the occurrence of rescue effect depended on the doses delivered to the irradiated cells, as well as to the proportion of cells being irradiated, both of which would affect the release of TNF-α by the bystander cells. Insufficient amounts of TNF-α released by bystander cells would lead to insufficient amounts of NF-κB produced in the irradiated cells, which would lead to failed suppression of p53 in irradiated cells, and as a result lead to no rescue effect. As such, whether a cytokine (TNF-α in our case) behaved as pro-inflammatory depended on, for example, its amount. This agreed in general with the views that inflammation was actually characterized by the interplay between pro- and anti-inflammatory cytokines, and that the cytokine properties were significantly affected by many factors including the cytokine amount [54].

To be consistent with findings from previous works, it would be most appealing if the requirement of an increased expression of NF-κB in irradiated cells to mediate the rescue effect could also explain the associated reduction in apoptosis [16], DNA damages (surrogated by the number of 53BP1 or γ-H2AX foci) [16,18,19], genomic instability (surrogated by the number of MN formation) [16,17] and ROS level [17], as well as increase in cAMP levels in the irradiated cells.

NF-κB is a family of transcription factors comprising five members, namely, RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2), and these members can from a variety of homo- or hetero-dimers [22]. The heterodimer of p65 and p50 will be used as an example for our discussion in the following. Generally speaking, the inactivated dimers are confined to the cytoplasm in the cells by the IκB-family proteins such as IκBα. Various extracellular signals can activate the IκB-kinase (IKK) complex to phosphorylate IκB to release NF-κB. The free NF-κB enters the nucleus and binds to specific sequences of the DNA, and the

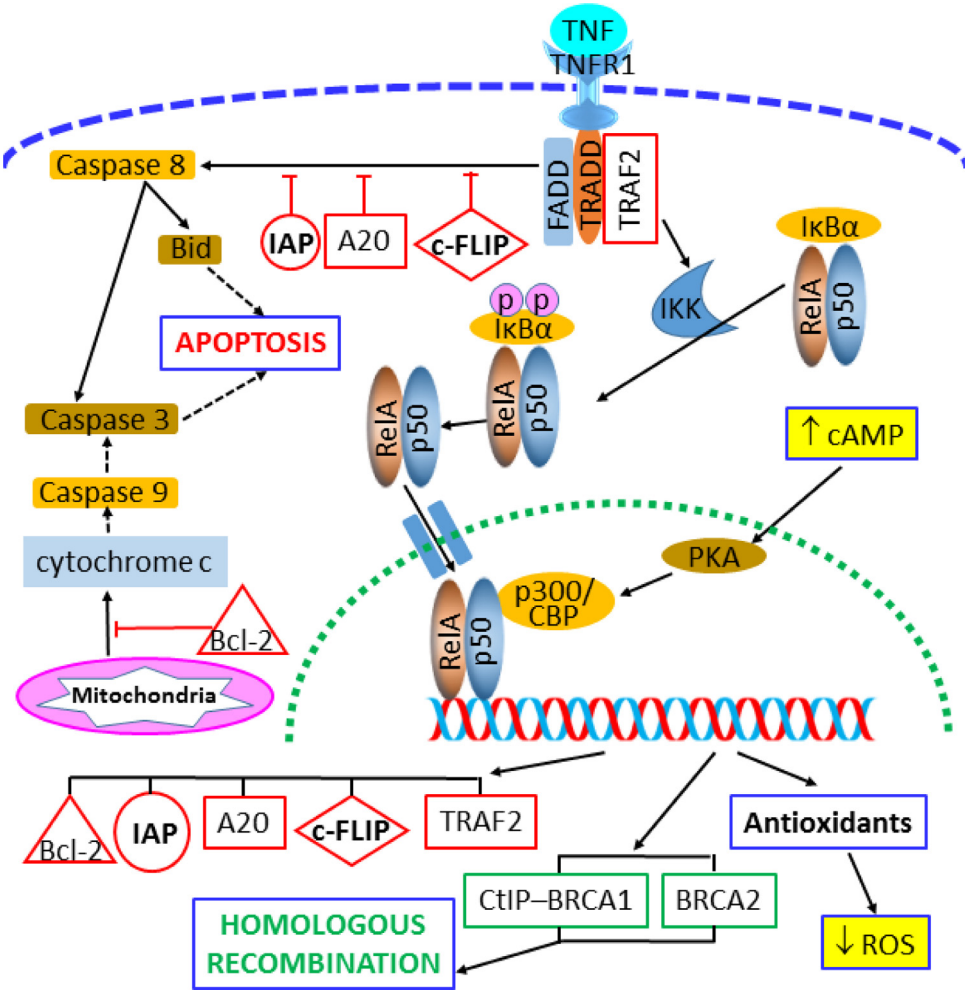

Fig. 11. Schematic diagram to show the involvement of activation of the nuclear factor-κB (NF-κB) response pathway in the rescue effect in irradiated cells. The scheme also explains the observations made by other groups before the present work (shown as yellow boxes), namely, increase in the cyclic adenosine monophosphate (cAMP) level and significant reduction in the reactive oxygen species (ROS) level in the irradiated cells. The prototypical heterodimer with RelA (p65) and p50, which are NF-κB family members comprising, has been used for illustration. Blue dotted line: cell membrane; green dotted line: nuclear envelope; dashed arrows: multiple steps involved. TNF: tumor necrosis factor; TNFR1: TNF receptor type 1; TRAF2: TNF receptor-associated factor 2; TRADD: TNFR1 associated death domain protein; FADD: Fas-associated protein with death domain; IKK: IκB-kinase; PKA: protein kinase A (or cAMP-dependent protein kinase); CBP: CREB binding protein; CREB: cAMP response element binding protein; IAP: inhibitors of apoptosis protein; A20: A20 zinc finger protein; c-FLIP: Cellular FLICE-like inhibitory protein; FLICE: FADD-like interleukin-1β-converting enzyme; bcl-2: B-cell lymphoma 2 protein; CtIP: C-terminal binding protein (CtBP)-interacting protein; BRCA1: breast cancer type 1 susceptibility protein; BRCA2: breast cancer type 2 susceptibility protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

DNA/NF-κB complex recruits proteins such as coactivators and RNA polymerase to facilitate the transcription (see Fig. 11).

In general, expression of NF-κB target genes can promote cellular survival, although there are some exceptions where NF-κB participates in cell-death induction. Magné et al. gave a review on the anti-apoptotic proteins regulated by NF-κB [23], including (i) inhibitors of apoptosis protein (IAP) family [24], (ii) TRAF (tumor necrosis factor receptor (TNFR)-associated factor) proteins [25], (iii) A20 zinc finger protein [26], (iv) c-FLIP (Cellular FLICE-like inhibitory protein, where FLICE stands for FADD-like IL-1β-converting enzyme) [27], and (v) anti-apoptotic proteins of the B-cell lymphoma 2 (bcl-2) family (see Fig. 11).

NF-κB has also been found to play important roles in DNA repair, in particular through the homologous recombination (HR). Volcic et al. [28] proposed multiple mechanisms of HR mediated by NF-κB. The authors showed that, as a first step, NF-κB stimulated the formation of (CtIP-BRCA1) complexes (where CtIP is C-terminal binding protein (CtBP)-interacting protein, and BRCA1 is breast cancer type 1 susceptibility protein) to trigger DSB end resection, which started the HR repair. In particular, formation of replication protein A (RPA) and Rad51 were promoted. RPA binds to single-stranded DNA (ssDNA) while Rad51 forms filaments on ssDNA to perform homology search and DNA strand invasion. As the second step, NF-κB transcriptionally up-regulated the breast cancer type 2 susceptibility protein (BRCA2) to channel the DSB repair into the HR pathway [29], and also up-regulated the ATM to ensure efficient HR through signaling to CtIP, BRCA1, BRCA2, Mre11, Rad50, and Nibrin [30,31]. In particular, the ATM downstream kinase Chk2 phosphorylates BRCA1 to promote HR over non-homologous end joining (NHEJ) [32]. BRCA2 also facilitated the loading of Rad51 onto the DNA. Nakamura et al. [33] also proposed that the collaborative action of CtIP and BRCA1 played a role in the elimination of covalent modifications from DSBs to facilitate subsequent DSB repair. Moreover, the stimulation of HR by NF-κB balanced the repression of HR by p53 [34]. In addition to the above mechanisms, some downstream components of NF-κB could also act upstream of NF-κB, which could result in signal amplification. For examples, BRCA1 could enhance the transcription by NF-κB [35] while ATM could phosphorylate IKKγ in the nucleus [22,36]. The signal amplification, together with the activation of multiple molecular targets by

NF-κB, might be designed for ensuring strong and rapid activation of HR [28].

Finally, a number of NF-κB-regulated genes are involved in regulating the intracellular ROS levels [37], and the cAMP-dependent PKA once having entered the nucleus will phosphorylate serine 276 on p65 to enhance its affinity for the transcriptional co-activators CBP/p300 [38], where CBP is the CREB binding protein and CREB is the cAMP response element binding protein.

There may be similarities between the rescue effects shown here and studies looking at bystander responses in modulated exposures, where in-field responses are not predictable from uniform exposure leading to increased survival and reduced DNA damage [52,55]. As described above, the capability of unirradiated normal cells to rescue irradiated cancer cells has a significant impact on the efficiency of radiation therapy. Now that activation of NF-κB pathway in irradiated cells has been identified as an essential step for execution of the rescue effect, it would be pertinent to study the effects of NF-κB on the efficacy of cancer treatments. In relation to the studied rescue effect, there was evidence that NF-κB led to resistance against genotoxic treatment in anti-cancer therapies [39,40]. In particular, it was proposed to explore whether NF-κB-induced accelerated removal of complex DSBs led to resistance to radiotherapy [39]. Similarly, Volcic et al. [28] proposed that NF-κB-dependent mechanisms which regulated HR could explain resistance to chemo- and radiotherapies, and at the same time the sensitization by pharmaceutical intervention of NF-κB activation. Incidentally, immuno-deficiency patients with mutations in the NF-κB pathway were predicted to be more sensitive to genotoxic therapies [41]. Altogether, the rescue effect mediated by activation of NF-κB pathway in irradiated cells can have far-reaching consequences on the design of radiation therapy procedures, and deserve much more extensive research in the future.

Conflict of interest

None.

Funding

None.

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