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Enhancement of sodium butyrate-induced cell death and apoptosis by X-irradiation in the human colorectal cancer cell line HCT 116

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Abstract. In this study, we aimed at evaluating the possible enhancing effect exerted by the combined use of sodium butyrate (SB) and X-rays on eradicating the human colorectal cancer cell line HCT 116 containing wild-type p53. We assessed the effect of this combination on the molecular pathways leading to cell death. HCT 116 cells were subjected to SB (1 mM) treatment followed by X-irradiation (5 Gy), and the effects on cell death, cell proliferation and cell cycle were examined. We also analyzed the apoptosis-indicating protein expression, mitochondrial membrane potential and intracellular superoxide formation. Treatment with SB alone significantly induced cell cycle arrest and apoptosis, whereas X-irradiation showed no effect on cell death despite its ability to block cell proliferation. Growth arrest and cell death were enhanced in the combined treatment groups. A marked reduction in the growth rate of the combined-treatment group was observed compared to that of the single-treatment groups. The apoptotic mitochondrial pathway was significantly enhanced with the combined use of the two agents. It was observed to be involved in the increased expression levels of p53 and p21, as well as in the release of cytochrome c and the alteration of the balance of anti- and pro-apoptotic Bcl-2 family proteins. Enhanced superoxide formation was also observed. However, the death receptor pathway was found to play no role in this phenomenon. These results suggest that X-irradiation promotes cell killing in synergy with SB treatment. Thus, the combined treatment led to a mutual potentiation of the killing effects of each agent.

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Introduction

Colorectal cancer is one of the three leading causes of cancer deaths worldwide (1). Most colorectal cancers are sporadic, with dietary risk factors implicated in their development. Despite curative surgery, patients still have a significant probability of disease relapse and poor survival. Much interest has been generated in the last few decades in adjuvant treatment that would eliminate microscopic diseases, thus preventing recurrent diseases (2).

Adjuvant chemoradiotherapy is an area of active research (3). Many trials have been conducted to improve the outcome and decrease recurrence possibility. It is without doubt that tracing the underlying molecular mechanisms within the adopted strategy for cell death is a cornerstone for the success of such trials.

Sodium butyrate (SB) is a four-carbon fatty acid and a natural component of the colonic milieu derived from the anaerobic microbial fermentation of diet-derived complex carbohydrates. In molecular terms, the action of butyrate is probably related to deacetylase inhibition, leading to the hyperacetylation of chromatin components such as histones and non-histone proteins and alterations in gene expression. SB has also been shown to induce apoptosis in a number of cancer cells (4). SB has received much attention as a potential chemopreventive agent and for its protective action against early tumorigenic events in colorectal cancer (5). However, during its first clinical trials, limited efficacy was found (6).

In addition to its potential as a single-modality anticancer agent, SB is effective in combination with radiotherapy (7). Extensive research has been carried out to outline the possible mechanisms by which butyrate enhances radiation injury. It appears that the mechanisms have been primarily proposed as gene activation by SB (8). The inhibition of the repair of X-ray-induced DNA double-strand breaks by SB (9), and remain unclear. Apoptosis induced by SB treatment in human colorectal adenoma and carcinoma cell lines is caspase-dependent (10). However, X-irradiation-induced apoptosis proceeds via the p53 pathway. The critical step is the increase in mitochondrial permeability to release cytochrome c under the control of pro- and anti-apoptotic proteins of the bcl-2

family (11). We propose that the combined treatment may have amplified effects on related pathways.

In this study, we applied the combined treatment to the human colon cancer cell line (HCT 116) and assessed whether X-irradiation affects butyrate-induced apoptosis in that butyrate sensitizes the cells to radiation. The results were very promising showing a synergistic effect leading to a pronounced enhancement of apoptosis. We also determined the inter-playing apoptotic pathways involved in cell death.

Materials and methods

Cell culture and treatments. Human colon carcinoma cells (HCT 116) (obtained from DS Pharma Biomedical Co., Ltd., Osaka, Japan) were grown in a modified McCoy's 5A medium (Gibco 16600) containing 10% heat-inactivated fetal bovine serum. The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37˚C and passaged using 0.25% trypsin three times a week. The experiments were performed using logarithmically growing cells divided into six groups as follows: control (C), 5 Gy X-rays (R5), 10 Gy X-rays (R10), sodium butyrate (SB), SB+R5 and SB+R10. Cells (~1x10^6) were inoculated in 6-cm-culture dishes. SB (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the SB-designated groups media at a final concentration of 1 mM.

On the second day, the irradiation-designated groups were exposed to X-rays at a rate of 5 Gy/min. Irradiation was carried out at room temperature using an X-ray apparatus (MBR-1520R-3, Hitachi Medico Technology Co., Kashiwa, Japan) operating at 150 kV and 20 mA at a dose rate of 5 Gy/min as determined by Fricke dosimetry. Analytical testing was carried out 24 h after irradiation, i.e., on the third day of the experiment, unless otherwise mentioned.

Survival curve studies. A trypan blue dye exclusion test was performed on all the groups for 5 consecutive days starting from the first day of the experiment. Cell suspensions were mixed with equal amounts of 0.3% trypan blue solution (Sigma, St. Louis, MO) in PBS. After a 5-min incubation at room temperature, the unstained (viable) cells and stained (non-viable) cells were counted using a Burker Turk hemocytometer. The percentage of dead cells was determined from the ratio of the number of stained cells to the total number of stained and unstained cells. The validity of this method was previously demonstrated (12).

Analysis of cell cycle. Cell phase distribution was determined using flow cytometry. HCT 116 cells (1-7x10^6) were harvested and washed with PBS. The cells were fixed overnight in 10 ml of 70% (-20˚C) ethanol at 4˚C, washed with PBS and incubated for 60 min at 37˚C with 25 mg/ml RNase (Wako Pure Chemical Industries). They were then treated with 50 μg/ml propidium iodide (Wako Pure Chemical Industries, Ltd.) and incubated for 30 min at 4˚C in the dark followed by flow cytometry (Epics XL, Beckman-Coulter, Miami, FL).

DNA fragmentation assay. The amount of DNA extracted from the cells was assayed using the Sellins and Cohen method with a few modifications (13,14). Briefly, ~3x10^6 cells were lysed using 200 μl of lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100 and pH 7.5) and centrifuged at 13,000 g for 10 min. Subsequently, DNA from each sample in the supernatant and pellet was precipitated in 12.5% trichloroacetic acid (TCA) at 4˚C overnight and quantified using the diphenylamine reagent after hydrolysis in 5% TCA at 90˚C for 20 min. The percentage of fragmented DNA for each sample was calculated as the amount of DNA in the supernatant divided by the total amount of DNA for that sample (supernatant plus pellet).

Measurements of caspase-3 and -8 activities. FLICE/Caspase-3 and FLICE/Caspase-8 colorimetric protease assay kits (MBL, Nagoya, Japan) were used to measure the activities of caspase-3 and -8, respectively. The proteins were extracted from ~1-2x10^7 cells, and prepared according to the manufacturer's procedures. Extracted protein (100 μg) was reacted with the Ac-DEVD-*p*NA substrate (caspase-3) or Ac-IETD-*p*NA substrate (caspase-8) at a final concentration of 200 μM, with 50 μl of 10 mM dithiothreitol (DTT) for 60 min at 37˚C in a final volume of 100 μl reaction buffer. Then the amount of the cleaved chromophore *p*-nitroanilide (*p*NA) was quantified spectrophotometrically at 400 nm using a spectrophotometer (Beckman Instruments Inc., CA).

Flow cytometry of mitochondrial transmembrane potential (MMP or Δψ). The cationic fluorophore, tetramethylrhodamine methyl ester (TMRM) (Molecular Probes, Eugene, OR), accumulates electrophoretically in mitochondria in response to Δψ and is released upon the loss of Δψ. TMRM at concentrations as low as 10 nM has been shown to represent Δψ loss in cells with a long-lasting opening of permeability transition pores (15). The cells were harvested 0, 6, 12, 24 and 48 h after irradiation, and stained with 10 nM TMRM for 15 min at 37˚C in PBS containing 1% fetal bovine serum, followed by immediate flow cytometry (excitation at 488 nm and emission at 575 nm).

Determination of superoxide ($O_2^{\bullet-}$) levels by dihydroethidine (DHE). To measure the relative levels of intracellular oxidative stress caused by different treatment regimens, the dye DHE, which is easily oxidized by $O_2^{\bullet-}$ to red-fluorescent ethidium (Eth), was used. The cells were collected 0, 6, 12, 24 and 48 h after irradiation and incubated with 5 μM DHE (Molecular Probes) in PBS for 15 min at 37˚C and immediately analyzed by flow cytometry (excitation at 488 nm and emission at 620 nm) (16).

Western blotting. Cells (0.6-1x10^7) per assay were collected, washed with cold PBS and lysed at a density of 1x10^7 cells/100 μl of RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40 (v/v), 1% sodium deoxycholate, 0.05% SDS, 1 μg/ml each of aprotinin, pepstatin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] for 20 min on ice. After brief sonication, the lysates were centrifuged at 12,000 rpm for 10 min at 4˚C. The protein content in the supernatant was measured using a DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Western blotting for p53, p21, Bcl-2, Bak, Bax, Bid, PCNA, procaspase-8 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), Bcl-xL, procaspase-3 (Cell Signaling Technology, Inc., Danvers, MA)

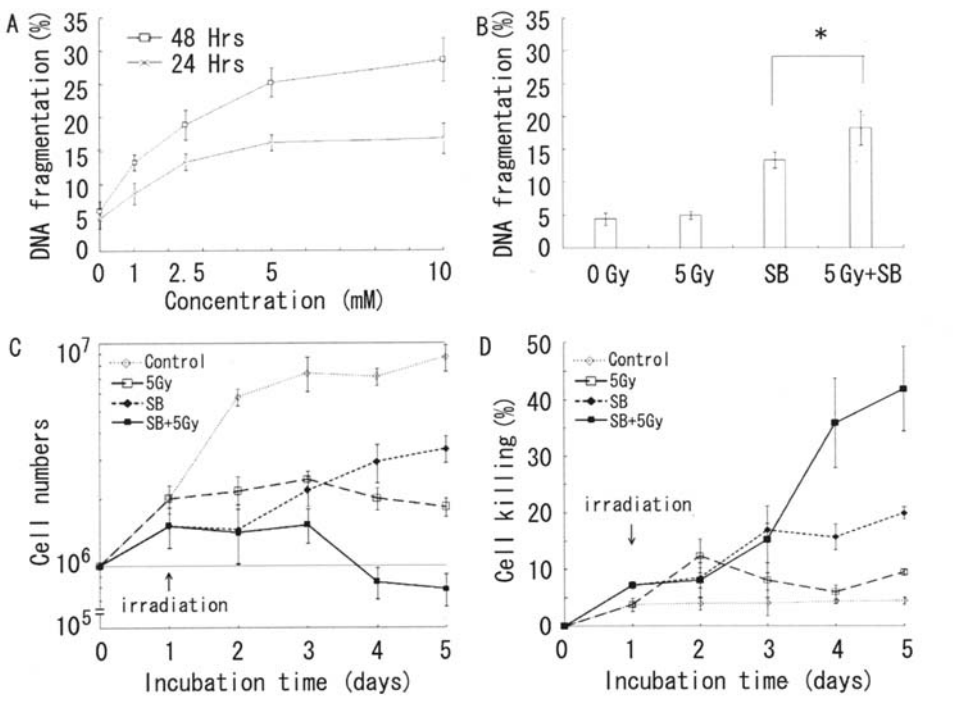

Figure 1. Effects of SB treatment and X-irradiation on DNA fragmentation, cell growth and cell death in HCT 116 cells. (A) Effects of treatment with SB at graded doses on DNA fragmentation. (B) Effects of X-irradiation (5 Gy), SB treatment (1 mM) and their combination. (C) Relationship between the cell numbers and incubation time (days). (D) Relationship between the cell killing percentages and incubation time (days), *p<0.05.

and anti-ß-actin mAb (Sigma-Aldrich, St. Louis, MO) was carried out (17). Using the secondary horseradish peroxidase (HRP) conjugated anti-rabbit and anti-mouse IgGs (Sigma-Aldrich), band signals were visualized on X-ray film using an ECL system (GE Healthcare, UK).

For the preparation of cytosolic extracts, the cells, suspended in an ice-cold solution containing 20 mM HEPES (pH 7.5), were disrupted using a Dounce homogenizer. Samples were centrifuged at 1,500 g for 5 min at 4˚C to remove the nuclei and intact cells. The supernatant was centrifuged again at 105,000 g for 30 min at 4˚C. The resulting supernatant was used as the soluble cytosolic fraction. The cytochrome c content in the cytosolic fraction was then determined.

Statistical analysis. All experiments were conducted in triplicate unless otherwise specified. All results are presented as means ± SD. Statistical analysis was carried out using a two-factor ANOVA test. P<0.05 was regarded as significant.

Results

DNA fragmentation analysis. The different treatment groups were subjected to DNA fragmentation analysis to evaluate the extent of apoptosis on the third day of the experiment. The effects of SB on apoptosis were examined, and the percentage of DNA fragmentation was found to increase in a dose- and time-dependent manner. Since 1 mM SB exerted a relatively low toxicity, it was used for further experiments. The groups subjected to irradiation alone showed a slight DNA fragmentation, whereas the SB-treated group exhibited significant DNA fragmentation (4.9±0.6 and 13.2±1.2%) (Fig. 1A and B). In contrast, the groups subjected to the combined treatment had showed a significant increase in the DNA fragmentation percentage compared to the SB group (18.1±1.8% at a dose of 5 Gy and 20.9±0.8% at a dose of 10 Gy), although no significant difference between the two irradiation doses was observed. Thus, the combined treatment with 1 mM SB and irradiation synergistically augmented the SB apoptotic effect (two-factor ANOVA, p<0.05), regardless of the irradiation dose.

Analyses of cell growth and viability. Generally, growth curves can help identify the extent and persistence of the lethal effects of a therapeutic agent. The SB group showed a significant decrease (3.9-fold) in the growth rate compared to the control group and so did the irradiated group over the 2 days following the X-irradiation with a sudden decrease on the third day, indicative of delayed cell death. The combination group exhibited a maximum decrease in the growth rate compared to the control, SB and irradiated groups (4.1-, 1.1- and 1.5-fold, respectively) with a similar delayed cell death (Fig. 1C and D). This increased amount of decrease in survival evidenced a possible synergistic effect, achieved by the combined therapy.

Cell cycle distribution. The cellular response to DNA damage involves checkpoint controls that delay cell cycle progression to provide time for either repairing damaged DNA, or triggering apoptosis. The analysis of the SB group showed a sub-G1 and G2/M blockade characteristic of cell cycle arrest induced by SB, whereas the irradiated groups showed only a high G2/M arrest. Notably, the combined treatment groups exhibited sub-G1 and -G2/M blockades, similar to the SB

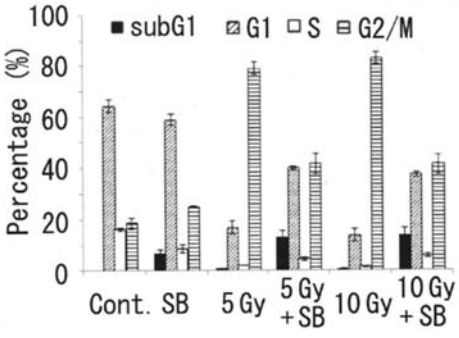

Figure 2. Cell cycle distribution after SB treatment (1 mM), X-irradiation (5 or 10 Gy) or their combination in HCT 116 cells. Cell cycle distribution of the cells after SB treatment, X-irradiation and their combination at 48 h.

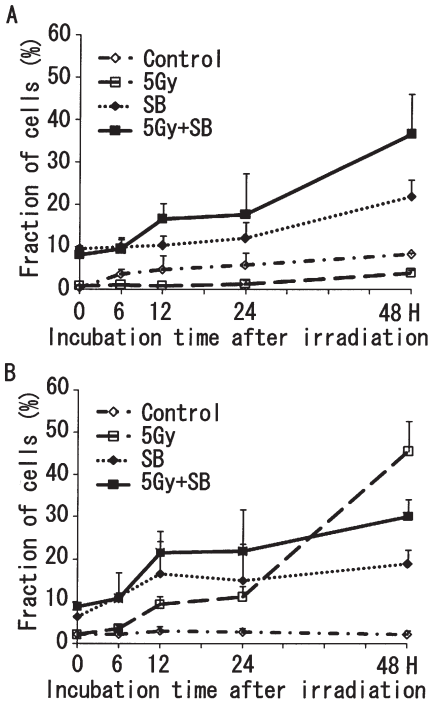

Figure 3. Flow cytometry of mitochondrial transmembrane potential (MMP) and superoxide generation in HCT 116 cells. (A) Relationship between the fractions of cells showing a low MMP detected using the fluorescence dye TMRM and incubation time (days). (B) Relationship between the fractions of cells showing a superoxide generation detected using a fluorescence dye, DHE and incubation time (days).

group but different to proportionality where the sub-G1 phase is enhanced and more cells are accumulated in the G2/M phase (Fig. 2). The common action modality may be one reason for the enhancement.

Mitochondrial transmembrane potential (MMP or Δψ) and superoxide formation patterns. To determine whether the mitochondrial pathway is related to the enhanced apoptosis by combination, we measured the changes in MMP and reactive oxygen species (ROS), which have proved to be responsible for outer mitochondrial membrane rupture (18). SB induced a significant decrease in MMP and an increase in superoxide production (2.1- and 5.6-fold compared to that of the control 24 h after irradiation, respectively). (Fig. 3A and B).

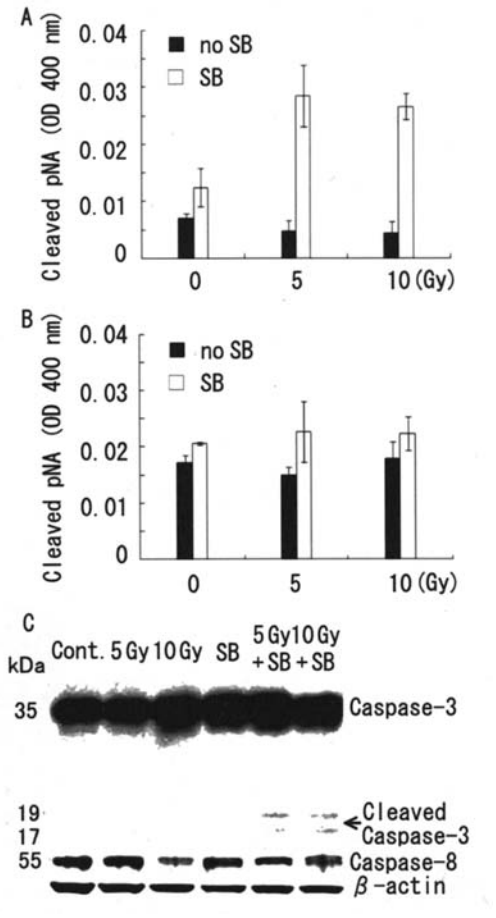

Figure 4. Enzyme activities and protein expression of caspase-3 and -8 in HCT 116 cells after X-irradiation with or without SB treatment. (A) Caspase-3 activity. (B) Caspase-8 activity. (C) Expression of caspase-3 and -8 detected by Western blotting.

However, irradiation led to no significant decrease in MMP but induced a gradual, slight increase in superoxide production followed by a surge during the second day after irradiation. The (SB + R) group showed a decreased MMP and an increased superoxide production (3.0- and 8.1-fold compared to that of the control 24 h after irradiation, respectively), similar to SB but at higher proportions. This similarity augments the hypothesis that radiation enhances the SB-induced apoptosis. These results also suggest that the enhancement of apoptosis involves the mitochondrial pathway under a combined treatment.

Enzymatic activity of caspase-3 and -8. To clarify whether the enhanced apoptosis is due to the caspase-dependent mitochondrial pathway, we studied the change in caspase-3 activity. The enzymatic activity of caspase-3 increased by 1.7-fold (SB) and ~4.0-fold (SB + R) versus that of the control group, despite the lack of a significant increase in the irradiation groups (Fig. 4A). To confirm these results the protein expression was also detected by Western blotting (Fig. 4C). No cleaved components were detected in the ion-alone-treated groups. This suggests that caspase-3 is partially responsible for the synergistic effect in the combination groups.

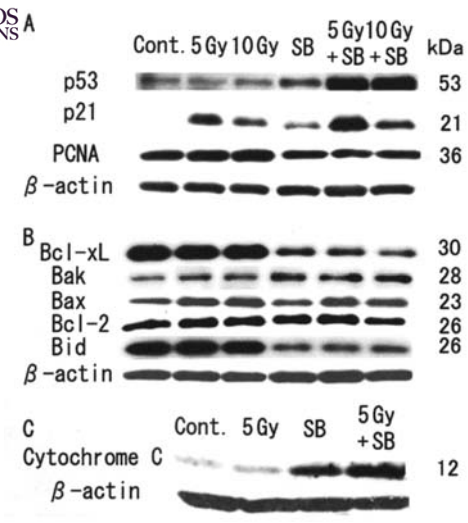

Figure 5. Western blotting of protein expression. HCT 116 cells were X-irradiated at a dose of 5 or 10 Gy followed by incubation with 1 mM SB for 24 h. Changes in the protein expression levels were then examined. (A) p53, p21, cyclin D1 and PCNA. (B) Bcl-2 family proteins. (C) Cytochrome c.

To further determine whether the activated pathway is intrinsic or extrinsic, we studied the activity of caspase-8. Although the increase in activity is statistically significant, the increase is very slight when compared to the control group (Fig. 4B). Furthermore, caspase-8 levels were almost the same among all the groups, and no cleaved components were detected (Fig. 4C). Thus, it seems that caspase-8 is not the primary-activated caspase protein, and that the extrinsic pathway has little effect on the enhanced apoptosis.

Expression of apoptosis-related proteins. To investigate the mechanisms of cell death and cell growth arrest, we measured the expression levels of apoptosis-related proteins. Notably, the SB group exhibited large increases in p53 and p21 expression levels whereas the irradiated group showed a significant increase in the p21 expression level. The (SB + R) group expressed p53 and p21 proteins significantly (Fig. 5A). The PCNA protein expression was also down-regulated with SB treatment.

Within the Bcl-2 family, the proteins showed an increased Bak expression level and decreased Bcl-xL and Bid expression levels with SB treatment while Bax showed an enhanced expression with X-ray treatment. However, no change in the expression level of Bcl-2 was observed after either treatment (Fig. 5B). There was an overall increase in the production of apoptotic proteins with a concomitant decrease in the production of anti-apoptotic ones in the SB and X-ray-treated groups (Table I).

Cytochrome c was released in large amounts in the groups (SB + R) and SB, although this was also slightly detected in the (R) group (Fig. 5C). The result was consistent with the decrease in MMP.

Discussion

In this study, we determined whether radiation can exert any potentiation on SB-induced apoptosis in HCT 116 cells. We found that radiation synergistically promotes cell death induced by SB, and that the mechanism involves a pronounced activation of the apoptotic mitochondrial pathway with the specific alteration in the balance of anti- and pro-apoptotic Bcl-2 family proteins by different treatments.

Table I. Expression levels of proteins after specific treatment on the third day versus that in the control group.

Protein	SB	X-ray	SB + X-ray
P53	+	+	+
P21	+	+	+
PCNA	-	NC	-
Bcl-xL	-	NC	-
Bak	+	NC	+
Bax	NC	+	+
Bcl-2	NC	NC	NC
Bid	-	NC	-
Cytochrome c	+	+	+

+, increase; -, decrease and NC, no change.

Butyrate sensitizes clone A and D human colon cancer cells to radiation (7). Herein, we adopted HCT 116 cells, a colon cancer cell line with wild-type p53, and exposed them to SB one day before irradiation and maintained them even after irradiation had been completed for the entire course of the experiment. The SB dose used was 1 mM. This dose has been shown to cause minimal DNA fragmentation and apoptosis (13.2±1.2%) in a dose- and time-dependent manner. Under such conditions, we determined that the combination induced stronger growth inhibition and a more pronounced cell death, as shown by the growth curves and killing rates. We also observed that there was a marked increase in the number of sub-G1 phase cells compared to the SB-treated group. Although X-ray-alone treatment did not significantly induce cell death, we observed the synergistic effect on cell death compared to the SB-alone treatment (18-13%, $p<0.005$) in DNA fragmentation assay. Therefore, we should further study why the combination treatment induces enhanced cell death.

Members of the Bcl-2 family of proteins are central regulators of mitochondrial integrity and apoptotic cell death (19). Mammals possess an entire family of Bcl-2 proteins that includes pro- and anti-apoptotic members. The ratio of anti- to pro-apoptotic molecules such as Bcl-2/Bax constitutes a rheostat that sets the threshold of susceptibility to apoptosis for the intrinsic pathway, which utilizes organelles such as the mitochondrion to amplify death signals. Bax or Bak is an essential gateway to the mitochondrial dysfunction required for cell death in response to diverse stimuli (20). In our study, enhanced Bak expression was observed with SB treatment, whereas enhanced Bax expression was observed with X-ray treatment. From our data, the expression levels of Bak and Bax increased with the combined treatment, probably contributing to the enhanced cell death. This is consistent with the result of the cytochrome c release, decreased MMP, caspase-3 cleavage and DNA fragmentation.

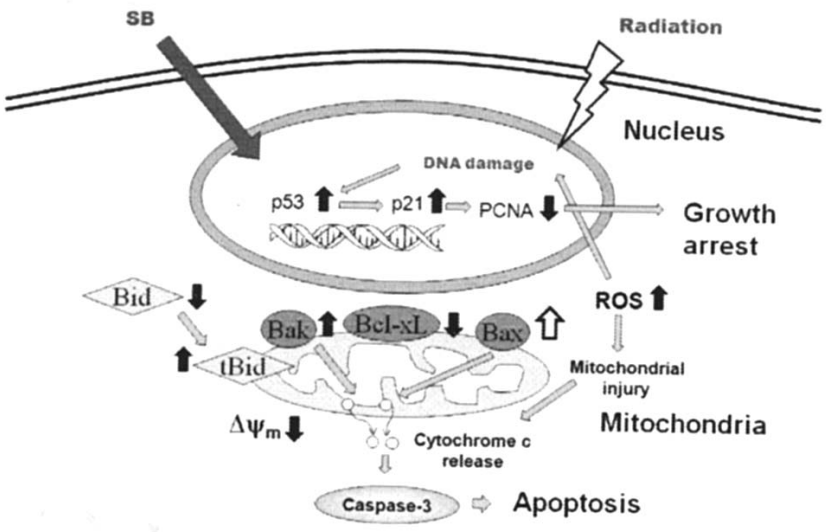

Figure 6. Mechanisms related to the enhancement of cell death by treatment with SB combined with X-irradiation. SB treatment and X-irradiation regulate p53 and p21 proteins to arrest cell growth. However, SB treatment alone changes the expression of Bcl-xL, Bak and Bid whereas X-irradiation alone enhances Bax expression. Since caspase-3 cleavage and cytochrome c release are markedly enhanced, the enhanced cell death is associated with the stronger activation of the mitochondria caspase pathway. Due to SB treatment or X-irradiation the superoxide formation also contributes to the enhancement of apoptosis when the two agents are combined. The upward arrows indicate stimulatory modification and the downward arrows indicate inhibitory modification. The white arrow besides the term Bax emphasizes the action of the X-rays.

Of note is that the Bid expression level markedly decreased in all the SB-treated groups. Bid is cleaved and activated to tBid after activation, which induces the oligomerization of Bak and Bax, inducing cytochrome c efflux (21). The change in the expression of Bid may be involved in the enhanced cell death in this study. However, we cannot confirm the factor responsible for Bid activation, because one of the upstream proteins, caspase-8, is not activated efficiently. Therefore, a death receptor may not be the major target during the treatment.

In contrast, the anti-apoptotic protein Bcl-xL was down-regulated in all the SB-treated groups, whereas Bcl-2 showed a constant expression level in all the groups. The antagonistic relations of Bcl-2 (22) or Bcl-xL/Bax (23) and Bcl-xL/Bak (24) have been demonstrated. Impairment in the expression of the anti-apoptotic proteins suggests that the enhanced effects in the combination groups and the attenuated effects in the irradiation groups are due to the change in the degree of antagonism. For the latter, we also found that the percentage of low MMP is lower than normal. This is also evidence of the inhibition of the mitochondrial pathway. There was an overall increase in the production of the apoptotic proteins with a concomitant decrease in the production of the anti-apoptotic ones in SB and X-ray-treated groups. We can conclude that enhanced cell death is achieved by the enhanced activation of the mitochondrial pathway as a result of the superiority of the pro-apoptotic Bcl-2 family members.

Concerning the upstream factors of the Bcl-2 family, p53 is one of the most important proteins. It can form complexes with the protective Bcl-xL and Bcl-2 proteins (25) and regulate Bax and Bak (26). In this study, SB induced a high p53 expression level. A higher p53 expression level was observed in the combination group. However, no notable change was detected in the irradiation group. This suggests that p53 expression is according to the cell death effect and that the absence of p53 expression in the irradiation group results in resistance to X-ray radiotherapy of colorectal HCT 116 cells. This can also be considered one of the bases of the combined treatment.

In this study, cell growth arrest was also observed and a significant change was noted in the combined group. P21 is necessary for the p53-mediated G1 (27) and G2 (28) arrests. Antiproliferative effects require p21 gene expression (29). To elucidate the mechanism of enhancement, we examined the p21 expression by Western blotting. The p21 expression level was significantly elevated in the SB and irradiation groups and further increased in the combination group, which is confirmed by the cell growth curve. Since p21 was shown to inhibit cell cycle progression by inhibiting the functions of cyclin/CDK complexes, and the PCNA function in G1 and G2 arrests (30), we further analyzed the PCNA and cyclin D1 expression. PCNA was down-regulated in the SB-treated group, while no change was observed in the irradiation group. As for cyclin D1, we observed an increase in the SB-treated groups (data not shown). Furthermore, in the cell cycle analysis, only the G2/M blockade was observed in all the groups. These results suggest that the growth arrest occurred in the G2 but not the G1 phase, which explains the increase in the cyclin D1 expression level. As to the effector of the growth arrest, PCNA is at least a promoter. Further work is needed for clarification.

The enhancement of intracellular superoxide formation is closely related to mitochondrial dysfunction and cell apoptosis (31). In our study, a close correlation was shown in that the decrease in MMP is associated with the enhancement of the superoxide formation except for the X-ray treatment. The explanation may be that SB can inactivate some protection against oxidative damage to the mitochondrial. Alternately, this effect is responsible for the enhanced cell death in the combination group.

SPANDIDOS PUBLICATIONS have proven the presence of mutual potentiation by butyrate and radiation adjuvant therapy. We also explored the interplaying molecular pathways that culminate in cell death (Fig. 6). With the combined treatment, the apoptotic mitochondrial pathway was enhanced significantly, and found to be involved in the increased expression levels of p53 and p21, the release of cytochrome c and the alteration in the balance of anti- and pro-apoptotic Bcl-2 family proteins. An enhanced superoxide formation was observed. However, the death receptor pathway was found to play no role in this phenomenon.

Acknowledgements

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