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BIOLOGY CONTRIBUTION

SUSCEPTIBILITY OF CANCER CELLS TO β-LAPACHONE IS ENHANCED BY IONIZING RADIATION

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Purpose: To reveal the interaction between β-lapachone (β-lap) and ionizing radiation (IR) in causing clonogenic death in cancer cells and to elucidate the potential usefulness of β-lap treatment in combination with radiotherapy of cancer.

Methods and Materials: FSaII tumor cells of C3H mice were used. The cytotoxicity of β-lap alone or in combination with IR *in vitro* was determined using clonogenic survival assay method. The IR-induced changes in the expression and the enzymatic activity of NAD(P)H:quinone oxidoreductase (NQO1), a mediator of β-lap cytotoxicity, were elucidated and the relationship between the NQO1 level and the sensitivity of cells to β-lap was investigated. The combined effect of IR and β-lap to suppress tumor growth was studied using FSaII tumors grown subcutaneously in the thigh of C3H mice.

Results: β-Lap caused clonogenic death of FSaII tumor cells *in vitro* in a dose- and time-dependent manner. When cells were treated first with β-lap and then exposed to IR *in vitro*, the resultant cell death was only additive. On the contrary, exposing cells to IR at 2.5 Gy first and then treating the cells with β-lap killed the cells in a synergistic manner. Importantly, the 2.5 Gy cells were sensitive to β-lap as long as 10 h after irradiation, which was long after the sublethal radiation damage was repaired. Irradiation of FSaII cells *in vitro* with 2.5 Gy significantly increased the expression and enzymatic activity of NQO1. The growth delay of FSaII tumors caused by an intraperitoneal injection of β-lap in combination with 20 Gy irradiation of tumor was significantly greater than that caused by β-lap or 20 Gy irradiation alone.

Conclusion: The sensitivity of cells to β-lap is dependent on NQO1 activity. IR caused a long-lasting increase in NQO1 activity in cancer cells, thereby sensitizing cells to β-lap and treatment of experimental mouse tumors with IR and β-lap suppressed tumor growth in a synergistic manner. The combination of β-lap and radiotherapy is a potentially effective regimen for the treatment of human cancer. **© 2005 Elsevier Inc.**

β-lapachone, Ionizing radiation, NQO1, FSaII tumors.

INTRODUCTION

β-Lapachone (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b] pyran-5,6-dione) (β-lap) was originally isolated from the bark of the Lapacho tree (*Tabebuia avellanedae*) growing in South America (1). This drug has attracted considerable interest in recent years because of its potent cytotoxicity against various cancer cell lines through a mechanism that works independent of the cell cycle or p53 status (1–8). β-Lap–induced cell death *in vitro* has been previously attributed to activation or inhibition of Topoisomerase I, inhibition of Topoisomerase II-α, and suppression of NF-κB activity (1). However, more recent studies have clearly indicated that none of these changes were the key determinant of cell death caused by β-lap, particularly *in vivo* (9).

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Recently, NQO1 (NAD(P)H:quinone oxidoreductase) has been reported to be a key player in β-lap–induced cell death (9). NQO1 catalyzes a two-electron reduction of β-lap to the hydroquinone form of β-lap, i.e., β-lap(HQ), using NADH or NAD(P)H as electron donors (9). The resulting β-lap(HQ) is unstable and consequently reoxidizes in the presence of O_2 to original oxidized β-lap, causing a futile cycling between the quinone and hydroquinone forms. This futile cycling of the drug causes a progressive depletion of NADH or NAD(P)H levels (3, 5, 9–12). In breast cancer cells, one β-lap molecule oxidized as many as 40 or more molecules of NAD(P)H (9). The depletion of NADH or NAD(P)H then leads to a massive release of Ca^{2+} from the endoplasmic reticulum into the cytosol, thereby depleting ATP levels (3–5) and depolarizing mitochondrial membranes causing release of cytochrome C (3, 5, 13). It has been suggested that the aforementioned disturbance in ionic homeostasis and the intracellular environment triggers apoptosis via calpain-like protease activation (3–5, 9), resulting in cleavage of vital proteins and fragmentation of DNA. Another proposed mechanism underlying β-lap cytotoxicity is that when β-lap(HQ) is oxidized to one-electron-reduced intermediate (i.e., semiquinone β-lap$(SQ)^{\bullet -}$), reactive oxygen species (ROS) are generated causing cell death (1, 9). Unlike the apoptosis caused by most DNA-damaging agents, the β-lap–induced apoptosis is unique in that caspases and p53 are not involved (2, 4, 5, 10). However, human breast cancer cells exposed to low continuous doses of β-lap have been reported to activate caspase-mediated apoptotic pathways, which is similar to other cytotoxic agents (5). It is likely that under certain conditions, β-lap may induce caspase-dependent apoptosis probably through the production of ROS, which causes caspase-dependent apoptosis. It should be noted that both the depletion of NADH or NAD(P)H levels and ROS generation by β-lap are consequences of the reduction of β-lap, which is dependent on NQO1 activity. Another proposed mechanism for the β-lap cytotoxicity is that the drug causes apoptosis by activating an S-phase checkpoint, thereby perturbing cell-cycle progression (14, 15).

Interestingly, β-lap has been reported to react synergistically with Taxol (6), mitomycin C (16), genistein (17), and ionizing radiation (IR) (7, 8, 18–20) *in vitro* against cultured cancer cells. It has also been reported that β-lap inhibits the repair of potentially lethal radiation damage by converting repairable single-stranded DNA breaks into repair-resistant, double-stranded DNA breaks (8, 20). Thus β-lap has been thought to act as a radiation sensitizer by inhibiting DNA damage repair. Previously, NQO1 was isolated as an IR-inducible transcript, xip3 (21, 22). In the present study, we observed that IR sensitizes cancer cells to β-lap by causing a long-lasting elevation of NQO1 activity. It thus appeared that the synergistic interaction of IR and β-lap in killing cancer cells was due to an increase in cellular susceptibility to β-lap, probably in addition to β-lap–induced radiosensitization.

Importantly, the NQO1 level in many human tumors is markedly greater than that in normal tissues (1, 23, 24). The fact that NQO1 level in tumors can be increased further by IR suggests that the combination of β-lap and radiotherapy may be a potentially effective approach to selectively damage tumors relative to normal tissue.

METHODS AND MATERIALS

β-lapachone, cells and clonogenic survival assay

β-Lap (3,4-dihydro-22,2-dimethyl-2H-naphthol[1,22b]pyran-5,6-dione) was purchased from a commercial source (Sigma, St. Louis, MO) or prepared by us as described previously (4). It was dissolved in dimethyl sulfoxide at 10 mM, diluted to desired concentration in RPMI 1640 medium, and used to treat cells with β-lap alone or in combination with IR *in vitro*.

FSaII cells, a fibrosarcoma of C3H mice (25), were used. Cells were maintained in RPMI 1640 medium (GIBCO BRI, Grand Island, NY) supplemented with 10% bovine calf serum (Hyclone Laboratories Inc., Logan, UT), penicillin (50 units/mL), and streptomycin (50 μg/mL) at 37°C in a humidified 95% air-5% CO_2 atmosphere. The population doubling time of FSaII cells used in the present study was 23 ± 3 h *in vitro*. For experiments, cells in exponential growth phase were dispersed to single cells by treatment with 0.25% trypsin for 10 min, washed twice with medium containing 10% bovine calf serum, and appropriate numbers of cells were seeded in 25 cm^2 plastic tissue culture flasks with 5 mL RPMI 1640 medium. After an overnight attachment period, the effects of β-lap alone or in combination with IR exposures were examined. For clonogenic survival assays, mock or experimentally treated cells were cultured for 7–8 days after treatment, and colonies were fixed with a mixture of methanol and acetic acid (10:1 v/v) and stained with 1% crystal violet. Colonies containing more than 50 cells were scored.

Determination of sublethal radiation damage repair

FSaII cells in exponential growth phase were irradiated with 5 Gy either in a single dose or two doses of 2.5 Gy separated by 1–10 h. During the interval of two irradiation exposures, cells were maintained in a 37°C incubator. The irradiation of cells was done with a ^{137}Cs irradiator (Model 68, J.L. Shepherd and Associates, Glenwood, CA) at a dose rate of 0.9 Gy/min. Clonogenic survival of the cells irradiated with a single dose or fractionated doses was determined, as mentioned previously.

Effect of dicoumarol on β-lap cytotoxicity

We assessed the potential involvement of NQO1 in the cell death caused by β-lap alone or in combination with IR against FSaII tumor cells using dicoumarol [3–3′methylene-bis(4 hydroxycoumarin)], an inhibitor of NQO1. Cells were incubated with 5 μM β-lap alone or with 50 μM dicoumarol for 4 h at 37°C and the clonogenic cell survival was determined. The effect of dicoumarol on the combined effect of IR and β-lap was studied by incubating the cells with 5 μM β-lap and 50 μM dicoumarol for 4 h beginning immediately after 5 Gy irradiation.

Immunostaining and confocal microscopic study of NQO1

Cells were cultured on tissue culture chamber slides and irradiated. The cells were rinsed with phosphate-buffered saline (PBS), fixed with acetone:methanol (1:1) for 20 min, and blocked with 1%

bovine serum albumin. Cells were then incubated with anti-NQO1 antibody (1:100 dilution in PBS) for 2 h followed by incubation with secondary antibody conjugated with FITC (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) for 1 h. After washing the labeled cells four times with PBS, NQO1 was visualized with a laser scanning confocal microscope (BioRad), and photomicrographs were taken.

Western blot analysis for NQO1

Cells were washed twice with ice-cold PBS and dissolved in solubilizing buffer (pH 7.4, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 20 mM Tris-HCl, 150 mM NaCl, I mM EDTA, 1 mM sodium orthovandate, 1 mM sodium fluoride, 2 mM phenylmethyl sulfonyl fluoride, 10 mM iodoacetamide, 10 μg/mL aprotinin, and 10 μg/mL leupeptin). Aliquots containing 50 μg of protein were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated polypeptides were then transblotted onto Hybond-P (Amersham Life Sciences, Inc., Arlington Heights, IL) in transfer buffer (192 mM glycine, 25 mM sodium dodecyl sulfate, and 10% methanol). Blots were then blocked with 3% nonfat dry milk in pH 7.4 TBST, and incubated with anti-NQO1 antibody (1:100 dilution, sc-16464; Santa Cruz Biotech, Inc, Santa Cruz, CA) and treated with horseradish peroxidase-conjugated anti-goat immunoglobulin G secondary antibody (1: 1000 dilution, Santa Cruz Biotech, Inc.). Immunoreactive bands were visualized using chemiluminescence. Equal sample loading was confirmed by reprobing the same blots with mouse monoclonal antiserum against α-tubulin.

NQO1 enzymatic activity assays

Cellular extracts for NQO1 activity assays were prepared before and after 2.5 Gy irradiation. Cells were harvested by trypsinization (0.25% trypsin and 1 mM EDTA), washed twice with ice-cold PBS, phenol red free Hanks' balanced salt solution, then resuspended in PBS, pH 7.2, containing 10 μg/mL aprotinin. Cell suspensions were sonicated four times using 10-s pulses on ice (Fisher Sonic Dismembrator), and S9 supernatants were harvested by centrifugation at 14,000 $\times$ *g* for 20 min. The resulting S9 supernatants were collected and aliquoted into microcentrifuge tubes and stored at -80°C until NQO1 enzyme activity was assessed (9).

NQO1 enzyme activities were assessed as previously described (26). Reaction medium contained 77 μM cytochrome C (practical grade, Sigma Chemical Co., St. Louis, MO) as substrate and 0.14% bovine serum albumin in Tris-HCl buffer (50 mM, pH 7.5). NQO1 activity was measured using NADH (200 μM) as the immediate electron donor and menadione (10 μM) as the intermediate electron acceptor. Each assay was performed in the presence and absence of dicoumarol, and the activity which was reduced by dicoumarol was taken as NQO1 activity. Assays were done at 37°C using a Beckman DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA), and were initiated by addition of S9 supernatants. Enzyme activities were calculated as nmoles cytochrome C reduced/min/μg protein, based on the initial rate of change in OD at 550 nm and an extinction coefficient for cytochrome C of 21.1 mM/cm.

Effect of β-lap alone or in combination with X-irradiation on tumor growth

Exponentially growing FSaII tumor cells in culture were harvested, washed, and suspended in serum-free RPMI medium. About 0.05 mL of the suspension containing 2×10^5 tumor cells was injected subcutaneously into the right thighs of female C3H mice weighing 20–23 g. When tumors grew to 7–8 mm in diameter, a group of mice were intraperitoneally injected with 50 mg/kg β-lap dissolved in 0.2 mL of HP-β-CD (β-hydroxypropyl-β-cyclodextrin) (27). A second group of mice bearing tumors of equivalent volumes were treated with 20 Gy X-irradiation in a single exposure. Finally, a third group of tumor-bearing mice received combined treatment: mice were injected intraperitoneally with β-lap at 50 mg/kg and the tumors were treated with 20 Gy X-irradiation 30 min after drug injection. The mice that did not receive β-lap injection received an injection of 0.2 mL of solvent, HP-β-CD, only.

For the irradiation of tumors grown in the thigh, mice were anesthetized with an intraperitoneal injection of a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine. This anesthesia method has been routinely used in our laboratory and causes little cardiovascular change in rodents (28). Except for the tumor area, the whole body of the anesthetized mice was covered with a 4-mm-thick lead shield and the tumors were irradiated with 250-kV orthovoltage X-rays (added filtration of 1.0 mm Al and 0.35 mm Cu) (Philips Medical System, Brookfield, WI) at 1.4 Gy/min. Control animals were mock-irradiated.

Tumor diameters were measured with a caliper, and tumor volumes were calculated using the formula: $V = a^2b/2$, where a was the shortest tumor diameter and b was the longest tumor diameter measured. For each experimental group, between seven and nine tumors were used and the average volumes of the tumors were obtained. The mice were euthanized by cervical dislocation while under anesthesia when tumor size reached 1.3–1.5 mm^2. All experiments were performed following protocol approved by the University of Minnesota Institutional Animal Care Use Committee (Protocol number: 0112A13064).

RESULTS

β-lap cytotoxicity against FSaII cells

Figure 1 shows changes in % survival of clonogenic FSaII cells treated with different concentrations of β-lap for varying lengths of time. When cells were incubated with 2.5 μM β-lap, survival declined only slightly during the first 6 h, but decreased rapidly thereafter. In contrast, incubation of cells with 5 μM β-lap for only 2 h significantly decreased survival. The clonogenic cell survival decreased to 12.8% and 1.5% after incubation with 5 μM β-lap for 4 h and 6 h, respectively. Incubation of cells with 10 μM β-lap for 6 h resulted in less than 0.03% survival.

Irradiation increases the β-lap–induced clonogenic cell death

Figure 2 shows the survival curve of FSaII cells treated with various doses of IR in combination with a 4-h treatment of 5 μM β-lap, applied either before or immediately after IR exposure. The Do of the radiation survival curve of control cells (radiation therapy only) was 125 cGy and that of the cells treated with β-lap for 4 h, washed, and irradiated was 123 cGy. This result indicated that the radiosensitivity of cells was not affected by prior treatment with β-lap. On the other hand, the Do of the radiation survival curve of

cells treated with β-lap for 4 h immediately after radiation exposure was 82.5 cGy. It was concluded that β-lap treatment and IR reacted in a synergistic manner when β-lap treatment was applied after radiation exposure, whereas the two treatment regimens reacted additively when β-lap treatment was applied before radiation exposure. The effect of adding β-lap to cells immediately before IR and washed 4 h later (data not shown) was identical to the effect of treating the cells with β-lap for 4 h starting immediately after radiation exposure.

To further analyze the interaction between IR and β-lap in reducing cancer cell clonogenicity, we irradiated FSaII cells with 2.5 Gy and then treated them with 5 μM β-lap for 4 h starting at various times after IR exposure. Figure 3 shows that 2.5 Gy alone and β-lap treatment alone reduced cell survival to 38.3 ± 5.6% and 12.3 ± 1.3%, respectively. The dotted line shows the expected % survival (4.7%) if 2.5 Gy IR and 4 h treatment with 5 μM β-lap reacted additively. When cells were irradiated and immediately treated with 5 μM β-lap for 4 h, cell survival decreased to 1.5 ± 0.4%, indicating that the combined effect of IR exposure and 5 μM β-lap in reducing clonogenic survival of FSaII cells was significantly greater than the additive effect. Importantly, a 4-h treatment with β-lap applied beginning 3 h or 5 h after IR exposure was as effective as that applied immediately after irradiation in reducing cell survival. The combined effect of IR and β-lap was greater than additive even when β-lap treatment was started 10 h after IR exposure, but it diminished to the additive level when the time interval between the radiation exposure and the β-lap treatment was increased to 24 h.

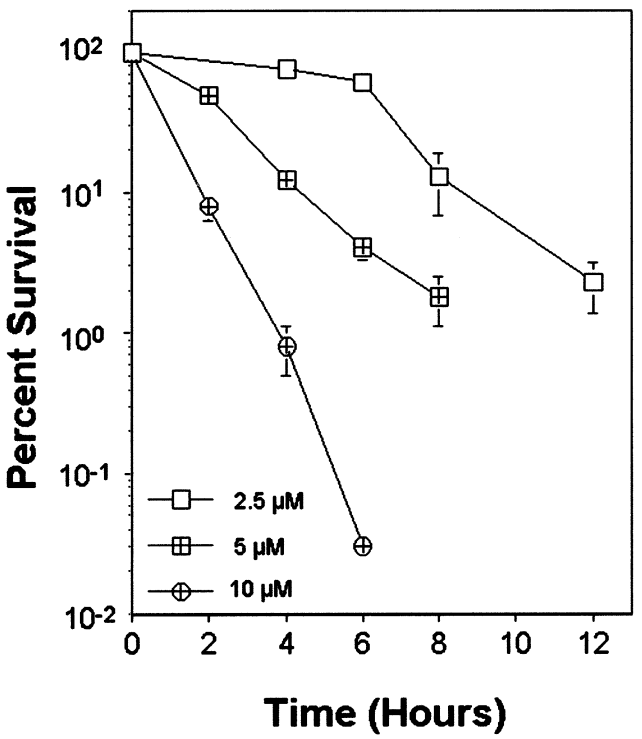

Fig. 1. Cytotoxicity of β-lapachone (β-lap) on FSaII cells. Cells were incubated with different concentrations of β-lap at 37°C for varying lengths of time, rinsed with fresh medium, and cultured for 8–9 days with regular medium. The number of colonies formed with more than 50 cells were counted and the percentage of survival calculated. Each data point shows the average of five to seven experiments with duplicate cultures ± standard error.

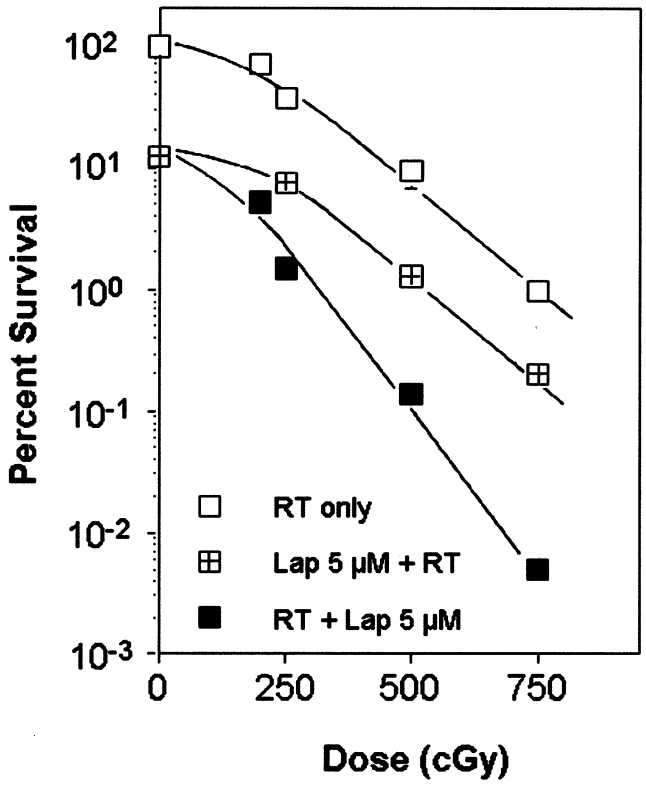

Fig. 2. Survival curves of FSaII tumor cells treated with ionizing radiation alone (radiotherapy only) or with β-lapachone (β-lap) treatment. Cells were incubated with 5 μM β-lap for 4 h, rinsed, and then irradiated (lap 5 μM + RT) or cells were irradiated first, then incubated with 5 μM β-lap and rinsed (RT + lap 5 μM). After the treatments, cells were cultured for 8–9 days, the number of colonies were counted, and percentage survival was calculated. Averages of seven to nine experiments with duplicate cultures ± 1 standard error are shown.

Dicoumarol suppresses the effect of β-lap alone or in combination with IR

The effects of dicoumarol on the clonogenic death of FSaII tumor cells caused by 5 μM β-lap alone or in combination with 5 Gy irradiation are shown in Fig. 4. The 4-h incubation of cells with 50 μM dicoumarol was slightly toxic, reducing the cell survival to about 75.1 ± 5.8%. The 4-h incubation with 5 μM β-lap reduced the cell survival to 9.4×10^{-2}, whereas the 4-h incubation with 5 μM β-lap with 50 μM dicoumarol resulted in cell survival of 18.8×10^{-2}. Irradiation with 5 Gy followed by a 4-h incubation with 5 μM β-lap decreased the cell survival to 6×10^{-4}, whereas concomitant incubation of cells with 5 μM β-lap and 50 μM dicoumarol immediately after 5 Gy irradiation reduced the cell survival to 40×10^{-4}. These results

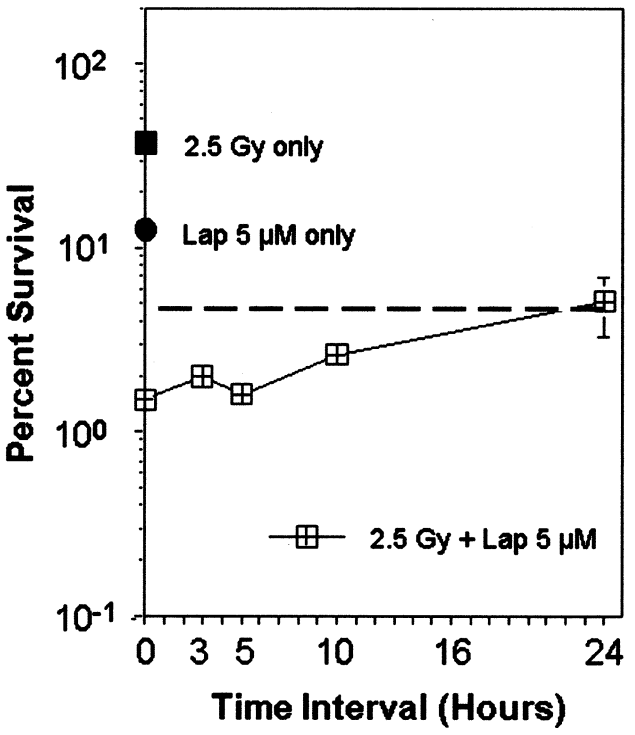

Fig. 3. Effects of β-lapachone (β-lap) treatment applied at different times after irradiation of cells. FSaII cells were irradiated with 2.5 Gy and incubated with 5 μM β-lap for 4 h starting at different times after ionizing radiation, as indicated. After the treatments, cells were rinsed and cultured for 8–9 days and the colonies were counted. The average of six to eight experiments with duplicate cultures ± 1 standard error are shown.

demonstrated that inhibition of NQO1 activity markedly inhibits the cell death caused by β-lap alone or in combination with IR.

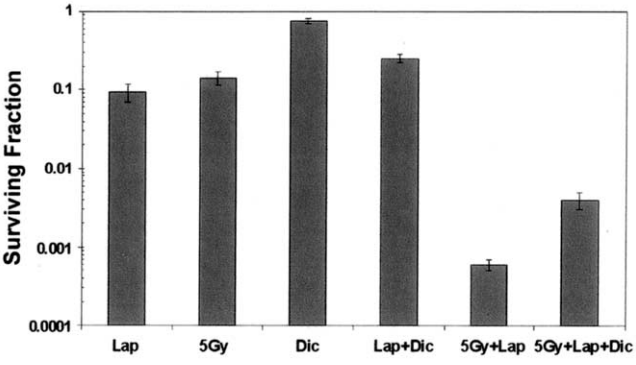

Fig. 4. Effect of dicoumarol on the cell death caused by β-lapachone (β-lap) alone or in combination with irradiation in FSaII cells. Lap = treated with 5 μM β-lap; 5 Gy = irradiated with 5 Gy; Dic = treated with 50 μM dicoumarol; Lap + Dic = treated with 5 μM β-lap +50 μM dicoumarol; 5 Gy + Lap+Dic = irradiated with 5 Gy and then treated with 5 μM β-lap +50 μM dicoumarol. The averages of six experiments with duplicate cultures ± 1 standard error are shown.

IR increases NQO1 levels

Because NQO1 has been reported to be a major determinant for β-lap–mediated cell death (9, 10), and because this protein can be induced in some cells by IR (22), we examined changes in NQO1 levels in FSaII tumor cells after IR. Figure 5 shows confocal micrographs of cells stained with anti-NQO1 antibody at various times after an exposure to 2.5 Gy IR. NQOI levels increased by 2 h after irradiation and further increased 4–8 h after IR. The NQO1 level 24 h after IR was slightly less than that at 8 h after IR exposure. Figure 5b shows a Western blot for NQO1 levels in FSaII tumor cells at various times after an exposure to 2.5 Gy. Consistent with changes in expression of NQO1 by confocal analyses, the NQO1 expression in the Western blot increased by 2 h after IR, peaking at 4–8 h, and then gradually declined. Finally, the enzymatic activity of NQO1 in FSaII tumor cells, calculated as nmoles of cytochrome C reduced/min/μg protein, was also found to markedly increase after an exposure of cells to 2.5 Gy IR (Fig. 6). NQO1 activity increased threefold 4–8 h after 2.5 Gy irradiation, then began to decline although the enzyme activity was still greater than twofold the control level 8 h after 2.5 Gy IR. The enzyme activity returned to control level 24 h after IR.

Sublethal radiation damage is repaired rapidly in FSaII cells

Irradiation of FSaII cells with a single dose of 5.0 Gy reduced the cell survival to 9.5 ± 2.8%, whereas 5.0 Gy IR in two equal fractions of 2.5 Gy separated by 1 h decreased the cell survival to 34.5 ± 3.1%. The cell survival further increased to 38.1 ± 3.5% and 42.3 ± 4.9% when the interval between the two radiation exposures was increased to 5 h and 10 h, respectively. These results demonstrated that most of the sublethal damage caused by 2.5 Gy IR was repaired within 1 h in FSaII cells.

IR increases anti-tumor activity of β-lap

Figure 7 shows changes in the volume of FSaII tumors after various treatments. An intraperitoneal injection of 50 mg/kg β-lap suppressed tumor growth and enhanced the effect of IR to suppress the tumor growth. The numbers of days required for a fourfold increase in mean tumor volumes for control, β-lap alone, IR alone, and IR + β-lap groups were 5.3 ± 0.2, 7.1 ± 0.3, 11.1 ± 0.5, and 18.4 ± 0.3 days, respectively. Consequently, for the fourfold increase in tumor volume, the growth delay (difference between the control and treated groups) caused by β-lap alone, IR alone, and the combination of β-lap and IR treatment were 1.8 days, 5.8 days, and 13.1 days, respectively. The growth delay by β-lap alone was statistically significant ($p < 0.00009$), and the growth delay caused by β-lap plus IR was significantly longer than that caused by IR alone ($p < 0.01$). β-Lap treatment did not appear to increase the radiation-induced damage in normal tissues adjacent to the treated tumors, although the normal tissue damage was not quantitatively determined in the present study.

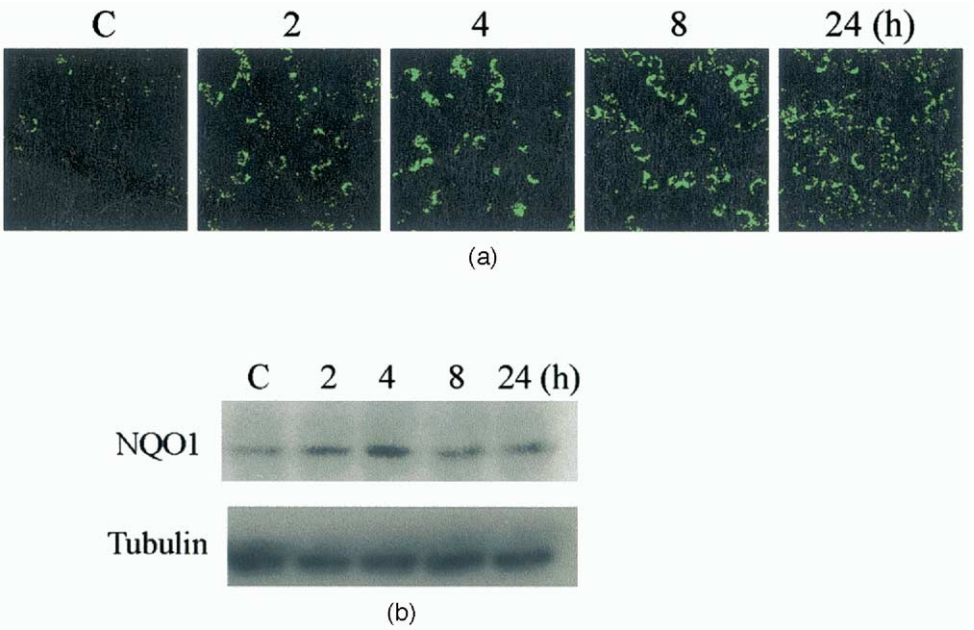

Fig. 5. The effect of 2.5 Gy irradiation on the expression of NQO1 in FSaII cells. (a) Confocal microscopy of fluorescein isothiocyanate (FITC)-stained NQO1 in FSaII cells. Cells were labeled with anti-NQO1 antibody followed by an incubation with secondary antibody conjugated with FITC. The labeled cells were imaged with a confocal microscope at 40×. (b) NQO1 expression in irradiated cells as determined by Western blot analyses using an NQO1-specific antibody. Shown in (a) and (b) are representative microphotographs of experiments repeated four times.

DISCUSSION

We have observed in the present study that β-lap is cytotoxic against FSaII tumor cells of C3H mice and that IR and β-lap synergistically react in causing tumor cell death *in vitro*. An intraperitoneal injection of β-lap to C3H mice bearing FSaII tumors in the hind leg suppressed tumor growth particularly when it was combined with IR. The synergistic effect of IR and β-lap against cancer cells appeared to be due mainly to a radiation-induced increase in the activity of NQO1, a mediator of β-lap toxicity.

It has been demonstrated recently that the cytotoxicity of β-lap is directly correlated to NQO1 expression in tumor cells (1, 9, 10). It has also been reported that NQO1 causes a futile cycling between the oxidized and two-electron reduced forms of β-lap, i.e., β-lap(HQ), utilizing NAD(P)H or

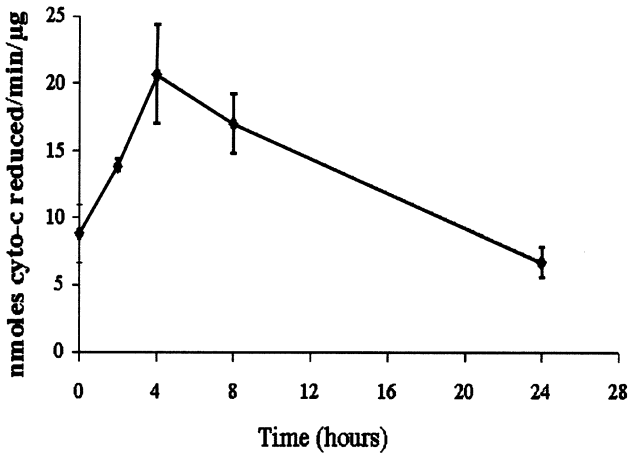

Fig. 6. Enzymatic activity of NQO1 after 2.5 Gy irradiation in FSaII tumor cells. At various times after irradiation, cells were sonicated and the NQO1 activity in S9 supernatants was determined. The NQO1 activities are expressed as nmoles cytochrome C reduced/min/μg protein. Data shown are an average of six determinations with duplicate cultures with ± 1 standard error.

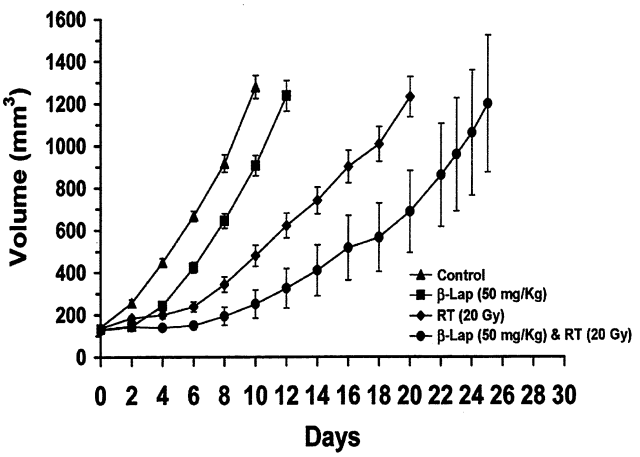

Fig. 7. Growth of FSaII tumors in the thighs of C3H mice after various treatments. Radiation therapy (RT) (20 Gy) = tumors were exposed to 20 Gy of X-ray in a single dose; β-lapachone (β-lap) (50 mg/kg) = host mice were injected intraperitoneally with β-lap at 50 mg/kg; β-lap (50 mg/kg) and RT = host mice were injected intraperitoneally with β-lap at 50 mg/kg; 30 min later tumors were exposed to 20 Gy of X-rays in a single dose. The averages of seven to nine tumors with 1± standard error are shown.

NADH as electron sources, thereby depleting NAD(P)H and NADH (3, 5, 9). The severe depletion of NAD(P)H and NADH from the futile cycling appeared to trigger apoptosis. Interestingly, the β-lap–induced apoptosis was reported to be p53-independent (2, 4, 5, 10). Another possible mechanism underlying the β-lap–induced cell death is that some of β-lap(HQ) formed through mediation of two-electron reductase NQO1 does not directly oxidize back to β-lap but oxidizes first to one-electron-reduced semiquinone β-lap(SQ)$^{\cdot}$ in the presence of oxygen, thus inducing redox cycling and generating cytotoxic reactive oxygen species (1, 9, 13). Although circumstantial evidence clearly indicates that NQO1 plays a cardinal role in β-lap–induced cell death, whether the direct cause of cell death is due to depletion of NAD(P)H and NADH or generation of ROS may depend on the concentration of β-lap and the availability of oxygen.

It has recently been proposed that β-lap selectively induces apoptosis in transformed cells by activating an S-phase checkpoint and inducing E2F1 in the absence of DNA damage (14, 15). This hypothesis excludes the involvement of NQO1 activity in β-lap–induced cell death, contrary to our observation in the present study (Fig. 4) and in previous reports (1, 3, 9, 10). Furthermore, in our recent study using A549 human lung cancer cells and RKO human colorectal cancer cells, β-lap treatment did not increase S-phase population, and the β-lap–induced apoptosis was independent of cell-cycle phase (unpublished observation).

As shown in Fig. 4, dicoumarol markedly suppressed not only the cell death caused by β-lap alone, but also that caused by β-lap in combination with IR, demonstrating that NQO1 is an important determinant of cell death caused by combination of β-lap and IR in FSaII cells. The confocal microscopy study (Fig. 5a), Western blot study (Fig. 5b), and the biochemical study (Fig. 6) clearly demonstrated that IR causes a prolonged increase in NQO1 activity in FSaII cells. As shown in Fig. 2, when cells were treated with β-lap before IR, the slope (Do) of the radiation survival curve was similar to that of the cells treated with IR alone, indicating β-lap and IR reacted merely additively. On the contrary, the radiation survival curve of cells receiving IR first and then treated with β-lap was significantly steeper than the survival curve of the cells treated with IR alone. It is highly likely that the β-lap treatment applied immediately after IR caused radiosensitization by inhibiting the radiation damage repair. On the other hand, because substantial portions of the sublethal damage caused by 2.5 Gy irradiation in FSaII tumor cells were repaired in 1 h (see "Results"), the synergistic interaction between 2.5 Gy IR and β-lap treatment applied 3–10 h after the IR (Fig. 3) was due mainly to an increase in β-lap–induced cell death as a result of IR-induced increase in NQO1 activity. However, because the sublethal damage repair may not complete, although it was substantial several hours after 2.5 Gy IR, the possibility that the synergisms between IR and β-lap applied with several hours apart was caused in part by β-lap–induced inhibition of sublethal damage repair may not be ruled out.

An intraperitoneal injection of 50 mg/kg of β-lap delayed the growth of FSaII tumors by 1.8 days and 20 Gy IR delayed the tumor growth by 5.8 days for a fourfold increase in tumor volume (Fig. 7). When tumors were treated with IR plus β-lap, tumor growth was delayed by 13.1 days, which was about 7 days longer than the growth delay by IR alone ($p < 0.01$). That is, the net growth delays by β-lap in unirradiated and irradiated tumors were 1.8 days and 7.3 days, respectively, which demonstrated that the irradiated tumors were far more sensitive to β-lap as compared with unirradiated tumors. In the present study, β-lap was injected intraperitoneally 30 min before tumor irradiation. Neither the concentration of β-lap in the tumors at the time of tumor irradiation nor the magnitude of increase in NQO1 in the tumors after 20 Gy IR was known in the present study. Nevertheless, it would be reasonable to expect that a considerable amount of β-lap was present in the tumors after irradiation and was activated by the IR-induced NQO1. Experiments to reveal the implication of timing of β-lap injection in relationship to tumor irradiation and also the effect of varying doses of β-lap to induce tumor growth suppression with minimal normal tissue damage are in progress in our laboratory.

In conclusion, IR of cancer cells causes a long-lasting increase in NQO1 activity, thereby increasing the sensitivity of cells to β-lap. In light of the fact that NQO1 is constitutively overly expressed intrinsically in many cancer cell lines (1, 23, 24) and that radiation exposures at doses as low as 2.5 Gy can significantly increase the activity of the enzyme, as demonstrated in the present study, further investigation on the feasibility of using β-lap in combination with radiotherapy to selectively kill human cancer cells appears to be warranted.

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