G₂/M is a critical phase to regulate X-ray-induced apoptosis in EL-4 mouse lymphoma cells

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The relationship between apoptosis induction by X-ray and by cell cycle regulation remains not well understood. In this study, using EL-4 mouse lymphoma cells we demonstrate that the majority of apoptotic cell death following X-irradiation is induced after G₂/M arrest. When cells were irradiated at a dose of 20 Gy, their viability began to decrease at 24 h. Gel electrophoresis of the extracted DNA showed multimers of 200 bp fragments, which are characteristic of apoptosis. An increase in the apoptotic fraction was observed when the X-irradiated cells began to accumulate in the G₂/M phase. The apoptotic rate was remarkably augmented by caffeine that inhibits the G₂ checkpoint system; this effect was clearly demonstrated by a suppression of the cyclin B1:cdc2 ratio. A remarkable decrease in bcl-2 expression may also be ascribed to the apoptosis-enhancing effect by caffeine. In contrast, an antimitotic drug, colchicine, did not affect the rate of DNA fragmentation. These results suggest that G₂/M is a critical phase in the regulation of apoptotic cell death and that X-ray-induced apoptosis can be triggered if mitotic events progress without repair of the damaged DNA. Despite X-irradiation's usual effect in increasing p53 expression, intracellular p53 levels in irradiated cells were significantly suppressed in the presence of caffeine. This demonstrates that the mechanism of X-ray-induced apoptosis is involved in the increased expression of p53 proteins, whereas enhancement of apoptotic cell death by caffeine is independent of p53 status.

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To repair damaged DNA and maintain the intact DNA structure in daughter cells, X-ray-irradiated cells stop the cell-cycle progression and go into a reversible arrest¹⁻³⁾. A moderate but not high dose of X-ray irradiation often induces apoptotic cell death in various cell lines, but its precise mechanisms are still unelucidated. In most cases, apoptosis can be induced when cells go through arrest without completing the repair of their damaged DNA. At present, two major cell-cycle checkpoints have been identified: One is in the late G₁ phase (the so-called "restriction point" or "G₁ checkpoint") which regulates cells entering into the S phase, and the other is in the G₂/M phase which ascertains the newly synthesized DNA to be intact and is concerned with the beginning of mitosis. Recent reports have indicated that the onset of apoptosis following X-ray irradiation is preceded by a period of G₂ arrest, and that the degree of G₂ arrest correlated well with cell viability^{4,5)}. If the degree of G₂ arrest can be altered by some chemicals that have a modulatory effect on cell-cycle progression, the role of the G₂ checkpoint in X-ray-induced apoptosis seems to become clearer. It has been reported that G₂-checkpoint inhibitors, such as caffeine, 2-aminopurine, IBMX, and 6-DMAP, can

induce premature chromosome condensation that enforce the cells to enter mitosis without completing the S phase⁶⁾. The X-irradiated cells override the G₂ checkpoint in the presence of these agents and suicidally progress into mitosis because their DNA was incompletely replicated or repaired. Although it is uncertain how the cell death program is activated after treatment with these agents, they do no harm to the cells in the absence of DNA damage.

The induction of apoptosis following DNA damage, including X-ray-induced DNA strand breaks, depends on the activation of p53. It is well-known that the level of p53 expression after treatment with DNA damaging agents is closely related to the rate of apoptosis, suggesting the importance of the G₁ checkpoint in the induction of apoptotic cell death⁷⁾. Recently it has been reported that fibroblasts from p53-knockout mice failed in the equal chromosome segregation to the daughter cells, which describes the involvement of p53 in G₂ checkpoint regulation⁸⁾. Since the magnitude of X-ray-induced cell damage is correlated with how rapidly the cells exit from G₂ checkpoint control, p53 may act as a modulator of apoptosis in the G₂ checkpoint as well as in the G₁ checkpoint. But other reports predict the existence of p53-independent pathways in X-ray-induced apoptosis^{9,10)}. In addition, some cell lines which fail to express p53 proteins have shown an obvious sensitivity to X-ray-induced apoptosis¹¹⁾. These contradictory reports demonstrate the

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Fig. 1 Effect of X-irradiation on cell growth and viability of EL-4 cells

Fig. 1A: Cells were cultured from 1×10^6 /ml at Day 0. Cell number was counted with a particle counter every 12 h and cell growth was plotted as the function of time after X-irradiation. Fig. 1B: Cell viability was measured by a trypan blue dye exclusion test. Symbols represent untreated control (■), 1 Gy (●), 10 Gy (▲), and 20 Gy (◆) X-irradiation.

difficulty in understanding the mechanism of X-ray induced apoptosis.

In this study, caffeine was applied to the X-irradiated cells to investigate the relationship between cell cycle regulation and X-ray-induced apoptosis. Apoptotic cell death after X-ray exposure was investigated especially in light of G_2 arrest modulation.

Materials and methods

Cell culture

EL-4 mouse T lymphoma cells were incubated in RPMI1640 medium (GIBCO) supplemented with 10% fetal bovine serum (Hyclone) at 37°C under a humidified atmosphere with 5% CO_2 . In a previous paper, the time required for passing through the S phase was calculated by the mid-S gating method, and the actual S phase-time of the actively dividing EL-4 cells was estimated to be about 6 hr¹².

X-ray irradiation

EL-4 cells in an exponentially growing phase were suspended in the medium at a concentration of 1×10^6 /ml in a 60×15 mm plastic culture dish. All X-ray irradiations were carried out at room temperature using a 150-kV Softex M-150W operating at 8 mA at a dose rate of 0.86 Gy/min. Immediately after irradiation, the cultures were returned to the CO_2 incubator.

Drug treatment

Caffeine (Wako Pure Chemical Industries), a cell-cycle checkpoint inhibitor, was dissolved in PBS at a concentra-

Fig. 2 Effect of X-irradiation on DNA fragmentation of EL-4 cells

DNA fragmentation at 24 h was analyzed by agarose gel electrophoresis. Cells treated with X-irradiation generated the ladder of 200 bp that are characteristic of apoptosis.

tion of 100 mM and was then added to the cell cultures at concentrations of 5 mM after X-irradiation. For examination of the response of the mitotic blocks to apoptosis, colchicine (Sigma) dissolved in DMSO was diluted and used at 200 nM. This dose of colchicine induces complete M-phase arrest but does not damage EL-4 cells.

Assay of cell growth and viability

Cells were initially plated at a density of approximately 5×10^5 per ml. Cells were serially sampled at the times indicated elsewhere and the number of cells was counted with a particle counter (ERMA PC-607, Tokyo). The cell's viability was observed using a trypan blue dye exclusion method.

Detection of DNA fragmentation by agarose gel electrophoresis

Twenty-four hours after X-irradiation, samples were harvested. Cells were lysed in cell membrane lysis buffer (CMLB; 0.32 M sucrose, 1% Triton X-100, 5 mM MgCl_2 , 12 mM Tris-HCl [pH 7.6]). The cell lysate was then centrifuged and resuspended in nuclear membrane lysis buffer (NMLB; 4 M guanidine hydrochloride, 12 mM Tris-HCl [pH 7.6], 12 mM EDTA, 375 mM NaCl, 0.5% sodium N-lauroyl sarcosinate, 0.1 M β -mercaptoethanol) at 55°C for 10 min. DNA was precipitated by addition of a 2.5 volume of ethanol. The DNA pellet was washed with 70% ethanol and resuspended in DNA preservation solution (10 mM Tris [pH 8.0] and 0.1 mM EDTA). After treatment with RNase, the DNA was electrophoresed on

Fig. 3 Effect of X-irradiation on cell cycle of EL-4 cells. Following 20Gy X-ray exposure, the position of the cell in the cycle was analyzed by flow cytometry. DNA histograms were analyzed by using DNA software, and the percentages of G₁ (—■—), S (—●—), and G₂/M (—▲—) populations were calculated.

a 2% agarose gel at 50 V for 1 h. The gel was stained with ethidium bromide for 20 min and photographed under ultraviolet light with FP-3000B film (Fuji Film). DNA fragments of apoptotic cells are characterized by multimers of 200 bp in the internucleosomal DNA cleavage.

Flowcytometric analysis of DNA fragmentation and cell cycle

Cells were processed by using Cycle TEST DNA reagent kit (Becton Dickinson) according to the instruction manual. Measurements of fluorescence signal were made with a FACStar^{plus} flow cytometer (Becton Dickinson) equipped with a 488 nm argon-ion laser. Data were acquired using selective gating to exclude doublet cells and analyzed with Consort 30 software. DNA fragmentation was recognized as a DNA population content of less than 2C (G₁) peak. In each experiment, the data from 5,000 to 10,000 cells were collected, stored, and analyzed. The fraction ratio of cells in each cell-cycle compartment was calculated with DNA software by using a polynomial model.

Immunoblotting analysis of cyclin B1, p34^{cdc2}, p53, and bcl-2

After being fixed by 10% trichloroacetic acid, whole cell extract was prepared according to the method described elsewhere¹². For Western blot analysis, cell extracts from each sample were separated on 7.5% sodium dodecyl sulfate (SDS)/polyacrylamide electrophoresis (PAGE) gel and were transferred to a polyvinylidene difluoride membrane (Clear Blot Membrane-p; ATTO Co.) by a semidry electroblotter (Horizeblott AE-6675, P/N type; ATTO Co.). The proteins were detected by using the alkaline phosphatase system kit (Kirkegaard & Perry Laboratories, Inc.) according to the instruction manual. Antibodies to cyclin B1 (clone GNS-1), p34^{cdc2} protein kinase (clone A17), p53 (clone PAb240), and bcl-2 (rabbit polyclonal antibodies) were purchased from PharMin-

Fig. 4 Effect of caffeine and colchicine on cell cycle and X-ray-induced apoptosis of EL-4 cells

Fig. 4A: EL-4 cells treated with 20Gy X-ray were cultured with 5 mM caffeine (cell cycle checkpoint inhibitor; left) or 200 nM colchicine (mitosis-blocking agent; right). The percentages of G₁ (—■—), S (—●—), and G₂/M (—▲—) populations were plotted every 4 h. Fig. 4B: Time course of the rate of apoptotic population is plotted as the function of time after X-irradiation. Remarkable increase in the apoptotic fraction is observed in cultures with caffeine but not in those with colchicine. Symbols represent X-ray exposure alone (—■—), X-ray + caffeine (—●—), and X-ray + colchicine (—▲—).

gen. After color development, the membrane image was digitized, incorporated into a Macintosh computer, and analyzed by using NIH image software (ver. 1.58). When SDS/PAGE was running, a control protein sample was used as a marker for comparing different groups.

Results

Effect of X-ray irradiation on cell growth and survival

After treatment with X-ray irradiation, EL-4 cells were cultured in a CO₂ incubator. Cell number (Fig. 1A) and surviving fraction measured by trypan blue dye exclusion (Fig. 1B), were observed every 12 h. Control cells showed an exponential growth rate and reached a concentration of approximately 1×10^7 cells/ml at 48 h. After 1 Gy irradiation, a remarkable suppression of cell growth was observed. In the case of 10 Gy or 20 Gy irradiation, cell

Fig. 5 Effect of caffeine on cyclin B:cdc2 ratio and bcl-2 levels in X-irradiated EL-4 cells

Fig. 5A: 20Gy X-irradiated EL-4 cells cultivated with or without 5mM caffeine were sampled every 4h, and whole-cell extracts were prepared. Each sample was separated on SDS-PAGE and analyzed by Western blot with monoclonal antibodies to cyclin B1 and p34^{cdc2}. The color intensity of each band was calculated, and cyclin B:cdc2 ratio was plotted as the function of time after treatment with X-ray. Symbols represent X-ray exposure alone (■) and X-ray + caffeine (●). Fig. 5B: bcl-2 expressions at the indicated time were plotted. Symbols indicate untreated 20Gy X-ray exposure alone (■), and X-ray + caffeine (●).

Fig. 6 Effect of caffeine on p53 levels in X-irradiated EL-4 cells. Cells were analyzed in a similar manner to Fig. 5. Photo reproduction of Western blot analysis with monoclonal antibodies to p53 is indicated (top). The color intensity of each band was calculated, and ratio to the value of control sample was plotted as the function of time after X-irradiation (bottom). Symbols represent 20Gy X-ray exposure alone (■) and X-ray + 5mM caffeine (●).

growth was completely suppressed during the observation period. Cell viability in the 20Gy irradiated group began to fall from 24h, and approximately 30% of the cells were killed at 48h. 10Gy treated EL-4 cells showed a remarkable growth arrest but did not reveal a decrease in cell

viability. Therefore, the 20Gy treatment was considered suitable for evaluating the apoptotic effect of X-ray in the EL-4 cells.

Morphology and DNA gel electrophoresis of X-irradiated EL-4 cells

Microscopic examination of the EL-4 cells 24h after 20Gy irradiation showed chromatin condensation, nucleic collapse, and the formation of small cell fragments (apoptotic bodies) which are characteristic to apoptotic changes (data not shown). Agarose gel electrophoresis of the extracted DNA from these cells showed ladders of 200 bp (Fig. 2).

Effect of X-ray irradiation on cell cycle and DNA fragmentation

The cell cycle distribution of the 20Gy irradiated EL-4 cells was observed up to 36h (Fig. 3). The rate of the S-phase fraction gradually increased after irradiation and reached about 55% at 12h. Then, a rapid decrease in the S-phase fraction and a reciprocal increase in the G₂/M phase were observed from 16h, when cell viability began to fall. Cell cycle arrest at the G₂/M phase persisted from 24 to 36h.

Effect of the cell cycle modulators, caffeine and colchicine, on cell-cycle progression and apoptosis of X-irradiated EL-4 cells

In the caffeine-treated cells, unlike the results of Fig. 3, accumulation into the G₂/M phase was transient and the G₂/M blockade lasted for only 18h. Regression of the G₂/M fraction was observed from 24h after treatment with caffeine (Fig. 4A, left). In contrast, in the case of colchicine treatment continuous accumulation of cells into the G₂/M phase was observed from 12h and the G₂/M fraction reached almost 80% at 28h (Fig. 4A, right).

The treatment of X-irradiated cells with caffeine induced a remarkable increase in the apoptotic cell fraction, whereas colchicine induced only a slight increase of DNA fragmentation compared to the control group (20Gy X-irradiation alone) (Fig. 4B). The apoptotic rate in the caffeine-treated cells reached 46% at 24h and 54% at 36h. From these results, it is suspected that the overriding a G₂/M block induced by caffeine enhances the apoptotic cell death of X-irradiated cells, but mitotic arrest by colchicine has no effect on the modulation of apoptosis.

Expression of cyclin B, p34^{cdc2} and bcl-2 proteins

To investigate the relationship between cell-cycle progression and the induction of apoptotic cell death in X-irradiated cells, intracellular levels of cyclin B and cdc2 kinase proteins were observed by Western blot analysis. Previous reports indicated that the cyclin B:cdc2 ratio

correlates well with maturation promoting factor (MPF) activity, which is a main engine in the progression of the cell cycle from the G₂ to M phase¹³. The cyclin B1:cdc2 ratio of 20Gy X-irradiated cells revealed a continuous increase, which is consistent with the accumulation of the cells in the G₂/M phase. In contrast, the cyclin B1:cdc2 ratio after treatment with caffeine was remarkably suppressed to the precultivation level (**Fig. 5A**).

The intracellular levels of bcl-2, a protein that inhibits apoptosis, gradually increased after 20Gy X-irradiation and at 36h reached about 1.5-fold of the beginning value. In contrast, those after treatment with caffeine significantly decreased from 18h and reached almost 0.5-fold at 36 h (**Fig. 5B**). This significant increase in apoptotic fraction after treatment with X-ray + caffeine (**Fig. 4B**) may be partially ascribed to the decrease of bcl-2 expression.

Expression of p53 proteins after X-irradiation

To investigate the involvement of p53 proteins in X-ray-induced apoptosis and the effect of caffeine, a cell-cycle checkpoint inhibitor, on the progression of apoptotic cell death, p53 levels were evaluated by Western blot analysis (**Fig. 6**). After 20Gy X-irradiation, a rapid accumulation of p53 proteins was observed at 4-12h, after which the p53 level began to decrease gradually from 16h. In contrast, the p53 protein levels in the caffeine-treated cells had increased at 4h but rapidly decreased thereafter. In this case, p53 levels recovered to almost their normal range by 20h. These results suggest that apoptosis induced by X-irradiation is closely correlated with an increased expression of p53 proteins, whereas enhancement of apoptotic cell death by caffeine is not mediated by p53 proteins.

Discussion

Radiation is often used for the treatment of solid tumors, such as lung cancers, esophageal cancers, breast cancers, and various types of lymphomas. In the past two decades, the mechanism of cell killing by radiation was considered to depend mainly upon the mitotic capacity of the tumor cells. Lethal DNA double-strand breaks by X-irradiation give rise to the irreversible damage of the tumor cells causing them to no longer maintain normal replication¹⁴. In this model, lethal mutations or dysfunctional chromosome aberrations eventually lead to progeny cell death, usually after several mitotic cycles^{5,15}. Recently, apoptosis has been believed to represent a major component of the clinical and experimental effects of radiation. The apoptotic cascade can be triggered by various stimuli, and DNA double-strand breaks by X-irradiation seem to be highly effective in signaling apoptosis.

In this study, we clearly demonstrated the role of the G₂/M phase in the regulation of X-ray-induced apoptosis by using EL-4 lymphoma cells which express wild-type p53 protein. First, the time course of X-ray-induced apoptosis was observed from the viewpoint of cell-cycle progression. The rate of apoptotic fraction seems to positively correlate with the magnitude of G₂/M arrest (**Fig. 3, 4**). After X-ray irradiation, cells temporarily accumulate to the S-phase since at this phase they decrease their cell cycle speed and take enough time to repair the damaged DNA. Soon after most of the cells go into the G₂/M phase and are arrested, the final check of the synthesized DNA is performed. Caffeine is known as a cell-cycle modulator which overrides the G₂ checkpoint and inhibits repair of damaged DNA¹⁶. On the other hand, colchicine is one of the antimetabolic drugs that act by interfering with the exchange of tubulin subunits between the microtubules and the free tubulin pool¹⁷. To determine whether the G₂ or mitotic checkpoint plays a key role in the X-ray-induced apoptosis, we investigated the relationship between cell-cycle progression and the DNA fragmentation of X-irradiated cells by cultivating them with 5mM caffeine or 200nM colchicine (**Fig. 4**). As expected, the apoptotic fraction was enhanced by the cancellation of cell-cycle arrest. In contrast, colchicine, an M-phase blocker, did not affect the apoptotic fraction. Therefore, we hypothesized that DNA repair at the G₂ checkpoint has the important purpose of deciding the fate of the X-irradiated cells; cells succeeding in DNA repair can go to the next phase without apoptotic changes, but once they fail to repair DNA, they fall into apoptosis¹⁸.

Nagasawa et al. clarified the relationship between γ -ray-induced G₂/M delay and cellular radiosensitivity by using CHO K1 and human tumor cell lines. It was concluded that the delay time may be related to the level of unrepaired DNA damages which accumulate in the cells awaiting mitosis⁴. On the other hand, Palayoor et al. described that some G₂ phase blocking agents—such as 12-O-tetradecanoylphorbol-13-acetate (TPA), N6, 2'-O-dibutyryl adenosine 3', 5'-cyclic monophosphate (DBcAMP), and 3-aminobenzamide—induced a remarkable accumulation in the G₂/M phase and less apoptosis in the X-irradiated cells⁶. Accordingly, X-ray induced apoptosis seems to be triggered in the vicinity of the G₂/M phase, but it is unclear whether apoptosis does occur while cells halt in the G₂ checkpoint phase. Abend et al. reported that apoptosis of radiosensitive HL-60 leukemia cells occurs mainly in the early G₂ phase¹⁹. They employed a new approach that characterizes apoptotic cells occurring probably during early G₂ and bearing a rapidly reduced number of kinetochores in contrast to mitotic cells. In their experiments, X-irradiated HL-60 cells showed an accumulation in late G₂ during the entire

duration of the examination, but a larger fraction of cells were released from the G₂ block without decreasing cell viability. Taken together, the G₂ checkpoint mechanism plays an important role in the regulation of the X-ray-induced apoptotic pathway and decides which cell fraction should be eliminated by apoptosis.

Expression of the p34^{cdc2} protein is relatively stable throughout the cell cycle, although its activity is variable by its phosphorylation state. Previous reports have indicated that the cyclin B1:p34^{cdc2} ratio represents a substantial cdc2 kinase activity which moves the cells from the G₂ to M phase¹². From the viewpoint of cell-cycle regulation, therefore, we observed the cyclin B1:p34^{cdc2} ratio not only in X-irradiated cells but also those after treatment with caffeine (**Fig. 5A**). In the X-irradiation-only group, the ratio gradually increased and reached almost 2-fold of the beginning value. In the group of X-irradiation + caffeine, on the other hand, the cyclin B1:p34^{cdc2} ratio was consistently suppressed during the observation period. These results suggest that caffeine reduced the MPF activity of X-irradiated cells and disturbed cell-cycle progression from the G₂ phase to mitosis. It has been reported that caffeine reduces the S-phase fraction but does no harm in the absence of DNA damage⁸. In the presence of caffeine, therefore, instead of performing a usual repair process, only cells with severe genetic damage are eliminated by apoptotic cell death.

It is well-known that p53 is able to induce the genetic program for both G₁ arrest and apoptosis. But recently evidence has been presented that p53 is also involved in the control of the G₂ phase in addition to its role in G₁ arrest and apoptosis induction. In our experiments, remarkable upregulation of p53 level was observed immediately after X-irradiation, and a high level of this protein continued 12 to 20 h after irradiation when both G₂/M accumulation and apoptosis induction were observed (**Fig. 6**). Guillouf et al. reported that γ -irradiation activated a function of the temperature-sensitive mutant transgene p53, promoting increased apoptosis relative to parental, null p53 M1 myeloblastic leukemia cells⁵¹. The kinetics of apoptotic cell death induced by γ -irradiation correlated with the rapidity of exit from γ -ray-induced G₂ arrest. Therefore, they concluded that wild-type p53 positively modulated the exit from the γ -ray-induced G₂ checkpoint and that this new function for p53 is a component of the physiological pathway by which p53 exerts its role in apoptosis. On the contrary, in our results, caffeine strongly suppressed the p53 expression of X-irradiated cells despite that it remarkably enhanced the apoptotic fraction (**Fig. 6**). This may be attributed to the G₂-overriding activity of caffeine, and p53 protein's unimportance in the process of apoptosis as well as the exit from G₂ arrest. Alternatively, p53 levels might decrease since cell viability

was suppressed by an apoptotic mechanism.

After treatment with caffeine, the Bcl-2 expression of X-irradiated cells was significantly suppressed from 12 h (**Fig. 5B**), which reflects the enhancement of X-ray-induced apoptosis by this agent. At the same time, reduced expression of p53 was characteristic of this type of apoptosis (**Fig. 6**). It has been reported that DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2²⁰. Therefore, the reduced expression of Bcl-2 protein by caffeine may promote the apoptotic fraction in a p53-independent manner, though the precise mechanism is still to be clarified.

Taken together, it has been shown that the G₂ checkpoint mechanism is involved in the execution of the apoptotic program following X-irradiation. The remarkable augmentation of apoptosis by caffeine revealed the importance of G₂/M arrest in the DNA damage-induced activation of the cell death program.

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