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Invited Review
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New concepts in radiation-induced apoptosis: 'premitotic apoptosis' and 'postmitotic apoptosis'

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Abstract

Formerly, the mechanisms responsible for the killing of cells by ionizing radiation were regarded as being divided into two distinct forms, interphase death and reproductive death. Since they were defined based on the classical radiobiological concepts using a clonogenic cell survival assay, biochemical and molecular biological mechanisms involved in the induction of radiation-induced cell death were not fully understood in relation to the modes of cell death. Recent multidisciplinary approaches to cell death mechanism have revealed that radiation-induced cell death is divided into several distinct pathways by the time course and cell-cycle position, and that apoptotic cell death plays a key role in almost every mode of cell death. This review discusses the mechanisms of radiation-induced apoptosis in relation to cell-cycle progression and highlights a new concept of the mode of cell death: 'premitotic apoptosis' and 'postmitotic apoptosis'. The former is a rapid apoptotic cell death associated with a prompt activation of caspase-3, a key enzyme of intracellular signaling of apoptosis. A rapid execution of cell killing in premitotic apoptosis is presumably due to the prompt activation of a set of pre-existed molecules following DNA damages. In contrast, the latter is a delayed apoptotic cell death after cell division, and unlike premitotic apoptosis, it neither requires a rapid activation of caspase-3 nor is inhibited by a specific inhibitor, Ac-DEVD-CHO. A downregulation of anti-apoptotic genes such as MAPK and Bcl-2 may play a key role in this mode of cell death. Characterization of these two types of apoptotic cell death regarding the cell cycle regulation and intracellular signaling will greatly help to understand the mechanisms of radiation-induced apoptosis.

Keywords: radiation-induced apoptosis - interphase death - reproductive death - premitotic apoptosis - postmitotic apoptosis - cell cycle - mitosis - caspase

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Introduction

Ionizing radiation is a potentially DNA-damaging agent and causes direct and/or indirect effect on the irradiated cells. The incidence of cell cycle disturbance, aberrant mitosis or cell death may rise according as the dosage of irradiation increases. Nowadays, the knowledge regarding the biological effects of radiation, especially in the field of cell death mechanisms has been greatly accumulating. In this context, apoptotic cell death is regarded as one of the major cell death forms after the cells are exposed to ionizing radiation [1, 2]. To better understand the modes of radiation-induced apoptosis, the characterization of each type of apoptotic cell death regarding the biochemical and molecular mechanisms in relation to cell cycle progression as well as morphological changes seems to be important.

Firstly, this review showed a traditional classification of radiation-induced cell death, interphase death and reproductive death. Then a recent progress regarding the modes of radiation-induced cell death was introduced, and several distinct pathways of radiation-induced apoptosis were discussed in relation to mitosis and cell cycle progression. Subsequently, based on a recent study, a new concept of radiation-induced apoptosis, 'premitotic apoptosis' and 'postmitotic apoptosis', was introduced, and the timing of apoptosis and biochemical changes were compared between these two distinct modes of cell death. Their characteristics were compared with those of the other types of apoptosis that were previously reported. In addition, the modes of radiation-induced cell death were analyzed from the cell cycle specificity point of view. This review also focused on the molecular mechanisms of intracellular signaling in radiation-induced apoptosis and on the gene-expression changes in delayed-mitotic type apoptosis, namely postmitotic apoptosis.

Interphase death and reproductive death

Traditionally, there are two well recognized forms of radiation-induced cell death; one is interphase death and the other is reproductive death [3-5]. Interphase death is usually defined as "cell death before reaching the first mitosis following to the exposure of ionizing irradiation". Yamada and Ohyama [4] described the characterization of radiation-induced interphase death of rat thymocytes and clearly demonstrated that this type of cell death is internally programmed (apoptosis). This mode of cell death is characterized by pyknosis, cell condensation/shrinkage and internucleosomal breakage of chromatin, all of which are hallmarks of apoptotic cell death. On the other hand, reproductive or mitotic cell death is characterized by the loss of clonogenic cell survival, and this mode of cell death is usually evaluated by a colony-forming assay. In this type of cell death, unrepaired or misrepaired DNA double-strand breakage directly acts some roles as the critical lesion, and inactivation of the cell, namely loss of clonogenicity, occurs during or after mitosis [6, 7]. Classically, reproductive cell death is defined as a failure to undergo further cell division in spite of the metabolic survival [8]. Although damaged cells do not necessarily die immediately, actual cell death after several cycles of cell division may be included. A critical level of genomic instability is considered to be a direct cue of the reproductive mode of cell death [1].

In the field of radiobiological research, the linear quadratic equation model, that is drawn from a clonogenic survival curve, is widely applied to describe the radiation sensitivity of the cell, especially in reproductive cell death and also to predict a dose-response relationship [9]. Although this model is very useful for comparing the radiation sensitivity among the different cells, it is independent of the mechanism responsible for radiation-induced cell inactivation. Recently, both modes of cell death have been proved to involve in apoptosis [3], and the importance of apoptosis as a determinant of radiation sensitivity has been claimed. There are an increasing number of reports in the literature suggesting that apoptosis plays a central role not only in interphase death but also reproductive or post-mitotic mode of cell death.

Several distinct pathways of radiation-induced apoptosis

Previously, Radford and Murphy examined the timing of gamma-irradiation-induced death in relation to cell cycle progression using a panel of mouse lymphoid or myeloid cell lines [10, 11]. They found that DNA double-strand breakage appeared to directly stimulate the destruction of cell lines susceptible to rapid interphase death, whilst the signal for delayed interphase and mitotic death appeared to be chromosomal aberrations. According to their results, cell death types were divided into three distinct pathways: (1) death was found to occur immediately after irradiation ('rapid interphase' death); (2) death was found to occur after arrest in G_2 phase ('delayed interphase' death); or (3) cells of the slow-dying lymphoid lines underwent one or more mitoses prior to death ('mitotic/delayed mitotic' death). Since several of the cell lines showed different timing of death dependent upon the radiation dose used, these differences seem to be useful indicators of the relative radiosensitivity of each cell line.

Recently, Endlich et al. performed a more precise morphological analysis to further define the modes of radiation-induced cell death by using a computerized video time-lapse (CVTL) microscopy [12]. The CVTL technique allows the investigator to record the precise relationship between cell death and other observable events in individual cells [13, 14]. After 4 Gy irradiation, 100% of the ST4 cells observed died by 'rapid-interphase apoptosis' (died with in 5-6 hr). In contrast, after a dose of 1 Gy, 87% of the irradiated cells died by rapid-interphase apoptosis, but 13% of the cells produced lineages in which progeny cells died by apoptosis after mitosis. The morphological events observed in irradiated ST4 cells that underwent postmitotic apoptosis appeared to be identical to those occurring in cells that underwent rapid-interphase apoptosis. In addition, the time range suggests that the cells are dying in S phase. Therefore, this type of apoptosis is called 'postmitotic interphase apoptosis'. On the other hand, L5178Y-S and MOLT-4 cells irradiated with 4 Gy showed a much slower induction of apoptosis. These cells revealed delayed apoptosis after aberrant cell division, and thus this mode of cell death is called 'delayed aberrant mitotic apoptosis'. Interestingly, rapid-interphase apoptosis was never observed for L5178Y-S or MOLT-4 cells irradiated with 4 Gy, suggesting that different biochemical pathways or triggering mechanisms may be involved in this mode of apoptosis.

New concepts: 'premitotic apoptosis' and 'postmitotic apoptosis'

Following an exposure to ionizing radiation, the cells undergo apoptotic cell death at various cell cycle stages. Regarding the timing of radiation-induced death in relation to cell cycle progression, two different forms of apoptosis, namely premitotic type [10, 15, 16] and postmitotic type [10, 17, 18], have been reported. Therefore, the term 'premitotic apoptosis' or 'postmitotic apoptosis' itself is not a novel one. However, the difference in the mechanisms between premitotic and postmitotic apoptosis following X-irradiation remained to be investigated. Recently, we demonstrated that irradiation of U937 cells at different X-ray doses can induce to undergo these two different types of apoptosis and that decision concerning these two types of apoptotic cell death may be made by the difference in the magnitude of cell damage following X-irradiation [19].

Previous studies of radiation-induced cell death of some lymphoma cell lines have produced somewhat conflicting interpretations. For instance, Shinohara and Nakano [3] reported that MOLT-4 cells underwent both interphase and reproductive death after exposure to ionizing radiation. Akagi et al. [20] concluded that interphase death of MOLT-4 cells includes both nuclear changes that were characteristic of apoptosis and cytoplasmic changes that were characteristic of necrosis. In contrast, Tauchi and Sawada [17] characterized radiation-induced death of L5178Y cells as apoptosis after mitotic failure. Endlich et al. [12] verified the induction of apoptosis after irradiation of ST4, L5178Y-S and MOLT-4 cells by visualizing apoptosis with a Hoechst 33324/PI staining. According to their report, early apoptosis occurred at 30 min in ST4 cells irradiated with 4 Gy and characterized by nuclear margination and fragmentation and the ability to exclude PI. On the contrary, late apoptosis was observed in MOLT-4

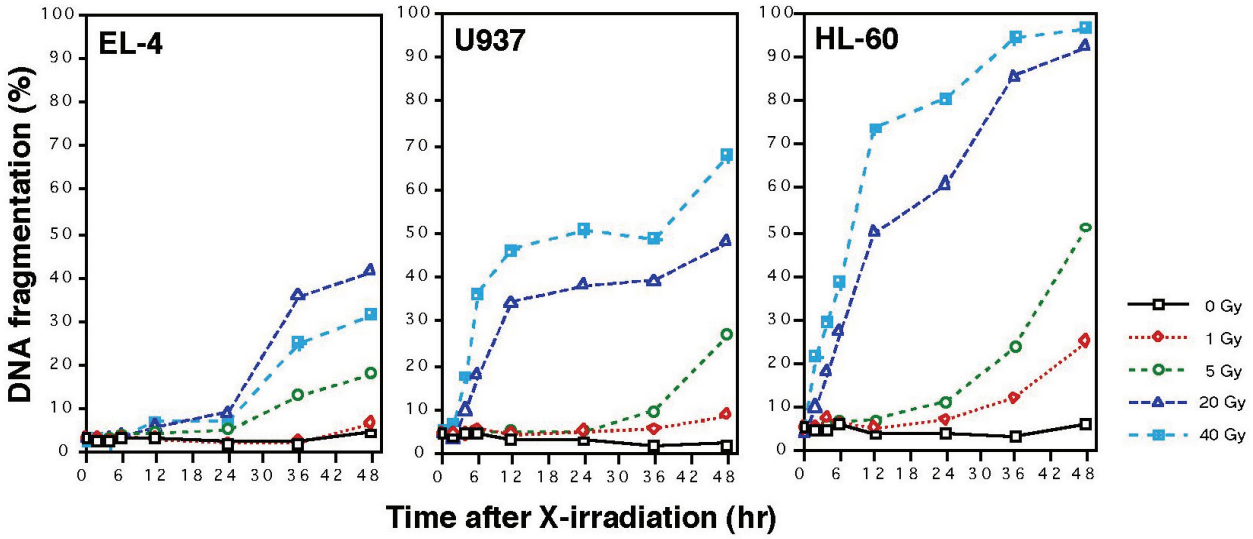

Fig. 1 Comparison of DNA fragmentation rates between three cell lines after various doses of X-irradiation. EL-4 lymphoma cells, U937 monoblastic leukemia cells and HL-60 promyelocytic leukemia cells were exposed to various doses of ionizing radiation, and time course of the DNA fragmentation rate (apoptotic fraction) was analyzed by flowcytometry. U937 cells showed two distinct types of apoptotic cell death; one is a rapid (within 4 to 6 hr) apoptosis which was induced by a dose of 20 Gy or more, and the other is a delayed (after 24 hr or later) apoptosis which was induced by a dose of 5 Gy or less. HL-60 cells revealed a very similar pattern to that of U937 cells though the sensitivity to apoptosis was much higher than U937 cells. In contrast, EL-4 showed only a delayed type of apoptotic cell death. Although the precise mechanisms are still unclear, the difference between these cells seems to be depending upon the signaling pathways of the apoptotic cell death as well as the DNA repair systems.

cells irradiated with the same dose of X-ray dose at 24 hr and was characterized by permeabilization of the cell membrane as indicated by PI uptake into cells. These differences may be ascribed to the sensitivity of individual cells and the dosage of ionizing radiation used in the experiments. Actually, in our experiments, EL-4, U937 and HL-60 cells revealed a characteristic pattern of DNA fragmentation, respectively (Fig. 1). EL-4 cells displayed a time course of only delayed apoptosis in response to 1 to 40 Gy irradiations. In contrast, U937 and HL-60 cells displayed a time course of both early and late apoptosis according to the doses of X-irradiation. These two cell lines showed a similar pattern of DNA fragmentation though HL-60 cells were more sensitive than U937 cells. DNA fragmentation of the high-dose (20 Gy or more) X-ray-treated cells increased from the very early phase and reached a plateau by 12 hr. In contrast, DNA fragmentation of the low-dose (5 Gy or less) X-ray-treated cells began to increase at 36 hr. The DNA histogram analysis revealed that the S phase fraction rapidly disappeared and that an increase of sub-G_1 fraction was detectable within a few hours after 20 Gy treatment. This is a typical feature of premitotic apoptosis. On the other hand, DNA histograms of the 5 Gy-irradiated group showed an obvious G_2/M blockade at 12-24 hr, then the cells were released from the blockade and entered G_1 phase at 36-48 hr when sub-G_1 fraction, namely an apoptotic population, became obvious (postmitotic apoptosis). In the colony-forming assay, only 3-4% of the 5 Gy-irradiated cells revealed clonogenicity and it was almost impossible to detect any clonies after 20 Gy irradiation. However, in the short-term DNA fragmentation assay, about one third of the 20 Gy-irradiated U937 cells were shown to undergo premitotic apoptosis within 6 hr, while all of the 5 Gy-irradiated cells survived until 24 hr and about a quarter of the cells showed postmitotic apoptosis at 48 hr.

To characterize more the differences in these two modes of apoptosis, the X-ray-induced

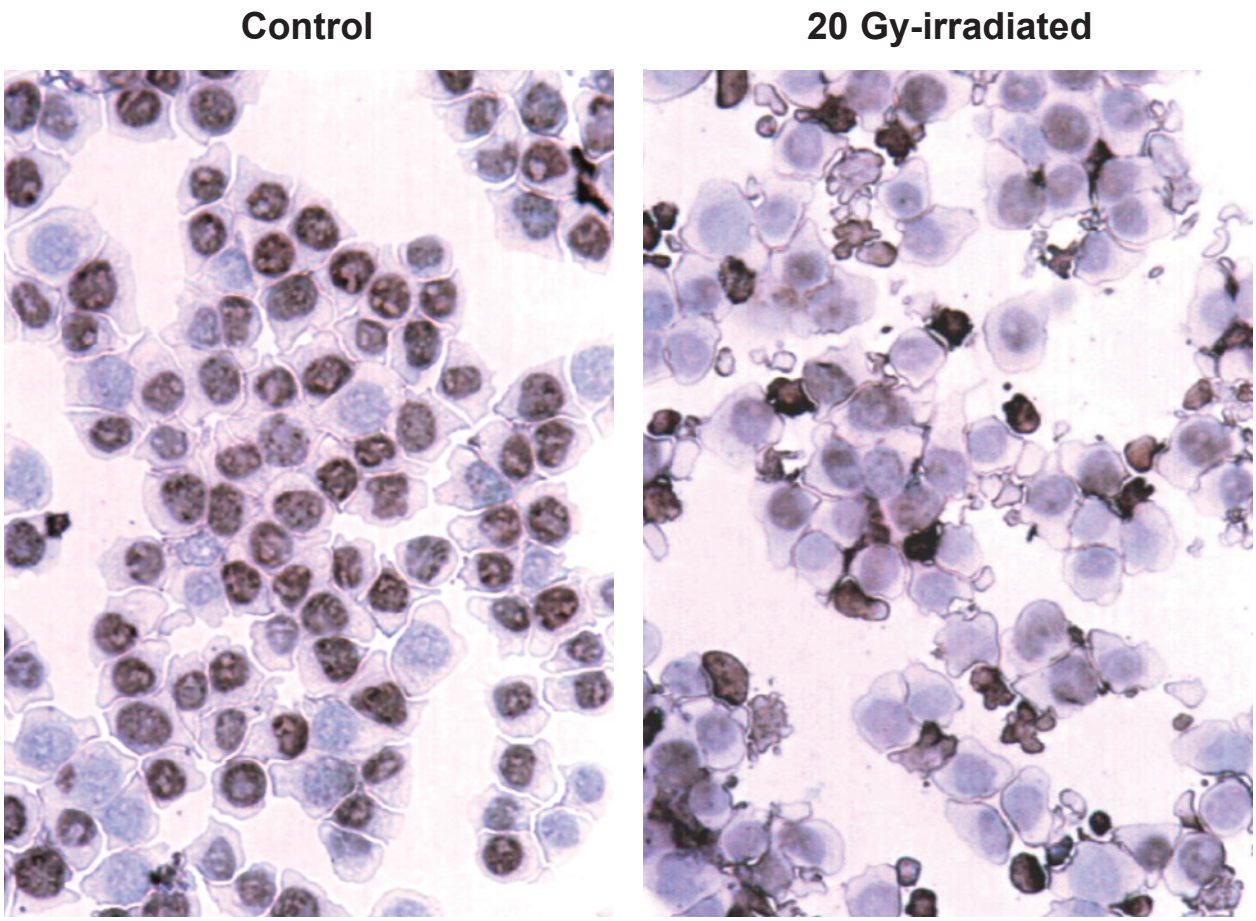

Fig. 2 S-phase specificity of the early type (premitotic) apoptosis. The S-phase fraction was labeled with BrdU immediately before irradiation, and the cells were harvested at 4 hr after irradiation. The cytospin specimens were stained by an immunohistochemical method. Most of the S-phase fraction in the control group showed a normal feature, and cells exhibiting apoptotic changes were not recognized. In contrast, 20 Gy-irradiated cells with a radiosensitizer revealed apoptotic changes, such as nuclear condensation and cell fragmentation, mainly in the S-phase cells. The cells positively stained by anti-BrdU were observed as those with dark-brown nuclei. The counterstain was performed by using hematoxylin. Original magnification, x400.

molecular events were examined in U937 cells. In the 20 Gy-irradiated group, all the sequential events related to intracellular signaling and execution of apoptosis, such as activation of caspase-3, cleavage of PARP, cleavage of actin filament and 200 bp-DNA ladder formation, were observed within a few hours. A reduction in the mitochondrial membrane potential ($\Delta\Psi_m$) was well correlated with the pattern of caspase activation, suggesting the involvement of the mitochondrial pathway in premitotic apoptosis. None of these changes were observed in the 5 Gy-irradiated group until 24 hr when the cells were still halted in a premitotic phase. This strongly suggests that the signaling pathway of apoptosis may be first triggered after cell division in the case of postmitotic apoptosis.

Regarding the cell-cycle specificity to apoptotic cell death of the irradiated cells, a DNA fragmentation analysis using cells synchronized to the G_1, S or G_2/M phases brought us a clear result. The DNA fragmentation was significantly enhanced in the S-phase-rich cells in premitotic apoptosis, whereas no remarkable difference in the DNA fragmentation rate between the three phases was recognized in postmitotic apoptosis. This result was also confirmed by a morphological analysis

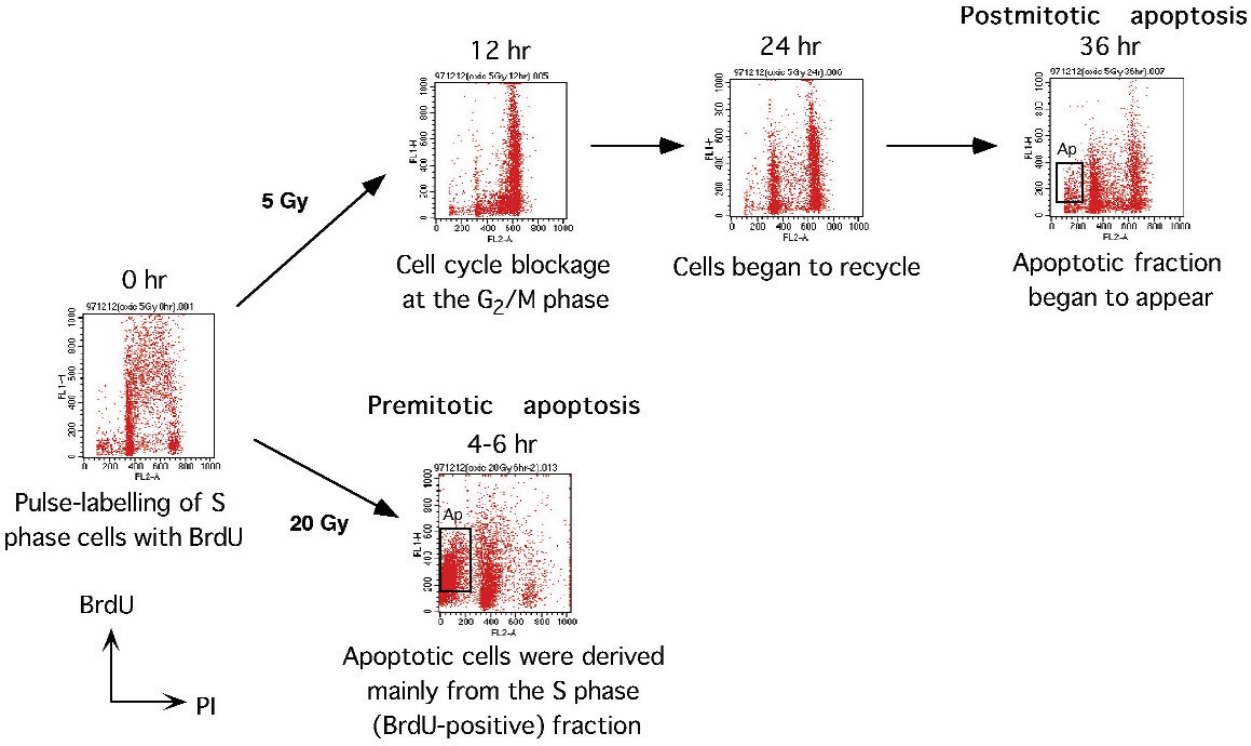

Fig. 3 Relationship between the apoptotic fraction and cell cycle in premitotic and post mitotic apoptosis. The S-phase cells were labeled with BrdU before irradiation and the appearance of apoptotic fractions in the labeled cells was serially chased. After 20 Gy irradiation, the cells rapidly (4-6 hr) fell into apoptosis (premitotic apoptosis). Most of the apoptotic cells were BrdU-positive, suggesting that the target in this case was the S-phase fraction. In contrast, after 5 Gy irradiation, a cell cycle blockage at the G_2/M-phase was observed from 12 to 24 hr. Then, the cells began to recycle from 24 hr, and after the cell division the apoptotic fraction began to appear (postmitotic apoptosis). In this case, some cells obviously died in the G_1 phase. Ap the apoptotic fraction positively stained by BrdU.

using a BrdU-labeling method (Fig. 2). At 4 hr after 20 Gy irradiation, apoptotic changes, such as nuclear condensation and cell fragmentation, were mainly observed in the BrdU-positive cells, and this type of apoptosis was enhanced by an addition of a radiosensitizing agent. This strongly support the idea that premitotic apoptosis is an S phase-specific event, whereas cell-cycle dependency is relatively low in postmitotic apoptosis.

The BrdU-labeling analysis also works as a good tool for investigating the relationship between apoptotic fraction and cell cycle. S-phase cells were specifically labeled with BrdU before irradiation and the appearance of apoptotic fractions in the labeled cells were chased (Fig. 3). In the 20 Gy-irradiated group, most of the sub-G_1 (apoptotic) population was BrdU-positive, which is compatible with the result of Fig. 2. In contrast, in the 5 Gy-irradiated group, cell-cycle blockade at the G_2/M phase was observed at 12 hr, but this blockade was released at 24 hr without an obvious increase in the apoptotic fraction. BrdU-positive cells in the sub-G1 fraction were first observed at 36 hr, and remarkable apoptosis was not observed until then. These results well describe the feature of postmitotic apoptosis. They are compatible with the report of Tauchi and Sawada [17] that apoptosis may be the ultimate form of cell death via mitotic failure caused by relatively small doses of radiation in L5178Y cells.

By using ST4, L5178Y-S and MOLT-4 cells, Endlich et al. [12] have shown the time course of cumulative percentages of cells initiating apoptosis for populations of cells. They clearly demonstrated that there are at least two distinct patterns of apoptosis regarding the time course of initiation of

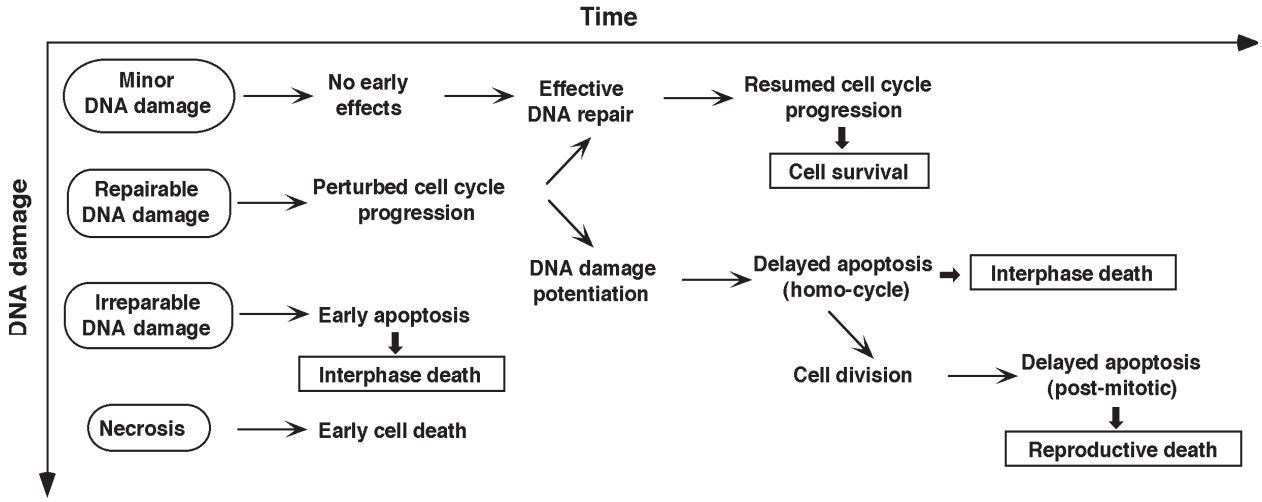

Fig. 4 Fate of the irradiated cells in relation to the initial damage. This scheme illustrates the relationship between the initial DNA damage (radiation dose) and the fate of the cells in relation to the cell cycle. Rewritten from the figure of Halicka *et al.* [1997] with some modifications.

apoptosis and duration of process. One is apoptosis in interphase (rapid-interphase apoptosis) that is observed in ST4 cells irradiated with 4 Gy. The other is apoptosis after aberrant mitosis (delayed aberrant mitotic apoptosis) that is observed in L5178Y-S and MOLT-4 cells irradiated with 4 Gy. In the former case, the onset of apoptosis was within a few hours and duration of process (from membrane blebbing to cell collapse) required 2-4 hr. In contrast, in the latter case, the cells collapsed at 2-23 hr after entering an abortive mitosis. The apoptosis pattern of the former case is compatible to 'premitotic apoptosis' and that of the latter case is considered to be very similar to 'postmitotic apoptosis'. Relationship among interphase death, reproductive death and various concepts of cell death types raised in this review is shown in Table 1. However, it remains unclear whether the case of ST4 cells after 1 Gy-irradiation (postmitotic interphase apoptosis) can also be included in 'postmitotic apoptosis' because there was no cell cycle delay and the cells were suggested to be dying in S phase.

Cell cycle specificity of apoptosis

Regarding the mechanism of induction of apoptosis in relation to cell cycle, Halicka et al. reported that three types of apoptosis could be distinguished in relation to the initial damage to the cell vis-a-vis cell cycle position [21]. They employed multiparameter flow cytometric methods that allowed us to identify apoptotic cells and position them with respect to their cell cycle phase. The first type of apoptotic cell death is homo-phase apoptosis where the cells underwent apoptosis during the same phase in which they were initially affected. The second one is homo-cycle apoptosis, where the cells underwent apoptosis during the same cell cycle in which they were initially affected, i.e., prior to or during the first mitosis. And the third one is post-mitotic apoptosis, where cells underwent apoptosis during the cell cycle subsequent to that in which the cell was initially affected most likely at the G_1 or G_2 checkpoints of this cycle. The initial damage can be divided into four ranges of genotoxicity, and the fate of the cell is decided according to the extent of this initial damage (Fig. 4). In the case of minor DNA damage, no early effects are conspicuous and an effective DNA repair successfully leads to a resumed cell cycle progression. When the cell receives a stronger DNA damage, several types of cell death are induced. Treatment with extremely high dose of anti-cancer drugs or ionizing radiation not only induces the DNA damage but also affects the function of the other molecules, such as proteins and enzymes in the cytoplasm, membrane

Table 1 Relationship among interphase death, reproductive death and various concepts of cell death types.

	Type of cell death			References
1	*Interphase death*		*Reproductive death*	[1] [3] [4] [5]
	Cell survival is evaluated by the dye exclusion test. Formerly apoptosis is included in this type of cell death.		Cell death is evaluated by the clonogenic assay. This type of death includes not only necrotic cells but also live cells with no reproductive capacity (loss of clonogenicity).	
2	*Rapid interphase death*	*Delayed interphase death*	*Mitotic/delayed mitotic death*	[10] [11]
	Cell death occurs immediately or within a few hours after irradiation. Triggered by DNA double strand break recognition system	Cell death occurs after a G_2-phase block. Dependent on G_2-phase lesions	Cell death occurs after one or more cell divisions. Induced by lethal chromosomal aberrations	
3	*Rapid-interphase apoptosis*	*Postmitotic interphase apoptosis*	*Delayed aberrant mitotic apoptosis*	[12]
	The interval from irradiation to cell collapse is within 5-6 hours. 100% of the radiosensitive ST4 mouse lymphoma cells undergo this type of apoptosis in response to ionizing radiation at doses of 2.5 – 4 Gy.	The majority of cells in the lineage die after two, three or five divisions. Probably cells are dying in S phase. The interval from irradiation to membrane blebbing shows a greater range for cells dying by this type of apoptosis than for cells dying by rapid-interphase apoptosis. After a dose of 1 Gy, 87% of the irradiated ST4 cells died by rapid-interphase apoptosis, and 13% of the cells produced lineages in which progeny cells died by this type of apoptosis.	Cell death is preceded by a long cell cycle delay. Large, abnormal cells form and attempt to divide at approximately 18-30 h after exposure to ionizing radiation, but division is aberrant or mitosis fails. After several rounds of membrane blebbing, gross shape distortions, fragmentations-refusions, and formation of apoptotic bodies, the cells collapse at 36-60 h after irradiation. L5178Y and MOLT-4 cells show this type of apoptosis.	
4	*Premitotic apoptosis*	*Postmitotic apoptosis*		[19]
	Cell death occurs before cell division. This type of apoptosis is associated with an immediate (within several hours) activation of caspase-3, a decrease in the mitochondrial transmembrane potential and DNA strand breaks. Apoptotic cell death occurs mainly in the S phase fraction. High-dose X-ray (20 Gy) induces a rapid and strong apoptosis, namely premitotic apoptosis, in U937 cells.	Cell death occurs after at least one cell division. The caspase activation and DNA strand breaks do not occur until cells complete the mitosis. Cell cycle arrest is observed at the G_2/M phase but cell death does not occur during or immediately after the cell-cycle blockage. No obvious difference in the susceptibility to cell death among the cell-cycle phases is observed. Low-dose X-ray (5 Gy) induces a slow and mild, namely postmitotic apoptosis, in U937 cells. The caspase-3 inhibitor Ac-DEVD-CHO blocks the DNA fragmentation and PARP cleavage in 20 Gy-irradiated cells but not in 5 Gy-irradiated cells.		

phospholipids, and so on. In this case, necrosis occurs within a short period of time. If the DNA damage is weaker than the necrotic case but an irreparable one, the cell can no longer continue the vital activity and an early apoptotic cell death occurs. In contrast, if the DNA damage is not so strong as to induce a direct cell death, the cell cycle progression once stops to repair the damaged DNA. The cell that successfully performed an effective DNA repair reenters the cell cycle and continues the normal growth. On the other hand, the cell that failed to conduct an effective repair and accumulated the DNA damage potentiation either falls into delayed homo-cycle apoptosis after a perturbed cell cycle progression or executes post-mitotic delayed apoptosis following to the cell division. According to the classical radiobiological definition, both early apoptosis and homo-cycle apoptosis may be classified into the interphase death, and post-mitotic delayed apoptosis is considered to be included in the reproductive death. However, all of them can not be applied to the radiation-induced apoptosis since the hypothesis drawn in Fig. 4 is mainly based on the results using anti-cancer drugs. Actually, regarding the cell cycle specificity of homo-cycle apoptosis after γ-irradiation, DNA strand breaks are only shown to occur specifically in the G_2 cells. As it is, the cell cycle specificity of the apoptosis depends both on the dose of irradiation and on the extent of subsequent repair of the damaged DNA.

As for the cell-cycle specificity of premitotic apoptosis, a BrdU-labeling technique clearly demonstrated that apoptotic U937 cells following 20 Gy irradiation were mainly derived from the S phase fraction (Fig. 3, bottom). This was presumably due to the fact that strong DNA damage was selectively brought to the S phase fraction in which the unwinding of the DNA helix occurs and the DNA is prone to be damaged by toxic substances. In this case, irreparable DNA damage might induce a very rapid-type apoptosis without any cell cycle progression or delay (homo-phase apoptosis). In contrast, in the case of repairable DNA damage (5 Gy), the cell cycle progression from G_2/M to G_1 phase was observed subsequent to the cell cycle delay at the G_2/M phase (Fig. 3, top). Some of the cells obviously died in G_1 of the subsequent cycle since an increase of the apoptotic fraction was first observed after the cells divided (post-mitotic apoptosis) and before they entered the S phase. Although it is impossible to follow the fates of all irradiated cells, both effective DNA repair and DNA damage potentiation seem to coexist because DNA fragmentation rate of 5 Gy-irradiated U937 cells at 48 hr remained about 25% (Fig. 1, center).

Molecular mechanisms involved in radiation-induced premitotic apoptosis and postmitotic apoptosis

The molecular mechanism of premitotic apoptosis is considered to be quite different from that of postmitotic apoptosis. As described in the above section, premitotic apoptosis is a very rapid type of cell death. Apoptosis is presumably triggered by a prompt activation of caspases, which play important roles as direct executioners of apoptotic cell death. Although the precise mechanisms how the caspases are rapidly activated following the exposure to ionizing radiation are still to be investigated, three models of radiation-induced apoptotic pathways have been proposed. Firstly, radiation-induced DNA damage may initiate apoptosis via p53 dependent mechanism by upregulating the expression of Bax, a pro-apoptotic molecule of the Bcl-2 family proteins [22-24]. Bax is a key molecule that regulates mitochondria-dependent apoptosis by inducing the formation of apoptosome, a complex molecule of cytochrome c, Apaf-1 and caspase-9 [25]. p53 may also upregulate the expression of Fas, the CD95 death receptor, and activate down stream caspases both through mitochondria-dependent and –independent mechanisms [26, 27]. Secondly, the biochemical changes such as an increase in the generation of reactive oxygen species and depletion of glutathione are seen in irradiated cells prior to the loss of surface membrane integrity [28]. Furthermore, reactive oxygen intermediates induced by ionizing radiation can trigger mitochondria pathway to release caspase-activating-factors [29]. Therefore, oxidative stress may play a direct role in radiation-induced apoptosis. Thirdly, interaction of ionizing radiation with cellular membranes induces rapid

upregulated genes

1 glutathione peroxidase 1
2 insulin-like growth factor binding protein 2 (36kD)
3 glutathione S-transferase pi

downregulated genes

4 chromosome segregation 1 (yeast homolog)-like
5 mitogen-activated protein kinase 6
6 BCL2/adenovirus E1B 19kD-interacting protein 2
7 cyclin C

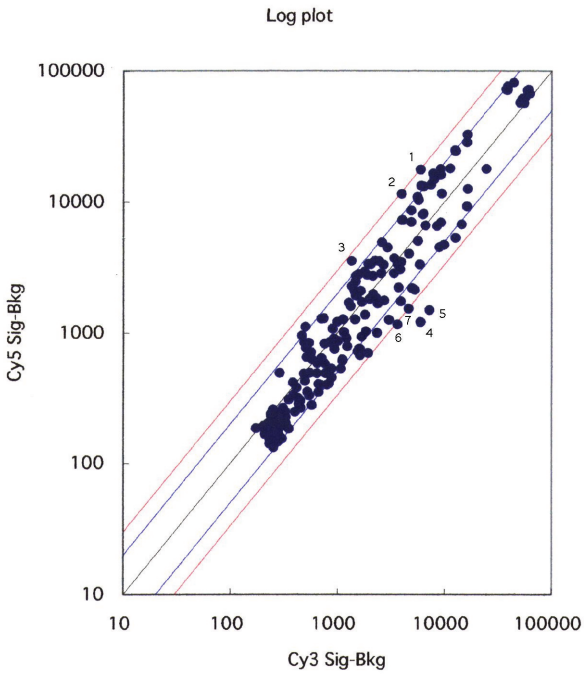

Fig. 5 DNA microarray analysis of the postmitotic apoptosis. The mRNA expression of U937 cells at 24 hr after 5 Gy exposure was compared with that of control (untreated) cells. cDNA from irradiated cells were labeled with Cy3 and that from control cells was labeled with Cy5. Labeled cDNAs were hybridized on the DNA chip and then the fluorescence signals were analyzed. In this figure, the fluorescence intensity was plotted as a logarithmic scale. The black, blue and red lines mean even, two-fold and three-fold expression levels, respectively. In this analysis, three upregulated (1-3) and four downregulated (4-7) genes were considered as significant.

sphingomyelin hydrolysis to ceramide [30], and activation of the pro-apoptotic SAPK/JNK pathway may occur downstream of membrane-derived ceramide signals [31, 32]. Subsequent phosphorylation of c-Jun upregulates the transcription of apoptosis-regulating genes. Cross talk between the SAPK/JNK pathway and caspase cascade also play some roles in radiation-induced apoptosis. Moreover, the ceramide formation and subsequent caspase activation may take part in the DNA damage-induced apoptosis [33]. In addition, MAPK and Akt activated by PI(3) kinase also play an important role in the mitochondrial pathway by phosphorylating Bad, which may promote survival by allowing heterodimerization of Bcl-X_L with Bax, thereby preventing the proapoptotic function of Bax [34].

Since premitotic apoptosis is a rapid mode of cell death, a prompt activation of preexisted cytoplasmic caspase-3 may be involved in this process. To verify this hypothesis, the caspase-3 inhibitor, Ac-DEVD-CHO, was added to the culture media and apoptosis-related events, such as PARP cleavage and DNA fragmentation were observed [19]. In the 20 Gy-irradiated U937 cells, the cleavage of PARP was suppressed only when Ac-DEVD-CHO was added before irradiation, and an addition of the caspase-3 inhibitor after irradiation could not reveal any protective effect against PARP cleavage even if it was added within 1 hr. This strongly suggests that the activation of caspase-3 is a very early event in premitotic apoptosis and that preexisted caspases play a key role in this mode of cell death. In addition, DNA fragmentation was considerably reduced in the 20 Gy-irradiated group when the casapase-3 activity was suppressed by Ac-DEVD-CHO, whereas in the 5 Gy-irradiated group (namely the case of postmitotic apoptosis), this inhibitor could not reduce the apoptotic rate. This suggests that the other pathway may be involved in

postmitotic apoptosis. Since postmitotic apoptosis requires a transient G_2/M blockade and also takes a longer incubation period (more than 24 hr) until executing the apoptotic cell death as compared with premitotic apoptosis, cell death in this case is presumably not only due to the primary damage but also may result from the accumulation of secondary changes that occurred during the cytostatic phase. Accordingly, downregulation of anti-apoptosis genes and upregulation of apoptosis-related genes are considered to be probably involved in the process of postmitotic apoptosis.

Genes involved in postmitotic apoptosis

To investigate the target genes involved in postmitotic apoptosis, a microarray analysis has been employed. The mRNA expression of the apoptosis-related genes was compared between 5 Gy-irradiated U937 cells and untreated control cells (Fig. 5). Since the DNA fragmentation of 5 Gy-irradiated U937 cells was not observed until 24 hr (Fig. 1, center) and also since changes of the expression of important genes seemed to be obvious around this phase, the difference in the gene expression at 24 hr between the two groups was investigated.

A remarkable downregulation of the genes involved in apoptosis, namely mitogen-activated protein kinase (MAPK) 6 and BCL2/adenovirus E1B 19kD-interacting protein 2 was observed in the 5 Gy-irradiated U937 cells. As mentioned above, both MAPK and Bcl-2 family genes play important roles in the mitochondrial pathway that may regulate a central signal transduction pathway in radiation-induced apoptosis. The kinetics and magnitude of SAPK/JNK activation demonstrate a close correlation with the induction of apoptotic nuclear changes [35]. Verheij et al. [32] have found that ionizing radiation employs the SAPK/JNK pathway to induce apoptosis in U937 cells, and that radiation-induced apoptosis is significantly inhibited in cells overexpressing dominant-negative mutants of the SAPK/JNK pathway. On the other hand, Ruiter et al. [36] have found that inhibition of the anti-apoptotic MAPK/ERK cascade by synthetic alkyl-lysophospholipids enhances radiation-induced SAPK/JNK-mediated apoptosis. These reports support the idea that the balance between pro- and anti-apoptotic effector kinases has been shown to determine whether a cell survives or undergoes apoptosis [37, 38]. In another report, inhibition of the MAPK pathway has been shown to significantly increase the ability of radiation to cause apoptosis 24 h after exposure [39]. The ability of DU145 prostate carcinoma cells to proliferate after irradiation became dependent on MAPK signaling. When cells were subjected to single doses or fractionated radiation exposure, continuous inhibition of the MAPK pathway significantly decreased clonogenic survival. This also demonstrates the importance of the MAPK pathway in the delayed-type of radiation-induced apoptosis. Therefore, a downregulation of anti-apoptotic MAPK and Bcl-2 may cooperate to accelerate the signaling of postmitotic apoptosis.

Regarding the expression of genes involved in cell cycle and mitosis, cyclin C and chromosome segregation 1 were significantly downregulated in the 5 Gy-irradiated U937 cells. Cyclin C belongs to the cyclin family of proteins that control cell cycle transitions through activation of specific catalytic subunits, the cyclin-dependent kinases. However, there is as yet no evidence for any role of cyclin C and its partner, cdk8, in cell cycle regulation. Rather, the cyclin C-cdk8 complex was found associated with the RNA polymerase II transcription machinery [40]. At present any conclusive roles of cyclin C in radiation-induced apoptosis are not clarified, but it may probably coordinate the function of apoptosis-related genes by regulating their transcription levels. In contrast, the suppression of genes involved in chromosome segregation may lead the irradiated cells to aberrant mitosis or mitotic failure that precede postmitotic apoptosis in the majority of the cases. Endlich et al. reported that the process of mitosis in the cells destined to die by apoptosis was usually asymmetrical, resulting in three or four daughter cells or cell fragments that formed separate lineages [12]. Since there was no consistent pattern, from cell to cell, in the degradation after an aberrant mitosis as far as the morphology was investigated, it is difficult to hypothesize what types of biochemical processes might be involved. The answer presumably depends upon what genes are up- or down-regulated in the segregated

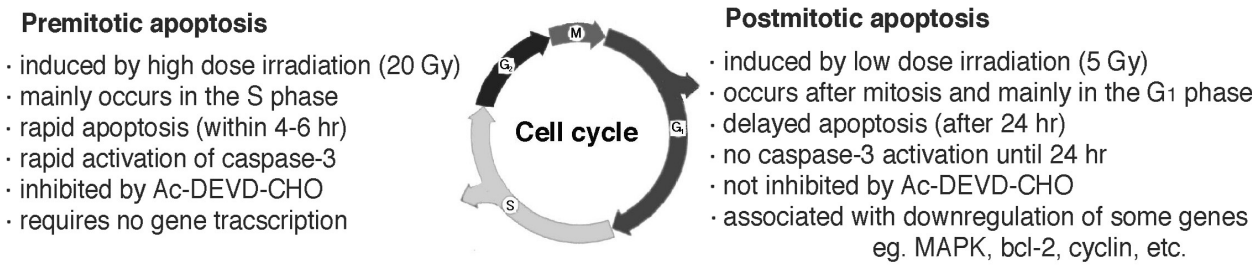

Fig. 6 Characteristic feature of premitotic and postmitotic apoptosis. The differences between these two types of apoptotic cell death are compared.

chromosomes. Maybe the results displayed in Fig. 5 present a typical pattern of the gene expression during the process of postmitotic apoptosis.

On the other hand, a marked upregulation of radical scavenger genes, such as glutathione peroxidase 1 and glutathione S-transferase pi (GST-π), was also observed in the 5 Gy-irradiated U937 cells. Radical scavengers play an important role in the cells defending themselves against free radicals that occur with irradiation [41]. Therefore, both glutathione peroxidase 1 and GST- π may be upregulated in the repair process of the damaged cells. Insulin-like growth factor binding protein 2 is also considered to assist the recovery from the damage and to promote the cell proliferation.

Since the difference in the gene expression was not investigated at the individual cell level, but was compared as a cell group in this DNA microarray analysis, it is not clear whether these transcriptional changes occurred in the same cell or in the different cell populations. However, the cells that have received repairable DNA damage may reveal the bi-directional destiny after perturbed cell cycle progression; one is a process of effective DNA repair which is associated with an upregulation of radical scavengers and growth factor receptors, the other is a process of DNA damage potentiation which is associated with a downregulation of anti-apoptotic genes and is followed by postmitotic apoptosis.

Conclusions

In this review, two distinct terms for forms of radiation-induced apoptosis 'premitotic apoptosis' and 'postmitotic apoptosis' were introduced (Fig. 6). The former was induced by high-dose X-ray and showed a rapid and strong apoptotic cell death. This type of apoptosis was associated with a prompt (within several hours) activation of caspase-3 and a decrease in the mitochondrial transmembrane potential. The rapid DNA cleavage in the nucleosomal level was presumably ascribed to the prompt activation of a set of pre-existed molecules that were involved in apoptosis. On the other hand, the latter was induced by low-dose X-ray and showed a slow and mild apoptotic cell death. The rapid activation of apoptosis-inducing molecules did not occur in this type of apoptosis. In stead of the rapid activation of caspase-3, in the postmitotic apoptosis, a downregulation of the genes that play inhibitory roles in apoptotic cell death was observed after cell divisions. Taken together, these two types of apoptosis revealed quite a different mode of cell death regarding the cell cycle. Since U937 cells can undergo both premitotic apoptosis and postmitotic apoptosis in response to the different dose of ionizing radiation, they will provide a good model for analyzing the intracellular signaling mechanisms of radiation-induced apoptosis.

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