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Review

Sixty years of follow-up of Hiroshima and Nagasaki survivors: Current progress in molecular epidemiology studies

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Abstract

This article provides an overview of the on-going molecular epidemiology studies among atomic-bomb survivors conducted at the Radiation Effects Research Foundation in Japan. The focus is on: (a) inter-individual variations in sensitivity to radiation-induced somatic mutations (*glycophorin A* (*GPA*) mutations) and their potential relevance to differences in susceptibility to radiation-related cancers and (b) the role of specific mutations/rearrangements in radiation-induced thyroid and colorectal cancers. The *glycophorin A* mutant fractions showed large differences between the survivors at each of the estimated bone marrow doses. Of note is the finding at doses ≥ 1 Gy; that the slope of the mutant fraction was significantly higher in the 'cancer group' than in the 'non-cancer group'. This study provided the basis for validating the use of γH2AX and reticulocyte micronucleus assays for evaluating radiosensitivity differences and genetic instability, respectively, in our studies in the coming years. Preliminary results from our molecular oncology studies on adult-onset papillary thyroid cancer provide evidence for the induction of *RET/PTC* rearrangements and *BRAF* point mutation (both known to be early stage events in adult-onset papillary thyroid cancer) but with a difference: cases associated with the rearrangements were more frequent at high doses, and developed sooner than those with *BRAF* mutation. In the case of colorectal cancer, the results suggest that radiation exposure might influence microsatellite instability (MSI) status through MSI-related epigenetic and genetic alterations—processes that might occur in the early stage of colorectal carcinogenesis.
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Keywords: Radiation; Atomic-bomb survivors; Somatic mutation; Oxidative stress; Colorectal carcinogenesis; Microsatellite instability; Thyroid carcinogenesis

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1. Introduction

Molecular epidemiology studies among atomic-bomb survivors (A-bomb survivors) seek to deepen our understanding of the mechanisms by which ionizing radiation affects human health. Their importance will be evident in future prevention of radiation-associated diseases: not only confined to establishing safeguards against radiation exposure, but also in developing the ways and means to identify high-risk individuals and more efficient approaches to prevent radiation-associated diseases. The mechanistic study group in our laboratory comprises two subgroups and the following research themes: radiation effects on immune functions (specifically, T-cell mediated immunity) and aging-associated persistent inflammation [1], a genome approach relative to individual risks of radiation-associated diseases, somatic mutability in relation to cancer occurrence and molecular oncology analyses of radiation-associated cancers. These studies are based on analyses of invaluable biological resources obtained from the two cohort studies conducted at the Radiation Effects Research Foundation (RERF): the Life Span Study (LSS) with 120,000 survivors, and the Adult Health Study (AHS) with 20,000 survivors, which has conducted biennual medical examinations among a subcohort of the LSS.

In this article, we report on current progress in selected research themes of our laboratory relating to two critical issues: (1) inter-individual variations in sensitivity to radiation-induced somatic mutation and (2) relationship between radiation exposure and various gene alterations in carcinogenesis. The first issue was addressed using biomarkers of individual radiation sensitivity and presumed radiation-induced genetic instability, among A-bomb survivors, based on our previous findings in the *glycophorin A* (*GPA*) mutant fraction (Mf) study, as well as biomarkers of inflammation and production of reactive oxygen species (ROS). In this *GPA* Mf study, we demonstrated large inter-individual variation in the *GPA* Mf response of survivors exposed to atomic radiation, as well as a significantly higher sloped *GPA* Mf dose–response curve for doses $\geq$1 Gy, when the cancer group was compared with the non-cancer group [2]. This study prompted two questions: (1) are individual sensitivities to radiation-induced genetic damage responsible for the inter-individual variation noted in dose-dependent *GPA* Mf response? And (2) is radiation-induced genetic instability involved in the dose-dependent increase of *GPA* Mf? To address these questions, we considered several biomarkers and assessed and verified the suitability of two of them, namely the γH2AX and reticulocyte micronucleus endpoints. Both of these biomarkers appear applicable to evaluation of individual sensitivity to radiation-induced genetic damage and instability [3,4]. In parallel with the somatic mutation study, we have observed dose-dependent increase of various inflammatory biomarkers among A-bomb survivors [5]. This enhanced and persistent inflammation needs to be examined in relation to the endogenous production of reactive oxygen species (ROS), linking inflammation and somatic mutations.

The second issue can be further broken down into two questions: which types of genetic alterations e.g., chromosome aberration or point mutation, preferentially occurred in radiation-associated cancer, and whether or not radiation influenced epigenetic alterations during carcinogenesis. Adult-onset papillary thyroid cancer seems to be a good model for examining and contrasting chromosome aberrations and point mutations, since the major initial event of this cancer is either *RET/PTC* rearrangements or *BRAF* point mutation, which appear to occur in a mutually exclusive manner. It is well recognized that colorectal cancer can be phenotyped according to microsatellite stability. Accordingly, we have begun to analyze microsatellite instability (MSI), along with methylation status of *MLH1*, using cancer tissue specimens from A-bomb survivors. Although these studies are still preliminary and continuing, we report here our interim results [6].

2. Radiation-induced genetic damage and inflammation

2.1. GPA Mf study in the AHS cohort

We have conducted a prospective study among a total of 1723 MN heterozygous AHS participants who were cancer free and without cancer history at the time (1988–1996) of *GPA* Mf measurements, in which mutated erythrocytes, namely hemizygous Mφ or Nφ cells, were counted by flow cytometry [2]. During a follow-up period that lasted until 2000, a total of 186 cancer cases were identified. The major findings from this study were: (1) a radiation dose-dependent increase of *GPA* Mf was observed in the total population, as well as in the cancer and cancer-free participants in Hiroshima and Nagasaki, and (2) the slope of the *GPA* Mf dose-response above 1 Gy was significantly higher in the Hiroshima cancer group than in the cancer-free group within the same high dose region (Fig. 1). These findings imply that inter-individual variations in *GPA* Mf might indicate individual differences in somatic mutability response to radiation exposure, and that individuals with higher mutability in *GPA* Mf response could have increased risk of radiation-associated cancer. It also suggests that the inter-individual variation in *GPA* Mf might involve differences in hematopoietic stem cell repair capacity of DNA double-strand breaks induced by high-dose irradiation. Alternatively, differences might exist between individuals in terms of persistent radiation-induced genetic instability within the hematopoietic system.

Another important message of Fig. 1 is that the wide variation in *GPA* Mf among individuals exposed to high radiation doses was not merely the result of random errors in measurement, or in dose dosimetry. It was also thought to primarily reflect inter-individual differences in sensitivity to radiation: higher responders to given radiation doses had higher probability of developing radiation-associated cancer. Of course, unidentified factors other than radiation sensitivity are possibly involved in this inter-individual variation, specifically considering the long period elapsed since atomic radiation exposure and other environmental factors influencing *GPA* Mf [7]. The large reduction in size of the stem-cell pool as a consequence of cell destruction by A-bomb irradiation might cause stochastic fluctuation of *GPA* Mf derived from a limited

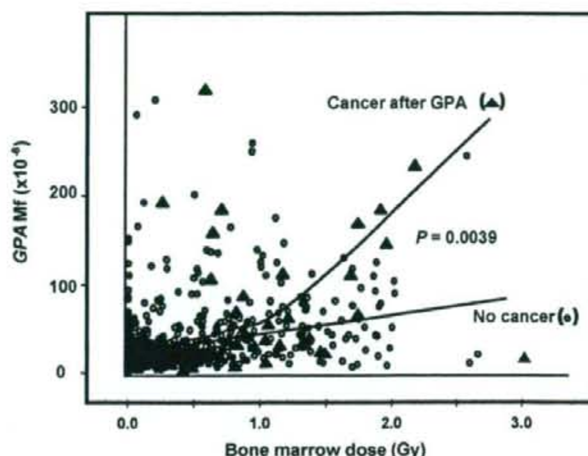

Fig. 1. Dose–response of *GPA* Mf to radiation dose (Gy) among MN heterozygous AHS participants in Hiroshima (modified from reference [1]). Non-parametric curve fitting for cancer (being identified by a follow-up study after *GPA* Mf measurement) and non-cancer groups revealed a significantly higher slope of *GPA* Mf dose–response found in the cancer group than in the cancer-free group in high dose region of ≥ 1 Gy (regression $P < 0.01$).

number of mutant stem cells [8]. Therefore, a new study will be required with use of biomarkers more specific to acute and delayed effects of radiation.

These results provided the basis to ask two further questions. One question is whether or not individual sensitivity to radiation-induced genetic damage was reflected by the noted inter-individual variation in *GPA* Mf as a function of radiation dose. The other is whether genetic instability was induced and contributed to the noted dose-dependent increase of *GPA* Mf. To answer these questions, we recently developed a γH2AX assay system to evaluate individual sensitivity to the immediate effects of set doses of radiation, as well as a reticulocyte micronucleus assay system to investigate delayed effects of radiation, namely genetic instability.

2.2. *Biomarkers for radiation sensitivity and genetic instability*

Various biomarkers measuring radiation-induced cellular damage, such as DNA strand-breaks, chromosomal damage and lethality, have been studied to assess individual sensitivity to radiation exposure in vitro. However, these bioassays require considerable labor and technical skill to achieve reliable and reproducible results. Thus, assay platforms that can more easily and reliably measure radiation-induced cellular damage need to be identified, especially to facilitate biomarker assays and associated risk estimation in a large study population. For this purpose, we thought that flow cytometry was the most applicable method because of its performance characteristics: i.e., high throughput and high-sensitivity.

It is known that histone H2AX, a subfamily of histone H2A, is phosphorylated and forms foci (γH2AX foci) at the sites of DNA double-strand breaks induced by ionizing radiation [9]. The number of γH2AX foci closely corresponds to that of DNA double-strand breaks in cells [10], and counting γH2AX foci has frequently been used as a more sensitive DNA damage marker than more conventional assays, such as pulse-field gel electrophoresis, neutral single cell electrophoresis (Comet assay), or DNA elution assay [11]. We recently have established a flow cytometry system for detecting γH2AX induced by in vitro irradiation, using cultured T lymphocytes, which are readily available from healthy individuals, and attempted to validate its suitability for analysis of individual radiosensitivities in human populations [3]. Because γH2AX focus formation appears not only in genomically damaged cells, but also in cells undergoing DNA synthesis and mitosis [12,13], improved assays to detect level of radiation-induced γH2AX foci were required. Toward this end, we simultaneously analyzed γH2AX expression and DNA content, and γH2AX levels were determined in cell fractions accurately gated for G0/G1 phase cells. Such cell-cycle-specific analysis is feasible for flow cytometry, but not for conventional fluorescence microscopy. Short-term (7 days) cultured T lymphocytes, but not uncultured, freshly isolated lymphocytes from peripheral blood, exhibited significant inter-individual differences in level of γH2AX induced by in vitro X-irradiation (Fig. 2). The reason why no obvious inter-individual differences were detected in the assay using uncultured lymphocytes remains elusive, but substantial differences in metabolic status, such as differences in the levels of radical scavenger proteins, between cultured and uncultured lymphocytes might result in differences in radiation sensitivity (discussed in reference [3]). Our assay system also provides good reproducibility, as well as a capacity to detect significant inter-individual differences between the responses of T lymphocytes from six healthy donors 6 h after 4 Gy of X-irradiation [3]. Variation in the level of γH2AX in cultured T lymphocytes of these individuals was about 1.5-fold. Differences in lymphocyte subsets either before or after culture were not responsible for this noted variance in individual radiosensitivity. Thus, our γH2AX assay system using cultured T lymphocytes appears to be useful for the rapid and reliable assessment of individual radiation sensitivity. We have already

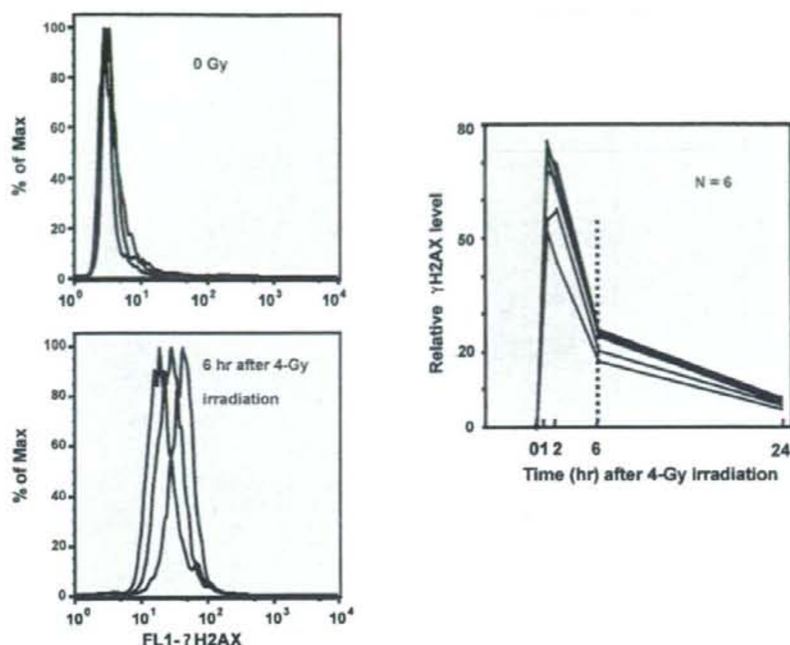

Fig. 2. Flow cytometry analysis of phosphorylated H2AX (γH2AX) in cultured T lymphocytes from three typical healthy individuals 6 h after 0 or 4 Gy of X-irradiation (left panels). Radiation-induced γH2AX expression levels in cultured T lymphocytes from six healthy individuals, which were measured 1, 2, 6 and 24 h after 4-Gy irradiation (right panel). There were significant inter-individual, but not inter-experimental, differences in the γH2AX levels 6 h after 4-Gy irradiation in three independent experiments using peripheral blood lymphocytes from six healthy donors (ANOVA, $P < 0.01$) [3].

confirmed that T lymphocytes cultured from cryopreserved lymphocytes are also useful for this radiation sensitivity assay. Evaluation of radiation sensitivity using cryopreserved lymphocytes from A-bomb survivors for whom *GPA* Mf has been analyzed is underway.

It has been recently reported that chromosomal alterations are indicated not only in cells that have been directly irradiated, but also in unirradiated neighboring cells, or descendants of irradiated cells [14]. Such delayed radiation effects, termed radiation-induced genetic instability effects, are thought to represent adverse cellular consequences, such as linked bystander and occasionally malignant transformation [14]. Although radiation-induced genetic instability is well characterized in vitro, evidence from in vivo studies has been limited due to lack of reliable bioassays capable of detecting and distinguishing "delayed type" chromosomal alterations induced by direct radiation effects versus those induced indirectly. Thus, to ensure identification of both delayed and direct radiation effects in vivo, we applied a flow cytometry-based reticulocyte micronucleus assay [15–17]. Because micronuclei in reticulocytes are chromosomal segments separated from entire chromosomes during the enucleation stage of erythrocyte maturation, and because the estimated lifespan of reticulocytes in vivo is as short as a few days [18], reticulocyte micronuclei observed more than 1 month after irradiation are not those that have been directly induced by radiation but those that have arisen in the course of normal erythopoiesis (Fig. 3). By utilizing this post-irradiation time discrepancy in the appearance of reticulocyte micronuclei, we were able to analyze delayed radiation effects separately from direct effects in whole-body X-irradiated mice [4]. In irradiated mice, we detected an acute effect from radiation dose as small as 0.1 Gy 2 days after irradiation, and a significant difference in the radiation-dose response between BALB/c and C57BL/6 mice (regression $P < 0.001$). As for delayed radiation effects, we also observed significantly increased frequencies of reticulocyte micronuclei in both BALB/c and C57BL/6 mice (1.6- and 1.3-fold increases compared with age-matched controls, Mann–Whitney, $P = 0.035$ and 0.039, respectively), 1 year after irradiation with 2.5 Gy of X-rays. Interestingly, there was also a significant mouse strain difference in the delayed radiation effect (Mann–Whitney, $P = 0.028$). It was therefore concluded that delayed effects of radiation on the murine hematopoietic system can persist in vivo for prolonged periods and that there were differences by mouse strain in susceptibility to such delayed radiation effects.

Two possibilities that are not mutually exclusive have been proposed for radiation-induced genetic instability [14]. One possibility is that cells not directly irradiated, but descended from irradiated cells, exhibit genetic instability. The other is that mediators, such as ROS and inflammatory cytokines, are released from irradiated cells and enhance DNA damage in unirradiated but neighboring cells to the irradiated cells (bystander effect). Our previous study indicated that low-grade inflammation may persist for more than 50 years after irradiation in A-bomb survivor populations [5]. It is intriguing to speculate that the reticulocyte micronucleus response, an

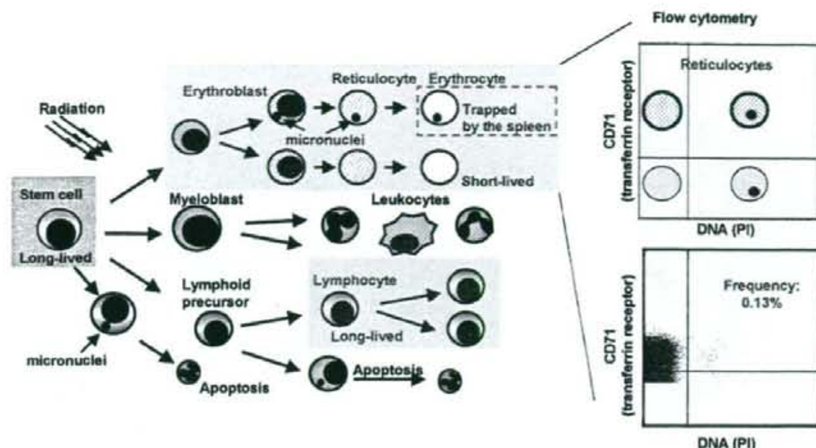

Fig. 3. A scheme of reticulocyte micronucleus assay thought to reflect the genetic damage involved in genetic instability induced by irradiation of the hematopoietic system. Circulating reticulocytes harboring micronuclei are differentiated from directly radiation-damaged precursor cells. Circulating reticulocytes with or without micronuclei will mature to erythrocytes in 3 days, resulting in elimination of directly damaged reticulocytes within a few days after irradiation. Therefore, micronuclei in reticulocytes after this acute phase are anticipated to indicate delayed radiation effects, namely genetic instability. The right panels show a schematic illustration of flow cytometry analysis of reticulocyte micronucleus and an actual flow cytometry pattern analyzing the reticulocyte micronucleus frequency in an irradiated mouse.

indication of genetic instability, is associated with the noted elevation of radiation-induced *GPA* Mf and with inflammatory status and ROS production in the survivors. Several flow cytometry methods measuring reticulocyte micronuclei in human blood samples have been developed [19,20]. Application of any one of these methods would allow us to further pursue molecular epidemiological investigations using this biomarker of genomic instability among the AHS population.

2.3. Biomarkers for inflammation and reactive oxygen species

Our immunology group has previously found that A-bomb radiation exposure enhanced persistent inflammation that is often accompanied with normal physiologic aging [5]. Further, inflammation-associated production of excessive levels of free radicals (e.g., ROS) is highly mutagenic. Therefore, endogenous ROS might be a factor to be analyzed with the aforementioned biomarkers. We have recently developed a total ROS assay system to determine the total amount of oxygen-centered free radicals derived from various ROS metabolites in a blood sample [21]. Our preliminary study showed that plasma ROS levels in AHS participants significantly increased with increased radiation dose (regression $P < 0.001$), even after adjustment was made for gender, age, smoking status and body mass (unpublished data).

3. Molecular oncology analyses of radiation-associated cancers

3.1. Adult-onset papillary thyroid cancer in the LSS cohort

Epidemiological studies on the LSS cohort of A-bomb survivors have identified a significant radiation-associated solid cancer risk [22]. However, the level of risk differed from organ to organ [22]. The excess relative risk of papillary thyroid cancer in survivors was 1.2 per Sv, and it increased with radiation dose [22]. A histopathological study has revealed that the thyroid cancer found in A-bomb survivors was largely conventional papillary in nature. This is also the case of spontaneous thyroid cancer in the Japanese at large. Solid variant papillary thyroid cancer was not found in A-bomb survivors, although this cancer has been frequently observed among post-Chernobyl children.

A major early event in papillary thyroid carcinogenesis is the constitutive activation of the MAP kinase signaling pathway caused by a single genetic alteration. This alteration has been identified as one among several possible, mutually exclusive, rearrangements of the *RET* and *NTRK-1* genes, and point mutations in the *RAS* and *BRAF* genes [23–25]. Among post-Chernobyl childhood thyroid cancers, the prevalent *RET/PTC3* rearrangement was closely associated with a short latency period after exposure and also with solid variant-type of the disease [26,27]. A low prevalence of *BRAF* mutation has been observed in childhood papillary thyroid cancer regardless of the presence or absence of past radiation exposure [28]. On the other hand, prevalence of *RET/PTC* rearrangements in radiation-associated adult-onset thyroid cancer, including radiation-therapy cases, was controversial [29,30]; *BRAF* point mutation ($BRAF^{V600E}$) was found at high frequency in papillary thyroid cancer among adult patients without history of radiation exposure [28]. In this regard, our previous experiments in vivo and in vitro showed that X-irradiation induced *RET/PTC1* rearrangement in scid mice within human thyroid tissue transplants, as well as in human thyroid cells cultured in vitro [31].

A logical first step for clarifying the mechanistic relationship between radiation exposure and the development of papillary thyroid cancer would be identification of the gene alterations that

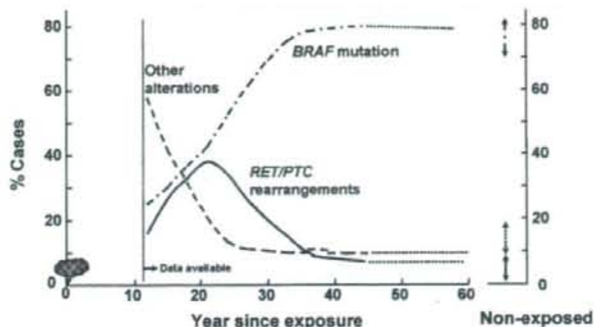

Fig. 4. A scheme of gene alteration types in adult-onset papillary thyroid cancer among A-bomb survivors varying with radiation dose and year elapsed since atomic radiation exposure.

preferentially occur during adult-onset radiation-associated thyroid carcinogenesis. Toward this end, we analyzed *RET/PTC* rearrangements and $BRAF^{V600E}$ point mutation among 50 adult-onset papillary thyroid cancer cases exposed to A-bomb radiation. Relationships to radiation dose, as well as years elapsed since A-bomb radiation exposure, were evaluated. When dividing the subjects into three groups harboring $BRAF^{V600E}$ mutation, *RET/PTC* rearrangements, and other unknown gene alterations, radiation dose (tertiles) responses of these groups differed: $BRAF^{V600E}$ mutation frequency significantly decreased in groups with increased radiation dose (Cochran-Armitage $P_{trend} < 0.001$) [6], while *RET/PTC* rearrangements showed significantly increased frequency with radiation dose (Cochran-Armitage $P_{trend} = 0.002$). Furthermore, other unknown gene alterations tended to be more frequent with increased radiation dose, suggesting that radiation-associated gene alterations (possible chromosomal rearrangements) other than *RET/PTC* rearrangements might be involved in the adult-onset thyroid cancers of A-bomb survivors who were exposed to high radiation doses. These findings also correspond with another significant observation in the same subjects: namely that the subjects with *RET/PTC* rearrangements developed cancer sooner following exposure than did the subjects with $BRAF^{V600E}$ mutation, as illustrated in Fig. 4 (Mann–Whitney, $P = 0.029$). Together, the interim results obtained thus far suggest an important role of *RET/PTC* rearrangements in adult-onset radiation-associated thyroid carcinogenesis.

3.2. Colorectal cancer from the LSS cohort

Radiation exposure was associated with increased risk of colon cancer (excess relative risk per Sv of 0.72, 95% confidence interval 0.29–1.28); interestingly, rectal cancer did not show apparent risk elevation upon radiation exposure [22]. In colorectal cancer, two major phenotypes, i.e., chromosomal instability and microsatellite instability (MSI), were correlated with different subsites of the colorectum. Specifically, high-MSI (MSI-H) cancer was frequently found at a specific subsite—the proximal colon [32]. Therefore, we hypothesized that the MSI phenotype might be associated with radiation exposure.

First, we determined MSI status in 24 colon and 11 rectal cancers in A-bomb survivors with defined radiation doses, in terms of six microsatellite markers. Five MSI-H cases were found among them, all in the proximal colon and none in the rectum. The median radiation dose of MSI-H colorectal cancer patients was significantly higher than that of microsatellite stable (MSS) and low-MSI (MSI-L) cancer patients (Mann–Whitney, $P = 0.042$). These observations suggest a possible link between radiation exposure and MSI.

Most MSI-H sporadic colorectal cancers showed loss of expression of the MLH1 DNA repair enzyme and the methylation of its promoter. The latter is probably a major cause for inactivation of this gene [33]. We examined as well the methylation of the CpG dinucleotides within the proximal region (−231 to −228) of the *MLH1* gene, since this CpG island is responsible for decreased mRNA and/or protein expression of the gene [34–36]. Using combined bisulfite restriction analysis [37], methylation of the *MLH1* gene was found in five patients, whose median radiation dose tend to be higher than that of subjects with *MLH1*-unmethylated genes; this finding, however, is of marginal significance (Mann–Whitney, $P = 0.06$). Methylation of the *MLH1* gene was significantly associated with MSI status in this study (χ^2, $P = 0.017$), as was the case in other studies: three patients showed both methylation of the *MLH1* gene and the MSI-H phenotype.

In addition to DNA methylation, we examined loss-of-heterozygosity (LOH) of the *MLH1* gene, which is also responsible for deficient expression of the *MLH1* gene [33]. We found that all five MSI-H cases carried LOH at the gene loci. Our preliminary findings imply that MSI colorectal carcinogenesis among A-bomb survivors might involve both epigenetic and genetic alterations of the *MLH1* gene.

During the past decade, it has become clear that there is a "serrated polyp pathway" associated with MSI [38]. This pathway is initiated by hyperplastic polyp formation, followed by serrated adenoma, and ultimately leading to invasive cancer. Loss of the MLH1 protein followed by acquisition of MSI occurs at the late stage [39], while point mutation of the *BRAF* gene ($BRAF^{V600E}$) is recognized as an early event [40]. We therefore examined $BRAF^{V600E}$ and found that four cases possessed this mutation. The median radiation dose of

BRAF-mutated cases was significantly higher than that of non-mutated cases.

Our results to date suggest that radiation exposure might influence MSI status through MSI-related epigenetic and genetic alterations—processes that may have occurred in the early stage of colorectal carcinogenesis. Further analysis with an increased number of cases is clearly required, however.

4. Future directions

Epidemiological follow-up study among A-bomb survivors for over half a century has provided invaluable knowledge about how atomic-radiation influenced disease outcome, and it will continue to do so in the years ahead. However, relatively little is known about the mechanisms of adverse health effects of ionizing radiation that arise late in life. For example, risk of various solid cancers increased with radiation dose and remain high, despite six decades since the exposure event. We still do not know why some organs are more sensitive to radiation exposure than others, and why cancer incidence within such organs remains high. Apart from determining risk for the population at large, a more elusive question is how to evaluate individual sensitivity to various radiation effects and how to handle risk estimation of individuals. The molecular epidemiology study at RERF seeks to find the answers to these important questions relating to the health effects of radiation.

It may be noted that the primary aim of molecular epidemiology is not necessarily to find association between radiation and disease outcome, but to assess the various radiation effects on cells, tissues, organs, and vital physiological systems and processes of the body, such as immunity and DNA repair, in terms of various biomarkers, some of which may be related to disease. The biomarkers to be used in our somatic mutability and molecular oncology studies are listed in Table 1, together with possible use of stored biological materials. Our somatic mutability study is positioned to look at the systemic effects of radiation. In fact, the observed relationship between *GPA* Mf, radiation dose, and solid cancers suggests that atomic radiation might exert long-lasting and systemic effects on the mutability of tissue cells of the body and not exclusively of hematopoietic cells, since increased *GPA* Mf at high doses was closely associated with cancers of various organs and not just the hematopoietic system. We anticipate that the results of the γH2AX and reticulocyte micronucleus assays will deepen our mechanistic understanding of radiation-induced somatic mutability.

Nevertheless, we still are looking at only the "shadow" of radiation effects. This is due, in part, to the fact that our survey has been and will continue to be performed well over 40 years after the bombings, and that the direct targets of radiation are stem/progenitor cells, the nature of which generally differs from that of differentiated cells. In the future, somatic mutability studies need to combine with stem cell biology studies, so that radiation effects on stem cells and early progenitors can be investigated in terms of genetic and epigenetic alterations, stem cell senescence, and genome maintenance.

Carcinogenic pathways appear to differ significantly between organ systems and specific types of pathologies, as evidenced by the markedly different cancer risks in various organs of the A-bomb survivors. Only a few molecular oncology studies on cancers among A-bomb survivors have been reported in the past. This is due in part to the difficulty in collecting cancer tissue samples as well as the difficulty in analyzing those long-term preserved formalin-fixed samples at the molecular level. As a result of our molecular oncology study, sophisticated methods have been developed for the analyses of archived, often decades old, tissue samples [41], and have examined the molecular events in the carcinogenic pathways of particular cancers, as influenced by radiation exposure. Results of our adult-onset papillary thyroid and colorectal cancer studies have suggested that radiation did not alter basic pathways, but preferentially induced specific events: e.g., preferential occurrence of chromosome aberrations in the early stage of papillary thyroid carcinogenesis, and MSI and its related molecular events as consequences, not causes, of colorectal carcinogenesis. Although sufficiently intense ionizing irradiation may be associated with genome-wide hypomethylation, comparable irradiation might be related to hypermethylation of specific genes. This may be the case in *MLH1* hypermethylation in colorectal cancer.

Our findings on thyroid and colorectal cancers remain preliminary. Given the potential implications of our preliminary findings, further work is warranted to assess more thoroughly the mechanisms of radiation-associated cancer. To pursue these molecular oncology studies, cancer tissue samples from

Table 1
The biomarkers to be used for RERF somatic mutability and molecular oncology studies

Biomarkers	Materials (other than fresh samples)	Related functions/phenotypes
GPA Mf	Paraformaldehyde- or DMS-fixed blood cells	Somatic mutability
γH2AX foci	Cultured T-lymphocytes (expanded from cryopreserved uncultured ones)	Radiation sensitivity
Reticulocyte micronuclei	Methanol-fixed blood cells	Acute and delayed genetic damage
Total ROS metabolites	Frozen serum or plasma	Cumulated ROS
RET rearrangements and *BRAF* mutation (papillary thyroid cancer)	Long-term preserved paraffin blocks	Chromosome aberration vs. point mutation, relative to radiation effects
MSI-related events (colorectal cancer) *MLH1* gene methylation Alterations in Ras signaling	Long-term preserved paraffin blocks	Microsatellite unstable carcinogenesis

A-bomb survivors, which are precious but presently spread over many hospitals, need to be collected in greater number and with greater efficiency.

We anticipate that our study will be linked to "molecular event-based risk estimation" of radiation-associated cancers, where the dose-risk relationships will be evaluated not only for a particular cancer-site but also for the fraction of this cancer harboring a specific molecular alteration, for example, radiation-induced risk of papillary thyroid cancer with *RET/PTC* rearrangements and MSI-positive colorectal cancer. These data will contribute not only to the mechanistic understanding of radiation-associated cancers but also to the future prevention of these cancers associated with natural, medical, occupational, and accidental exposure to radiation.

Acknowledgements

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Cytolethal Distending Toxin Induces Caspase-Dependent and -Independent Cell Death in MOLT-4 Cells▽

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Cytolethal distending toxin (CDT) induces apoptosis using the caspase-dependent classical pathway in the majority of human leukemic T cells (MOLT-4). However, we found the process to cell death is only partially inhibited by pretreatment of the cells with a general caspase inhibitor, z-VAD-fmk. Flow cytometric analysis using annexin V and propidium iodide showed that a 48-h CDT treatment decreased the living cell population by 35% even in the presence of z-VAD-fmk. z-VAD-fmk completely inhibited caspase activity in 24 h CDT-intoxicated cells. Further, CDT with z-VAD-fmk treatment clearly increased the cell population that had a low level of intracellular reactive oxygen. This is a characteristic opposite to that of caspase-dependent apoptosis. Overexpression of *bcl2* almost completely inhibited cell death using CDT treatment in the presence of z-VAD-fmk. The data suggest there are at least two different pathways used in CDT-induced cell death: conventional caspase-dependent (early) apoptotic cell death and caspase-independent (late) death. Both occur via the mitochondrial membrane disruption pathway.

Programmed cell death is critical for organ development and homeostasis in eukaryotes (24, 49, 52). In the past, the caspases were considered essential proteases for apoptosis. However, accumulating data suggest that caspase-independent cell death occurs in programmed cell death (4, 23) and, in certain conditions, the caspase-independent pathway is an important mechanism to protect organs when caspase-dependent cell death does not occur (4).

Viral or bacterial infection and cancer often influence programmed cell death pathways. This is true of death induced by the cytolethal distending toxin (CDT), one of the bacterial toxins produced by *Aggregatibacter actinomycetemcomitans*, a gram-negative pathogen implicated in the pathogenesis of juvenile and adult periodontitis (39, 42). CDT was demonstrated to induce apoptosis in various cell types, including the T-lymphocytic leukemia cell lines, Jurkat cells, and MOLT-4 cells (31, 35, 50).

CDT belongs to a family of toxins with cell cycle specific inhibitory activity blocking the G_2 to M phase through inactivation of the CDC2/cyclin B complex (for recent reviews, see references 17, 30, and 32). CDT is a complex of three subunits: CdtA, CdtB, and CdtC. CdtB induces double-strand breaks, acting as a DNase that triggers the CDT intoxication (9, 25). CdtA and CdtC have homologies to lectin-like domains that can bind to surface molecules on the target cells (7). CDT internalizes through a receptor-mediated endocytosis and subsequently reaches the nucleus by retrograde transport and active nuclear pore transport (15, 29). In the nucleus, the CDT-induced chromatin injury is found as double-strand breaks that may recruit a large protein complex, the PIDDosome, in which caspase-2 activation occurs (44). Previously, we demonstrated that CDT induces apoptosis and activates caspase-2 in two T-cell leukemia cell lines, Jurkat and MOLT-4 (31). Activated caspase-2 acts as an upstream initiator of mitochondrial membrane permeability (22). Increased permeability of the mitochondrial membrane releases proapoptotic molecules, including cytochrome *c*, to activate executive caspases, and this loss of the mitochondrial membrane potential leads to the production of reactive oxygen species (ROS) (34). In the presence of a caspase inhibitor, CDT-induced apoptosis was completely blocked for 16 h in Jurkat cells, suggesting that CDT-induced cell death was dependent on caspase activation (31). However, we found that some of the cells, after 24 h of CDT intoxication, undergo death in a manner different from conventional apoptosis using caspase activation. Here, we report the detailed features of this cell death and discuss the importance of caspase-independent cell death during late-stage CDT-intoxication.

MATERIALS AND METHODS

Purification of *A. actinomycetemcomitans* CDT. CDT holotoxin was purified by using a Ni-chelated agarose resin column as described previously where the C-terminal His_6-tagged CdtC was expressed using the pQE 60 expression vector in M15 *Escherichia coli* (Qiagen, Tokyo, Japan) that carried the *A. actinomycetemcomitans cdtA*, *cdtB*, and *cdtC* genes downstream of the T5 promoter (31). A mutant CDT with CdtB His274Ala (*cdtB* 274 histidine changed to alanine) was purified by using the same method. The mutant was constructed by using site-directed mutagenesis of the 274th histidine residue to an alanine in the *cdtB* gene of pQEcdtABC and was performed by using the overlap extension method (46). The primers used were 5′-ACA TCC GAT gcT TTT CCT GTT-3′ and 5′-AAC AGG AAA Agc ATC GGA TGT-3′ (mutated sites are shown as lowercase letters). The mutant DNA containing *cdtAB(H274A)C* was subcloned into the pQE60 vector (Qiagen).

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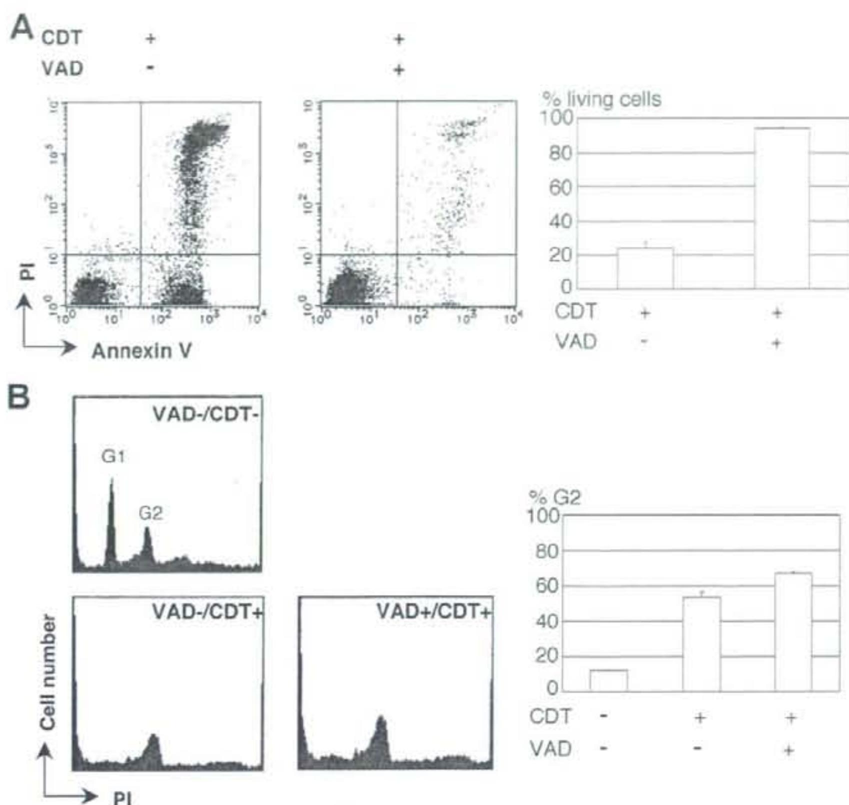

FIG. 1. Early effects of CDT intoxication on MOLT-4 cells in the presence of the general caspase inhibitor, z-VAD-fmk. The effects of CDT on MOLT-4 cells were examined 16 h after pretreatment of the cells with or without the general caspase inhibitor, z-VAD-fmk (100 μM), for 30 min. (A) MOLT-4 cells treated with CDT (100 ng/ml) for 16 h were observed after staining with PI and FITC-labeled annexin V for fluorescence by using a FACSCalibur. The percentages of living (annexin V-negative, PI-negative) cells are shown in the graphs along with the standard deviations. (B) The cell cycle was determined by staining the cells with PI after fixing the cells with 70% ethanol and RNase treatment. The percentages of the cells in the G_2 phase are shown in the graph, along with the standard deviations.

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Preparation of cells and culture conditions. The thymic T-cell leukemia cell line, MOLT-4, and the peripheral T-cell leukemia cell line, Jurkat, were cultured in RPMI 1640 with 10% fetal calf serum (FCS), 100 U of penicillin G/ml, and 100 μg of streptomycin/ml and incubated at 37°C using 5% CO_2 incubator. Cells (10^6 cells/ml) were treated with or without CDT (100 ng/ml) and cultured under similar conditions. In some experiment, cells were X-irradiated. Irradiation of cells was performed by an X-ray generator (Shimadzu HF-320; 220 kVp, 8 mA) with a 0.5-mm aluminum and 0.3-mm copper filter at a dose of ~0.8 Gy/min. Cells were irradiated in a plastic dish at room temperature. z-VAD-fmk, a general caspase inhibitor (MBL Nagoya, Japan), was used at 100 μM and was added 30 min before CDT treatment.

Establishing MOLT-4 cells stably overexpressing *bcl*-2. The complete coding sequence of *bcl*-2 (19), the gene governing antiapoptotic mitochondrial outer membrane permeabilization (MOMP), was amplified by using the PCR and subsequently cloned into an SFFV-neo vector (14). MOLT-4 cells were stably transfected with the plasmid SFFV-human *bcl*-2 or a control plasmid, SFFV-neo, using electroporation at 350 V with a capacitance of 960 μF with a GenePulser (Bio-Rad, Richmond, CA). Transfected cells were selected in medium containing 0.5 mg of G418/ml for 30 days. *bcl*-2 transfectants were found by using fluorescence-activated cell sorting (FACS) and Western analysis with Bcl-2 monoclonal antibody, 6C8 (BD Pharmingen, San Jose, CA), where we demonstrated Bcl-2 levels increased 10- to 20-fold greater than Bcl-2 present in untransfected or SFFV-neo-transfected MOLT-4 cells. The cells stably expressing Bcl-2 are referred to as MOLT-4bcl2 cells. The cells transfected with the control plasmid SFFV-neo served as a control and are referred to as MOLT-4neo cells.

Flow cytometry. Conformational change in the membrane by phosphatidylserine translocation and membrane hole formation was observed by counting the cell population stained with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (PI) as described previously (31). Briefly, CDT-treated cells (5×10^5 to 10×10^5) were centrifuged at $350 \times g$ for 2 min and washed three times with 500 μl of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 [pH 7.3]) with 1% FCS. The washed cells were resuspended in 180 μl of PBS containing 1% FCS, 0.5 μl of FITC-labeled annexin V, and 1 μl of PI using a MEBCYTO apoptosis kit (MBL, Nagoya, Japan). After 5 min at room temperature, 10,000 cells were scanned by using a FACScan (BD Biosciences, San Jose, CA). We performed a quadrant population analysis using CellQuest software (BD Biosciences). The live cell population was negative for both annexin V and PI (shown in the lower left quadrant).

Hydroethidine (HE) was used to measure the intracellular ROS, the superoxide anion ($O_2 \cdot ^-$) (16). HE (5 mM) was added to the PBS-washed cells (5×10^5 cells in 500 μl of PBS with 1% FCS). Cells were incubated for 20 min at 37°C. After a wash with PBS using centrifugation at $350 \times g$ for 5 min, the cells were resuspended in 200 μl of PBS containing 1% FCS and scanned using the FACScan. A gated population analysis was performed by using CellQuest software (BD Biosciences).

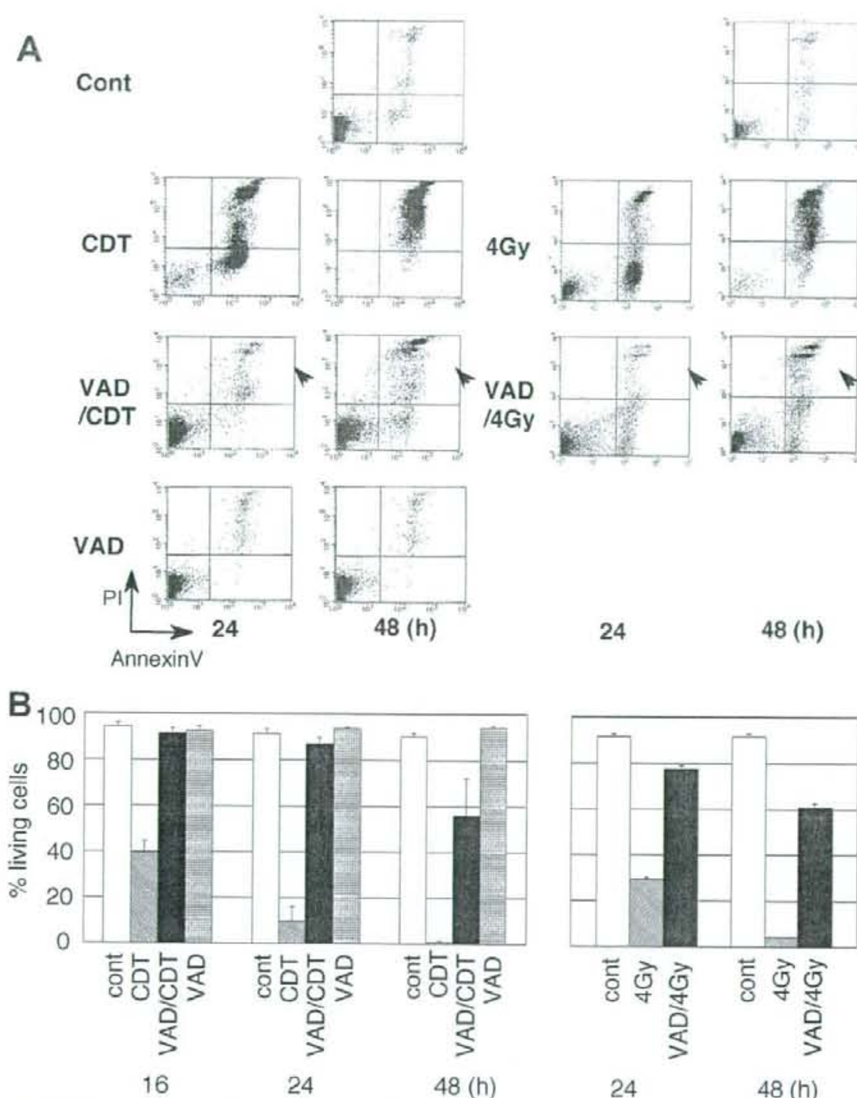

FIG. 2. Late effects of CDT in MOLT-4 cells in the presence of z-VAD-fmk. CDT-induced cell death was monitored in MOLT-4 cells at 24 h or 48 h after CDT treatment (100 ng/ml) in the presence or absence of z-VAD-fmk (100 μM). (A) Cell death was observed by using a FACScan after staining with PI and FITC-labeled annexin V. (B) Percentages of living (annexin V-negative, PI-negative) cells at 16, 24, and 48 h. As a control, radiation-induced cell death was monitored in MOLT-4 cells at 24 and 48 h with or without z-VAD-fmk. Arrowheads show that dead cell populations increased at 48 h even in the presence of z-VAD-fmk after treatment with CDT or radiation.

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The cell cycle was determined as follows. CDT-treated cells were washed twice with PBS and fixed with 70% ethanol for 2 h at 4°C. The fixed cells were washed twice with PBS and incubated with 0.25 mg of RNase A/ml for 15 to 60 min at 37°C. DNA in the RNase-digested cells was stained with 50 μg of PI/ml for 30 min at 4°C and analyzed by using a FACSCalibur flow cytometer (BD Biosciences).

Caspase assay. CDT-treated cells were harvested and washed with PBS. PBS-washed cells were lysed with lysis buffer (10 mM Tris-Cl [pH 7.4], 25 mM NaCl, 0.25% Triton X-100, 1 mM EDTA) and centrifuged at 15,000 × g for 10 min. The supernatant was diluted with lysis buffer at a protein concentration adjusted to 1 mg/ml. Then, 5 μg of total protein was incubated with 200 μl of caspase buffer (50 mM Tris-Cl [pH 7.2], 100 mM NaCl, 1 mM EDTA, 10% sucrose, 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 5 mM dithiothreitol) using 50 μM concentrations of the various fluorigenic substrate peptides. The peptides used were Ac-DEVD-7-amino-4-methyl cumarine (AMC) for caspase-3, caspase-7, and caspase-8; Ac-IETD-AMC for caspase-8, caspase-6, and Granzyme; Ac-LEHD-AMC for caspase-9; and Ac-VDVAD-AMC for caspase-2 (Peptide Institute, Inc., Osaka, Japan). The reaction mixture

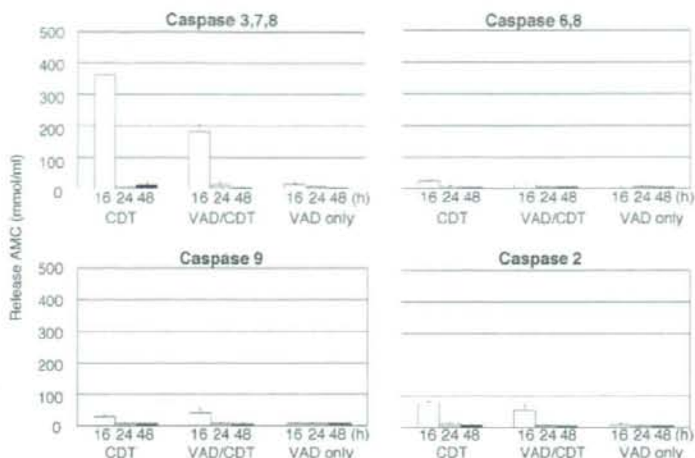

FIG. 3. Caspase activities in CDT-treated MOLT-4 cells in the presence of z-VAD-fmk. Caspase activities were monitored in MOLT-4 cells at 24 or 48 h after CDT treatment (100 ng/ml) in the presence or absence of z-VAD-fmk (100 μM). CDT-treated cell extracts (5 μg of total protein) were incubated with 50 μM concentrations of the various fluorigenic substrate peptides: AMC for caspase-3, caspase-7, and caspase-8; Ac-DQTD-AMC for caspase-7 and caspase-3; Ac-IETD-AMC for caspase-8, caspase-6, and Granzyme; Ac-LEHD-AMC for caspase-9; and Ac-VDVAD-AMC for caspase-2 (Peptide Institute, Inc., Osaka, Japan). The reaction mixture was incubated at 37°C for 60 min, where the release of AMC was measured by using a fluorometer (Shimazu RF-540) with an excitation at 380 nm and an emission at 460 nm.

was incubated at 37°C for 60 min where the release of 7-amino-4-methylcumarin was measured by using a fluorometer (Shimazu RF-540) with an excitation at 380 nm and an emission at 460 nm.

RESULTS

Early effect of CDT in MOLT-4 cells in the presence of the general caspase inhibitor, z-VAD-fmk. The effect of the general caspase inhibitor, z-VAD-fmk, on MOLT-4 cells was examined for 16 h after the cells were treated with 100 ng of CDT/ml. As shown in Fig. 1, when the cells were treated with z-VAD-fmk (100 μM) most cells retained the phenotype of living cells (annexin V negative, PI negative). This indicated the caspase inhibitor almost completely blocked CDT-induced apoptosis for at least 16 h but did not block the G_2/M arrest (Fig. 1B). We investigated DNA ladder formation in the presence of z-VAD-fmk in the CDT-treated MOLT-4 cells. z-VAD-fmk completely inhibited internucleosomal DNA fragmentation (data not shown). These results are consistent with our previous study using Jurkat cells (31).

Late effect of CDT in MOLT-4 cells in the presence or absence of z-VAD-fmk. We then assessed the effect of long-term exposure to CDT on MOLT-4 cells in the presence or absence of z-VAD-fmk. A 24-h exposure to CDT on MOLT-4 cells increased the population of annexin V-positive and PI-negative cells, as well as a population of annexin V-positive and PI-positive (dead) cells (Fig. 2A). Pretreatment with z-VAD-fmk significantly inhibited the increase of these populations at 24 h (Fig. 2A). At 48 h, however, a population of annexin V-positive and PI-negative cells and also a population of annexin V-positive and PI-positive cells increased compared to the numbers at 16 h (Fig. 1 and 2). At 48 h after CDT exposure in the presence of z-VAD-fmk, living cells defined as annexin V-negative and PI-negative cells decreased by 35% compared to nontreated cells (90.18% living) (Fig. 2B). This is very similar to the observation when MOLT-4 cells were X-irradiated (5). Ionizing radiation on MOLT-4 cells induced G_2/M arrest (37). Moreover, pretreatment of z-VAD-fmk inhibited the increase of dead cells at 24 h (Fig. 2A) (5). However, at 48 h after X-irradiation in the presence of z-VAD-fmk, the number of living cells decreased. With CDT treatment, caspase activities increased after 16 h (Fig. 3). This was apparent for caspase-3, caspase-7, and caspase-8. However, pretreatment with z-VAD-fmk significantly suppressed caspase activity, and this was likewise observed in Jurkat cells (31). Further, there was almost no caspase activity in MOLT-4 cells at 24 h after CDT exposure in the presence of z-VAD-fmk, even though 85% of the cells were alive (Fig. 2B and 3). This was also found at 48 h after CDT exposure in the presence of z-VAD-fmk. This suggests that CDT is able to induce cell death in the late stages of cell intoxication without caspase activation.

Comparison of the late effects of CDT to the mutant CdtB His274Ala CDT. Previous studies suggest CdtB shows sequence similarity to DNase I, where it shares conserved amino acids essential for Mg binding (9, 25). The 274th histidine residue in the CdtB subunit is one of the active sites where a mutation at 274th His to Ala abolishes CDT cytodistending and cell cycle arrest activities (28). This strongly suggests that CdtB acts as a nuclease. To determine whether the CdtB nuclease activity is responsible for CDT-induced late cell death, mutant CDT containing CdtB H274A was generated and added to the medium of MOLT-4 cells. Mutated CDT did not show cytokilling activity until 24 h, as expected (Fig. 4). By 48 h, ca. 20% of the cells treated with the mutant CDT were dead, and this late cell death was almost completely inhibited by pretreatment with z-VAD-fmk. This shows that the possible nuclease activity of CdtB is also important for late cell

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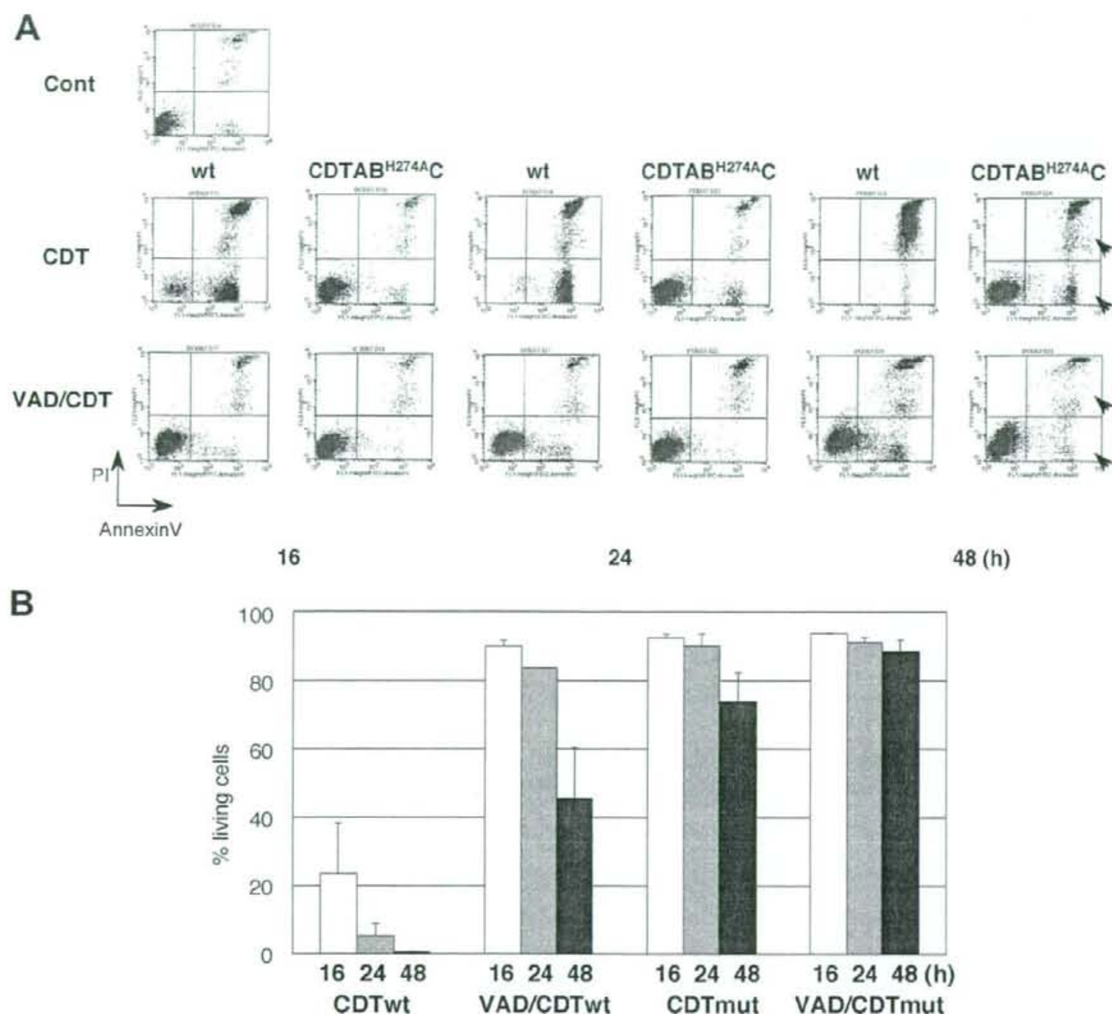

FIG. 4. Effects of mutated CDT on MOLT-4 cells in the presence or absence of z-VAD-fmk. CDT-induced cell death was monitored at 24 or 48 h in MOLT-4 cells after CDT treatment (100 ng/ml) or mutated CdtB His274Ala CDT. (A) Cell death was analyzed by using a FACScan after staining with PI and FITC-labeled annexin V. (B) Percentages of living (annexin V-negative, PI-negative) cells at16, 24, and 48 h. Arrowheads show that dead cell populations increased at 48 h after treatment with CDT but was not seen in the presence of z-VAD-fmk.

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death and was not inhibited by the general caspase inhibitor, z-VAD-fmk.

CDT-induced cell death in MOLT-4 cells overexpressing the *bcl2* gene. We previously demonstrated that CDT induces mitochondrial membrane permeability, resulting in an apoptotic cell death (early cell death) (31). To determine the role of mitochondria in late-stage CDT-induced cell death, we examined MOLT-4 cells forced to overexpress the *bcl2* gene (MOLT-4/bcl2). As shown in Fig. 5A, Bcl2 overexpression showed an inhibitory effect on the increase of the population of annexin V-positive, PI-positive cells using CDT intoxication. After 48 h of CDT intoxication, a 23% decrease in the living cell population was observed in CDT-treated MOLT-4/bcl2 cells (72% alive) compared to nontreated cells (95% alive) (Fig. 5B). This shows that CDT-induced cell death was significantly attenuated by the overexpression of *bcl2* in MOLT-4 cells. Further, pretreatment of MOLT-4/bcl2 with z-VAD-fmk completely inhibited CDT-induced cell death for 48 h (Fig. 5A [arrowheads] and B). This finding suggests that increased permeability of the mitochondrial membrane is a central factor in the overall cell death by CDT intoxication.

Appearance of a cell fraction with a low level of superoxide anion in CDT-treated MOLT-4 in the presence of z-VAD-fmk. Caspase-dependent apoptosis is characterized by an increase

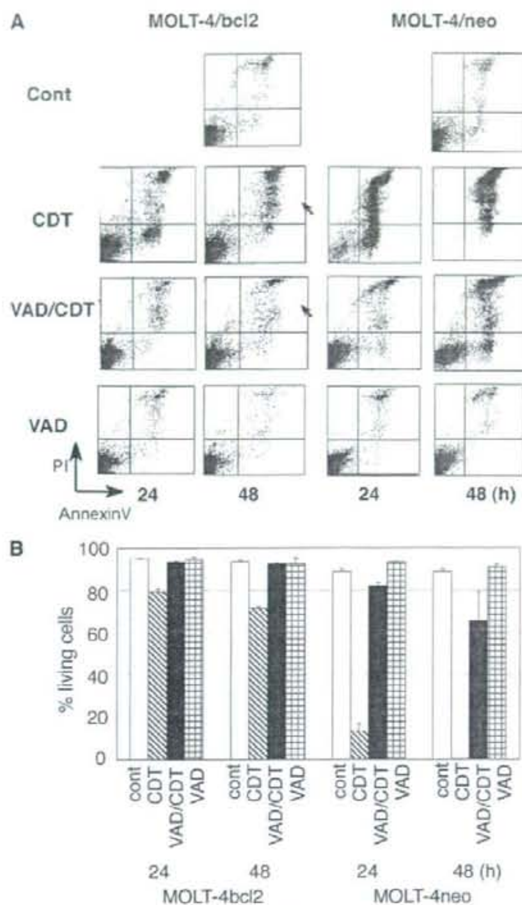

FIG. 5. Late effects of CDT in MOLT-4 cells overexpressing the *bcl2* gene in the presence or absence of z-VAD-fmk. CDT-induced cell death was monitored at 24 or 48 h after the treatment in MOLT-4 cells that were forced to overexpress the *bcl2* gene (MOLT-4/bcl2), MOLT-4 cells transfected with pSFFV-neo vector only (MOLT-4/neo), and parental MOLT-4 cells. (A) Cell death was analyzed by using a FACScan after staining with PI and FITC-labeled annexin V. (B) Percentages of living (annexin V-negative, PI-negative) cells at 48 h. Arrowheads show that dead cell populations increased at 48 h after treatment with CDT but was not seen in the presence of z-VAD-fmk.

in the intracellular superoxide anion and is related to mitochondrial membrane activity. We examined the levels of superoxide anion in CDT-treated cells using HE that reacts with superoxide anion. Concomitantly, FITC-labeled annexin V and PI were used to detect apoptotic and dead cell populations, respectively. We divided the cell population obtained by FACS into four groups (Fig. 6A): (i) S1 with normal levels of superoxide anion and nonapoptotic annexin V-negative, (ii) S2 with low levels of superoxide anion and annexin V negative, (iii) S3 with high level levels of superoxide anion and apoptotic annexin V positive, and (iv) S4 with low levels of superoxide anion (low HE) or at a normal level and apoptotic annexin V positive. Compensation between HE and PI was adjusted to separate the locations for the PI-positive and PI-negative (dead/live) populations in the quadrant analysis, where the PI-positive population is shown as dark red. As shown in Fig. 6, CDT treatment increased the S3 population to a maximum at 16 h, together with the appearance of a PI-positive dead cell population. Longer exposure increased the PI-positive population, as well as the S4 population. This shows that the CDT-induced early apoptosis was followed by cell death. We then assessed the population change in MOLT-4 cells exposed to CDT in the presence of z-VAD-fmk. z-VAD-fmk induced the appearance of an S2 population in CDT-treated MOLT-4 cells at 16 h, at which point the S3 population did not increase. At 48 h, the cells in the S2 population decreased and PI-positive population, as well as the S4 population, increased. MOLT-4/bcl2 cells exposed to CDT in the presence of z-VAD-fmk showed most cells remained in the S1 population even after 48 h of intoxication (data not shown). This, together with the caspase assay, clearly shows that CDT intoxication in the presence of z-VAD-fmk induced MOLT-4 cells into a caspase-independent nonapoptotic cell death, where MOMP played a significant role.

DISCUSSION

We show here that long exposure to CDT killed MOLT-4 cells even if caspase activation was inhibited by z-VAD-fmk. A similar observation was reported when MOLT-4 cells were X-irradiated in the presence of z-VAD-fmk (5). X-irradiation induces caspase-3-dependent apoptosis, but treatment of inhibitor of caspase-3 was not accompanied by any persistent increase in cell survival. Instead, irradiated cells treated by the inhibitor exhibited characteristics of a necrotic cell death. Similarly, our results show that caspase-dependent and caspase-independent pathways are involved in the CDT-induced MOLT-4 cell deaths. Similar responses of CDT-intoxicated MOLT-4 cells and X-irradiated MOLT-4 cells further support the idea that CDT intoxication triggers DNA damage.

Preincubation with z-VAD-fmk almost completely inhibited CDT-induced cell death in MOLT-4 cells overexpressing *bcl2* that increases the stability of the mitochondrial membrane. The data shows CDT-induced cell death is mitochondrion dependent and suggests the caspase-dependent and caspase-independent death pathways converge at the mitochondria (45). Reports demonstrate death signaling through the mitochondrial pathways induces MOMP to release several mitochondrial proteins including cytochrome *c* (13, 47, 48), where the Bcl-2 family of proteins are considered to be important regulatory factors for MOMP (8, 41). A possible reason why general caspase inhibitors fail to prevent cell death is because these inhibitors lack the ability to inhibit MOMP (8, 10, 21).

CDT is not the only bacterial toxin that induces cell death through multiple pathways. *Staphylococcus aureus* alpha-toxin, the major hemolysin, induces massive cell necrosis by forming pores in lipid bilayers at high doses (>6 μg/ml). At low doses, this toxin can induce DNA fragmentation and caspase activation, the typical classical apoptosis pathway, and also caspase-independent cell death (11). *Streptococcus pneumoniae* induces a rapid caspase-independent cell death in cultured bone mar-

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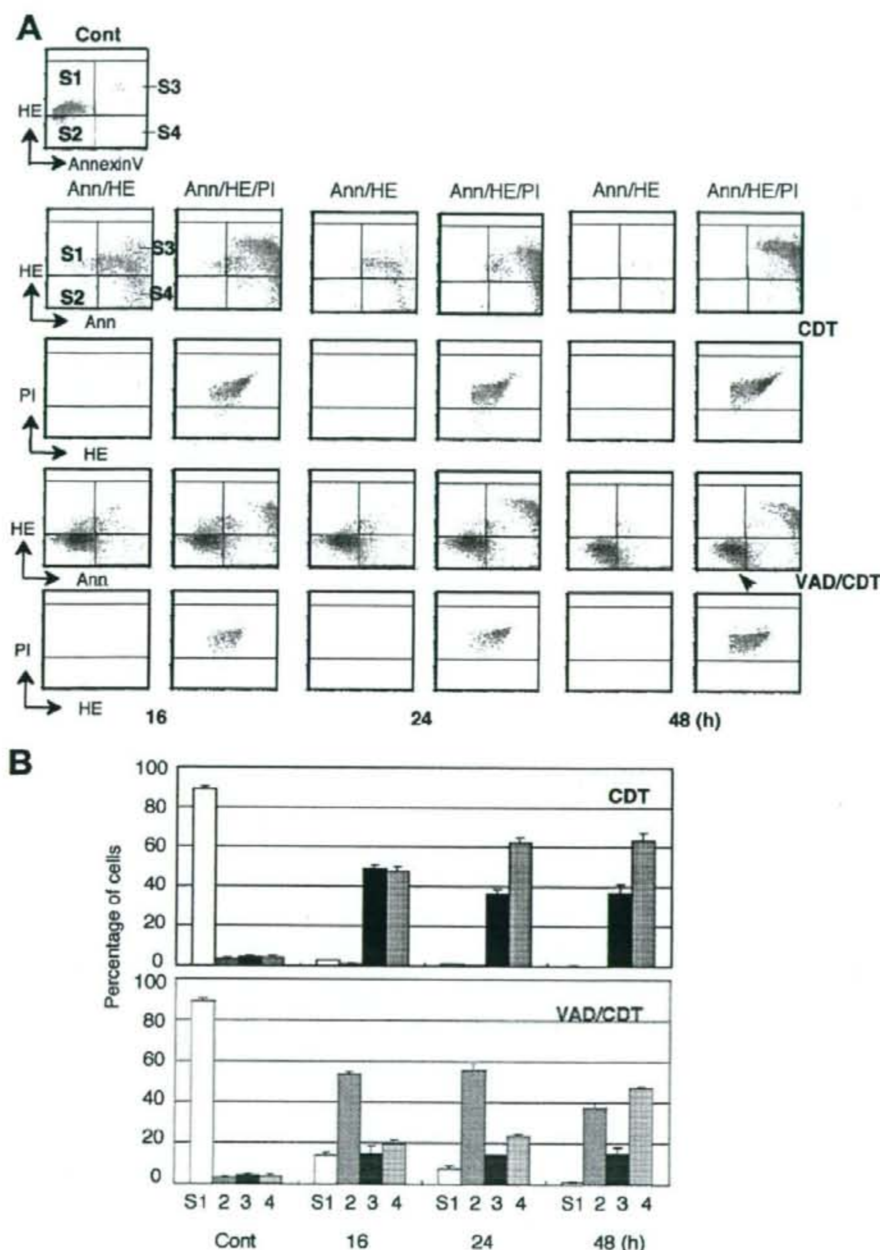

FIG. 6. ROS and phosphatidylserine translocation in CDT-treated MOLT-4 cells in the presence or absence of z-VAD-fmk. (A) The levels of ROS and phosphatidylserine translocation in the cytoplasmic membrane in the CDT-treated MOLT-4 cells were measured at 24 and 48 h by using a FACScan. To monitor the dead cells, the cells were subjected to double (Ann/HE) and triple (Ann/HE/PI) staining. Compensation between HE and PI was adjusted to separate the locations of PI-positive and PI-negative populations in the quadrant analysis. PI-positive populations were gated and are shown in the quadrant analysis of HE and annexin V double staining as a dark red color. S1, region of cells with normal levels of HE and annexinV; S2, cells with low levels of HE; S3, cells with high levels of HE and annexin V binding; S4, cells with low levels of HE and high levels of annexin V binding. The arrowhead shows an increased population in S2 by pretreatment with z-VAD-fmk, i.e., cells with a low level of ROS. (B) Percentages of cells distributed into the fractions at 16, 24, and 48 h, where CDT indicates the cells treated with 100 ng of CDT alone/ml and VAD/CDT indicates CDT-treated cells after pretreatment with z-VAD-fmk (100 μM).

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row-derived dendritic cells (6). This induction is dependent on the expression of pneumolysin, one of the major cytotoxins of *S. pneumoniae*. This is followed by the delayed onset of caspase-dependent cell death associated with the terminal maturation of dendritic cells. *Escherichia coli* heat-labile enterotoxin is composed of a single catalytically active A subunit and a pentameric B subunit that interacts with a receptor that mediates the uptake of the holotoxin into the target cells (38). Interestingly, the nontoxic B subunit induces both caspase-dependent and -independent cell death in CD8 T cells. The enterotoxin B subunit induces a rapid loss of mitochondrial membrane potential where cell viability is not affected by caspase inhibitors, suggesting some other intracellular signaling pathways are involved following interaction with the B subunit receptor. Another example is *Clostridium difficile* toxin B (33). *C. difficile* toxin B inactivates small GTPases, Rho, Rac, and Cdc42, which lead to caspase-3 activation in HeLa cells. Caspase inhibitors delayed cell death but did not alter the consequence.

Several classifications are proposed to differentiate types of cell death (1, 4, 12, 23). For example, Kroemer et al. (23) proposes four types: (i) classical apoptosis showing programmed cell death through caspase activation; (ii) apoptosis-like cell death resembling apoptosis but lacking total chromatin condensation, karyorhexis, and oligonucleosomal DNA fragmentation (20, 43); (iii) autophagic cell death with an accompanying accumulation of autophagic vacuoles in the cells (36, 51); and (iv) necrosis exhibiting pronounced swelling of the cytoplasmic organelles (18, 48).

Caspases, a group of cysteine proteases, normally act only during classical apoptosis; however, the activation of caspases is also observed in apoptosis-like autophagic cell death, and necrosis as well (36, 43, 48, 51). Further, caspases may be activated not only during the lethal process but also in nonlethal signal transduction (27). Paradoxically, accumulating evidence suggests several types of programmed cell deaths occur without caspase activation in parallel to caspase-dependent cell death as found in apoptosis-like cell death, autophagic cell death, and necrosis (2, 3, 13, 40). Fink et al. (12) propose four types of dying cells caused by infection with microorganisms: apoptosis, autophagy, oncosis, and pyroptosis. Apoptosis is a form of caspase-mediated cell death with particular morphological features, e.g., the apoptotic body, without inflammation. Oncosis is a prelethal process that occurs in ATP-depleted cells concomitant with morphological swelling and eventual membrane permeability. Autophagic cell death involves degradation of intracellular components using autophagic vacuoles. Pyroptosis is a pathway to cell death that involves interleukin-1-mediated inflammation.

Although several morphotypes have been proposed, a definitive classification of the types of cell death pathways has not been established. This is probably because there may be some signaling pathways overlapping and sharing the different death programs (4, 26). It has also been proposed that a dominant cell death morphotype may be determined by comparing the rapidity of the available death programs, i.e., the fastest and most effective pathway among them is dominant (4). In the case of CDT intoxication, the caspase-2-related classical pathway may be the fastest and most efficient pathway. Identification of molecule(s) involved in the CDT-induced caspase-independent and the MOMP-dependent pathway is required to further characterize this death pathway.

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Adaptation to Estradiol Deprivation Causes Up-Regulation of Growth Factor Pathways and Hypersensitivity to Estradiol in Breast Cancer Cells

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Abstract

Deprivation of estrogen causes breast tumors in women to adapt and develop enhanced sensitivity to this steroid. Accordingly, women relapsing after treatment with oophorectomy, which substantially lowers estradiol for a prolonged period, respond secondarily to aromatase inhibitors with tumor regression. We have utilized in vitro and in vivo model systems to examine the biologic processes whereby Long Term Estradiol Deprivation (LTED) causes cells to adapt and develop hypersensitivity to estradiol. Several mechanisms are associated with this response including up-regulation of ERα and the MAP kinase, PI-3-kinase and mTOR growth factor pathways. ERα is 4–10 fold up-regulated as a result of demethylation of its C promoter, is nuclear receptor then co-opts a classical growth factor pathway using SHC, Grb-2 and Sos. is induces rapid nongenomic effects which are enhanced in LTED cells.

The molecules involved in the nongenomic signaling process have been identified. Estradiol binds to cell membrane-associated ERα which physically associates with the adaptor protein SHC and induces its phosphorylation. In turn, SHC binds Grb-2 and Sos which results in the rapid activation of MAP kinase. These nongenomic effects of estradiol produce biologic effects as evidenced by Elk-1 activation and by morphologic changes in cell membranes. Additional effects include activation of the PI-3-kinase and mTOR pathways through estradiol-induced binding of ERα to the IGF-1 and EGF receptors.

A major question is how ERα locates in the plasma membrane since it does not contain an inherent membrane localization signal. We have provided evidence that the IGF-1 receptor serves as an anchor for ERα in the plasma membrane. Estradiol causes phosphorylation of the adaptor protein, SHC and the IGF-1 receptor itself. SHC, after binding to ERα, serves as the "glue" which tethers ERα to SHC binding sites on the activated IFG-1 receptors. Use of siRNA methodology to knock down SHC allows the conclusion that SHC is needed for ERα to localize in the plasma membrane.

In order to abrogate growth factor induced hypersensitivity, we have utilized a drug, farnesylthiosalicylic acid, which blocks the binding of GTP-Ras to its membrane acceptor protein, galectin 1 and reduces the activation of MAP kinase. We have shown that this drug is a potent inhibitor of mTOR and this provides the major means for inhibition of cell proliferation. The concept of "adaptive hypersensitivity" and the mechanisms responsible for this phenomenon have important clinical implications. The efficacy of aromatase inhibitors in patients relapsing on tamoxifen could be explained by this mechanism and inhibitors of growth factor pathways should reverse the hypersensitivity phenomenon and result in prolongation of the efficacy of hormonal therapy for breast cancer.

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