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Mcl-1 Depletion in Apoptosis Elicited by Ionizing Radiation in Peritoneal Resident Macrophages of C3H Mice

Yoshihisa Kubota,1* Keiji Kinoshita,* Katsutoshi Suetomi,* Akira Fujimori,* and Sentaro Takahashi†

Remarkably, apoptosis was induced by exposing peritoneal resident macrophages (PRM) of C3H mice, but not other strains of mice, to ionizing radiation. The molecular mechanism of this strain-specific apoptosis in PRM was studied. The apoptosis elicited in C3H mouse PRM 4 h after exposure was effectively blocked by proteasome inhibitors. Irradiation-induced disruption of mitochondrial transmembrane potential and the release of cytochrome c into the cytosol were also suppressed by a proteasome inhibitor but not by a caspase inhibitor. To determine whether the apoptosis occurred due to a depletion of antiapoptotic proteins, Bcl-2 family proteins were examined. Irradiation markedly decreased the level of Mcl-1, but not Bcl-2, Bcl-XL, Bax, A1, or cIAP1. Mcl-1’s depletion was suppressed by a proteasome inhibitor but not by a caspase inhibitor. The amount of Mcl-1 was well correlated with the rate of apoptosis in C3H mouse PRM exposed to irradiation and not affected by irradiation in radioreistant B6 mouse PRM. Irradiation increased rather than decreased the Mcl-1 mRNA expression in C3H mouse PRM. On the other hand, Mcl-1 protein synthesis was markedly suppressed by irradiation. Global protein synthesis was also suppressed by irradiation in C3H mouse PRM but not in B6 mouse PRM. The down-regulation of Mcl-1 expression with Mcl-1-specific small interfering RNA or antisense oligonucleotide significantly induced apoptosis in both C3H and B6 mouse PRM without irradiation. It was concluded that the apoptosis elicited in C3H mouse PRM by ionizing radiation was attributable to the depletion of Mcl-1 through radiation-induced arrest of global protein synthesis. The Journal of Immunology, 2007, 178: 2923–2931.

I t is now well established that the cells of the macrophage lineage play important roles in host defense due to their ability to phagocytose various particulate matters, involving microbes, and to participate in inflammatory and immunological processes. Tissue macrophages are terminally differentiated long-lived cells and are resistant to various toxic stimuli, including Fas, TNF-α, and multiple antineoplastic or cytotoxic agents (1–4). As an antineoplastic or cytotoxic agent, ionizing radiation has long been used in tumor therapy and bone marrow transplantation because many types of tumor and immune cells are known to be radiosensitive. Macrophages are also resistant to ionizing radiation, particularly regarding interphase cell death (5–7). Therefore, ionizing radiation-induced cell death has not been considered worthy of note in macrophages. The main focus has been on the radiation-induced functional activation of macrophages, rather than cellular inactivation (8). Primary macrophages or macrophage cell lines could be primed by ionizing radiation to produce NO in response to IFN-γ and/or LPS (9–12). Macrophages were also activated by irradiation to produce cytokines (13, 14) and IL (15). However, we recently reported that ionizing radiation surprisingly elicited remarkable apoptotic cell death in peritoneal resident macrophages (PRM)3 of C3H mice, but not other strains of mice (16). Several studies were performed to elucidate the mechanism of apoptosis in C3H mouse PRM exposed to ionizing radiation. Ionizing radiation is a typical DNA damaging (genotoxic) agent, and is known to induce apoptosis in various cell types via intracellular signal transduction pathways originating from DNA damage. In general, apoptosis elicited by genotoxic agents has been reported to be dependent on tumor protein 53 (TP53) (17–21), ataxia telangiectasia mutated (ATM) (22–24), or DNA-protein kinase catalytic subunit (25–29)-mediated cellular responses. However, we demonstrated that these responses were not involved in radiation-induced apoptosis in C3H mouse PRM. Another study elucidated that superoxide, but not other reactive oxygen species, played a major role in radiation-induced apoptosis in C3H mouse PRM (30). However, the molecular events occurring downstream of the generation of superoxide in radiation-induced apoptosis of C3H mouse PRM remained obscure. In this study, we demonstrated that Mcl-1, a member of the Bcl-2 family that is considered to have antiapoptotic properties in various neoplastic cells, is critical to radiation-induced apoptosis in C3H mouse PRM.

Materials and Methods
Preparation and irradiation of PRM

The materials and methods were described in detail in a previous article (16). Briefly, 6-wk-old female C3H/HeJ and C57BL/6J mice were purchased from a domestic breeder (Japan SLC). The C3H/HeJ (C3H) and C57BL/6J (B6) mice used were LPS-low and -high responders, respectively. Since even a low dose of LPS and cytokines can protect some types of cells from radiation-induced apoptosis, it is possible that some LPS

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2 Abbreviations used in this paper: PRM, peritoneal resident macrophage; ATM, ataxia telangiectasia mutated; GusB, glucuronidase β; HVJ, Hemagglutinating Virus of Japan; MEF, mouse embryonic fibroblast; RIPα, radioimmunoprecipitation assay; siRNA, small interfering RNA; TP53, tumor protein 53.

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contaminated the culture, conferring the radioresistance to B6 mouse PRM. However, in a preliminary study, no difference in radiation-induced apoptosis was observed between C3H/HeJ and C3H/HeN (LPS-high responder) mouse PRM under the experimental conditions of the present study, and a LPS antagonist, polymyxin B, did not sensitize B6 mouse PRM to elicit radiation-induced apoptosis. Therefore, it was concluded that genetic background relating LPS response did not affect radiation-induced apoptosis in PRM. Mice were housed in a controlled environment with free access to food and water. Animals younger than 24 wk old were used to obtain PRM. After the animals were anesthetized with diethyl ether and killed by exsanguination, the peritoneal cavity was lavaged with calcium- and magnesium-free PBS (Invitrogen Life Sciences). All the experiments with animals were conducted with the permission and according to the regulations of the Institutional Committees for Animal Safety and Welfare and in accordance with the Regulations on Appropriate Animal Breeding and Treatment, the Prime Minister’s Office of Japan. After centrifugation of the lavage fluid, cells were suspended in a culture medium, which was comprised of α-modified MEM (Invitrogen Life Sciences) containing 10% heat-inactivated FCS (JRH Biosciences), 100 U/ml penicillin, and 100 μg/ml streptomycin, plated in 35-mm tissue culture dishes (Falcon 3001; BD Biosciences) or 60-mm dishes (Falcon 3002), and cultured at 37°C in a humidified atmosphere of 5% CO2 in air. One hour after the incubation, the dishes were washed several times with fresh culture medium to remove the nonadherent cells. The cells that remained adhered to the dishes were referred to as PRM. Microscopic examination of the Giemsa-stained PRM showed that >95% of PRM were macrophages based on morphological criteria. PRM were irradiated with the 137Cs gamma source at a dose rate of ~8.5 Gy/min and then further cultured as described above. In some experiments, PRM were treated with hydrogen peroxide (Wako Pure Chemical) for 15 min, washed, and then further cultured or treated with cycloheximide throughout the incubation period.

Treatment with inhibitors

In some experiments, PRM were treated with various inhibitors 1 h before irradiation. Benzyloxycarbonyl-l-valyl-l-alanyl-l-aspartyl-l-yl-fluoromethane (z-VAD), benzyloxycarbonyl-l-leucyl-l-leucyl-l-leucinal (MG-132), lactacystin, and E-64-d were purchased from Peptide Institute. N-acetyl-l-leucyl-l-leucyl-l-norleucinal (ALLN) and BAPTA-AM were from Sigma-Aldrich.

Evaluating apoptosis microscopically

The apoptotic cell death induced in PRM by exposure to gamma irradiation or hydrogen peroxide was examined microscopically. Four hours after treatment, the culture medium was gently aspirated from the culture dishes, and a fluorescent dye solution containing acridine orange and propidium iodide at concentrations of 10 and 20 μg/ml in culture medium, respectively, was added to the dishes (31). A coverslip was placed over the cells, and they were observed under a fluorescence microscope. The percentage of apoptotic cells with condensed and fragmented nuclei and postapoptotic necrotic cells was calculated by observing at least 300 cells in individual samples.

Measurement of mitochondrial transmembrane potential

Mitochondrial transmembrane potential was evaluated as a Mitocapture Mitochondrial Apoptosis Detection kit (MBL), according to the manufacturer’s instructions. C3H mouse PRM exposed to irradiation, MG-132, and z-VAD were subjected to assays at 4 h. Images were taken under a fluorescence microscope using a bandpass filter to detect FITC and rhodamine. The cells with many aggregates giving off a bright red fluorescence represented those with a intact mitochondrial transmembrane potential and were enumerated.

Measurement of amount of cytochrome c released into the cytosol

The amount of cytochrome c in the cytosolic fraction was measured with a quantitative Cytochrome c ELISA kit (MBL) according to the manufacturer’s instructions. C3H mouse PRM at 4 h after irradiation were recovered from culture dishes, suspended at a concentration of 2 × 106 cells/ml with ice-cold preparation buffer (10 mM Tris-HCl (pH 7.5), 0.3 M sucrose, 10–3 M aprotinin, 10–7 M pepstatin A, 10–7 M leupeptin, and 1 mM PMSE) and homogenized extensively with a glass homogenizer (Weatom) until nearly all of the cells were disrupted. The homogenized samples were centrifuged at 10,000 × g for 30 min at 4°C, and the supernatant (referred to as the cytosolic fraction) was subjected to ELISA.

FIGURE 1. Apoptosis elicited by ionizing radiation in C3H mouse PRM is suppressed by proteasome inhibitors. A–E, C3H mouse PRM were left untreated or else treated with E-64-d, ALLN, MG-132, BAPTA-AM, or lactacystin 1 h before being irradiated at a dose of 40 Gy or treated with H2O2 at a dose of 4 × 10–5 M for 15 min. Apoptosis was evaluated 4 h after the irradiation or H2O2 treatment. Stars indicate a significant difference in apoptosis at that dose of the inhibitor from that with no inhibitor.

Western blot analysis

PRM were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and protease inhibitor mixture (Roche Diagnostic Systems)) on ice. Cell debris was removed by centrifugation at 10,000 × g for 10 min at 4°C. The protein concentration in cell lysates was determined using a Bio-Rad protein assay kit (Bio-Rad). An equal amount of protein (20 μg) for each sample was separated by 12% SDS-PAGE and subsequently transferred electrophoretically onto Immobilon-P membranes (Millipore). The membranes were blocked for 1 h at room temperature in blocking buffer (0.1% Tween 20 and 5% nonfat dry milk in TBS). They were then incubated for 2 h in blocking buffer containing primary Abs at a final concentration of 0.5 μg/ml. All of the primary Abs examined, rabbit polyclonal anti-Mcl-1 (S-19), rabbit polyclonal anti-Mcl-1 (K-20), rabbit polyclonal anti-Bax (N-20), rabbit polyclonal anti-Bcl-xS (S-18), rabbit polyclonal anti-A1 (FL-175), rabbit polyclonal c-IAP1 (H-83), rabbit polyclonal anti-Bcl-2 (N-19), rabbit polyclonal anti-Flip (H-202), rabbit polyclonal anti-GATA2 (H-116), and goat polyclonal anti-A1 (T-18), were purchased from Santa Cruz Biotechnology. The membranes were washed in TBS and incubated with secondary goat anti-rabbit or donkey anti-goat Ab labeled with alkaline phosphate (1/1000; Santa Cruz Biotechnology) for development with the 5-bromo-4-chloro-3-indoly phosphate/NBT liquid substrate system (Sigma-Aldrich).

RT-PCR

Quantitative PCR was used to quantify the mRNA expression in PRM. Total RNA was extracted from ~1 × 106 PRM with a RNeasy Mini kit.
From total RNA, cDNA was synthesized with a SuperScript III First Strand Synthesis System (Invitrogen Life Sciences). Quantitative PCR was performed using the PRISM7500 Real-Time PCR system with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays, which were preoptimized for each target, namely, Mcl-1, Bax, Bcl-2, and glucuronidase β (GusB), or GAPDH as an internal control (Applied Biosystems).

Detection of newly synthesized MCL-1 protein and global protein synthesis

PRM cultured in 60-mm dishes were washed with PBS 90 min after irradiation, starved in methionine/cysteine-free DMEM (Invitrogen Life Sciences) at 37°C for 30 min, then labeled with 100 μCi of [35S]cysteine/methionine (redivue Pro-mix L-[35S] in vitro cell labeling mix; Amersham Biosciences) per dish for 2 h in the presence of lactacystin at a concentration of 3 × 10⁻⁶ M for labeling newly synthesized proteins. Newly synthesized Mcl-1 protein was detected by autoradiography. In brief, a total of ~5 × 10⁶ cells was pooled as one sample. Radiolabeled cells were lysed in cold radioimmunoprecipitation assay (RIPA) lysis buffer (10 mM Tris-HCl (pH 7.4), 1% Nonidet P-40, 1 mM EDTA, 0.1% SDS, and 150 mM NaCl) with fresh proteinase inhibitors (Roche Diagnostic Systems), then aspirated through a 25-gauge syringe 20 times. Equivalent amounts of cell extract were adjusted to equal volumes with RIPA buffer and clarified by centrifugation. Lysates were then incubated with polyclonal Abs to Mcl-1 (S19; Santa Cruz Biotechnology) at 4°C overnight, and the immune complexes were precipitated with protein G-Sepharose beads (Amersham Biosciences) for 4–6 h. The immunoprecipitates were washed five times with RIPA buffer and incubated at 98°C for 10 min in SDS sample buffer. Samples were subjected to SDS-PAGE and subsequently transferred electrophoretically onto Immobilon-P membranes. The membranes were placed in contact with an imaging plate. The radioactive images recorded on the imaging plate were read with a laser beam scanner and analyzed with an attached computer (BAS2000; Fuji Photo Film). On the other hand, global protein synthesis was examined by measuring radioactivity incorporated in the TCA-insoluble fraction. In brief, radiolabeled PRM were disrupted by treatment with distilled water, detached from dishes by scraping with a rubber policeman, and recovered in plastic tubes. TCA was added at a final concentration of 10% (w/v). The TCA-insoluble fractions were trapped on paper filter, and the radioactivity was measured by liquid scintillation counter.

Culture of bone marrow-derived proliferative macrophages

Viable bone marrow mononuclear cells were purified by Ficoll-Paque (Amersham Biosciences) separation of bone marrow cells recovered from (Qiagen) by following the protocols recommended by the manufacturer. From total RNA, cDNA was synthesized with a SuperScript III First Strand Synthesis System (Invitrogen Life Sciences). Quantitative PCR was performed using the PRISM7500 Real-Time PCR system with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays, which were preoptimized for each target, namely, Mcl-1, Bax, Bcl-2, and glucuronidase β (GusB), or GAPDH as an internal control (Applied Biosystems).

FIGURE 2. Irradiation elicited a disruption of mitochondrial transmembrane potential and the release of cytochrome c into the cytosol, and MG-132, but not z-VAD, suppressed the effect of irradiation. A–C, Mitochondrial membrane potential was visualized with a MitoCapture Mitochondrial Apoptosis Detection kit. A, C3H mouse PRM irradiated at 40 Gy. B, C3H mouse PRM treated with MG-132 at a dose of 1 × 10⁻⁶ M and irradiated at 40 Gy. C, C3H mouse PRM treated with z-VAD at a dose of 5 × 10⁻⁵ M and irradiated at 40 Gy. D, C3H mouse PRM with many aggregates shedding a bright red fluorescence were counted, and values are expressed as a percentage of all cells. E, The amount of cytochrome c in the cytosolic fraction of C3H mouse PRM was quantified.

FIGURE 3. Mcl-1 disappeared in C3H mouse PRM treated with cycloheximide or ionizing radiation but not with H₂O₂. A–C, C3H mouse PRM were left untreated or else treated with cycloheximide for 4 h, treated with H₂O₂ for 15 min, or irradiated. Four hours after the initiation of treatment, apoptosis was evaluated. D, C3H mouse PRM left untreated or else treated with cycloheximide at 5 × 10⁻⁵ M, irradiated at 40 Gy, or treated with H₂O₂ at 4 × 10⁻⁵ M for 15 min were harvested 4 h after the initiation of treatment, and total cell lysate was subjected to Western blotting to detect Bcl-xL, Bax, Bcl-2, Mcl-1, cIAP1, and A1.
C3H and B6 mouse femoral bones. Approximately 10^9 cells suspended in 2 ml of culture medium were mixed with 18 ml of methylcellulose medium (METHOCULT GF M3534; StemCell Technologies) and plated in a 100-mm culture dish (Falcon 3003) to develop colonies of granulocytic/monocytic lineage. At 10 – 11 days of incubation, the cells were recovered from the methylcellulose medium and resuspended in culture medium containing recombinant murine GM-CSF at a concentration of 50 ng/ml (Genzyme) and plated in 35-mm culture dishes. After 2 h of incubation, nonadherent cells were removed by extensive washing with the culture medium. The cells remaining adhered to the dishes were referred to as bone marrow-derived proliferative macrophages. The cells were irradiated or treated with cycloheximide in the presence of recombinant murine GM-CSF. At 6 h after irradiation, the cells were observed microscopically to detect cell death or subjected to a Western blot analysis to detect Mcl-1 protein.

Culture of C3H mouse embryonic fibroblasts (MEF)
Pregnant C3H/HeJ mice were purchased from Japan SLC. The fetuses at 16 days of gestation were minced and treated with 0.05% trypsin-EDTA (In-vitrogen Life Sciences) at 37°C for a few minutes, after which FCS was added. Minced tissues and isolated cells were together washed with culture medium and plated in PRIMARIA tissue culture flasks (Falcon 3808). C3H mouse MEF were established after several passages. Exponentially growing C3H mouse MEF were exposed to gamma irradiation, UV light, or cycloheximide. At 6 h after the treatments, MEF were observed microscopically or subjected to a Western blot analysis.

Mcl-1 antisense oligonucleotides and small interfering RNAs (siRNA)
C3H mouse PRM were transfected with Mcl-1 antisense oligonucleotide or Mcl-1 siRNA. HPLC-purified Mcl-1 antisense oligonucleotide (5'-Alexa488-labeled OFZOOCAGCCTTTTZEZZZG-3', where Z, O, C, and F are phosphorothioate-T, phosphorothioate-C, phosphorothioate-G, and phosphorothioate-A, respectively) and Mcl-1 sense oligonucleotide (5'-Alexa488-labeled OFFFOAAAGGCTEEEFZG-3') were provided by Invitrogen Life Sciences. Mcl-1 siRNAs (siGenome SMART pool Upgrade Mouse MCL1, a mixture of four different siRNAs, those that proved to be theoretically and/or empirically effective in gene knockdown) and a control siRNA (siCONTROL NON-Targeting siRNA#1, 5'-UAGCGACUAAACACAUCAA-3') were purchased from Dharmacon.

A Hemagglutinating Virus of Japan (HVJ) envelope vector kit (trade name: GENOMONE) manufactured by Ishihara Sangyo was used for transfection. The inclusion of the antisense oligonucleotide and siRNA into the HVJ envelope and transfection of the HVJ envelope into PRM plated at a density of ~2 x 10^5 cells per 35-mm culture dish were conducted.
according to the instructions of the manufacturer. PRM were cultured for 8 h after the transfection, and then apoptosis was examined under a fluorescence microscope. In PRM transfected with the Mcl-1 antisense or sense oligonucleotide, apoptosis was identified by staining nuclei with Hoechst 33342 (Molecular Probe).

**Statistical analysis**

The mean and SE were calculated from the results of independent experiments. A χ² test was used to evaluate the statistical significance between two samples of interest. The value was regarded as significant if the probability was <0.05 (p < 0.05).

**Results**

**Suppression of radiation-induced apoptosis in C3H mouse PRM by proteasome inhibitors**

Gamma irradiation at a dose of 40 Gy or treatment with hydrogen peroxide at a dose of 4 × 10⁻⁵ M for 15 min induced significant apoptosis in C3H mouse PRM. The effect of several synthetic inhibitors on the apoptosis was examined (Fig. 1, A–D). ALLN (calpain inhibitor I) suppressed the radiation-induced apoptosis dose-dependently. E-64-d (an inhibitor for cathepsin B/H/L and calpain) showed a weak inhibitory effect at high doses. MG-132 (a proteasome inhibitor) was most effective at inhibiting the radiation-induced apoptosis, whereas BAPTA-AM (a selective chelator of intracellular calcium) was not effective at all. On the other hand, none of the inhibitors examined had an effect on the hydrogen peroxide-induced apoptosis. Lactacystin, a microbial product known to be a specific inhibitor for proteasomes (32, 33), also suppressed radiation-induced apoptosis in C3H mouse PRM (Fig. 1E). It is known that proteasome inhibitors activate apoptotic signaling pathways through the endoplasmic reticulum stress elicited by the accumulation of misfolded or unfolded proteins in the endoplasmic reticulum (34–36). Actually, overnight culture with proteasome inhibitors induced severe apoptosis in both C3H and B6 mouse PRM (data not shown). However, apoptosis in which the depletion of antiapoptotic rapid turnover proteins plays a great role might be transiently suppressed by proteasome inhibitors. The suppression of radiation-induced apoptosis with proteasome inhibitors in C3H mouse PRM suggested that the activation of proteasomes by irradiation enhanced the degradation of antiapoptotic proteins or that the synthesis of antiapoptotic proteins with short half-lives was down-regulated by irradiation. In either case, the amount of antiapoptotic proteins should be reduced by irradiation.

**Mitochondrial transmembrane potential and cytochrome c release**

Mitochondria are known to participate extensively in cases of apoptosis where mitochondrial transmembrane potential is disrupted and cytochrome c is released into the cytosol (37). Therefore, it was studied if mitochondria took part in radiation-induced apoptosis in C3H mouse PRM. Fig. 2, A–C, shows fluorescence microscopic images of C3H mouse PRM loaded with MitoCapture, a cationic dye that fluoresces differently depending on the mitochondrial transmembrane potential. Mitochondrial transmembrane potential and cytochrome c in C3H mouse PRM left untreated or irradiated were examined by Western blotting. H, Global protein synthesis was measured in C3H and B6 mouse PRM left untreated, irradiated, or treated with cycloheximide. The values for control PRM left untreated have been normalized to 100, and the stars indicate significant differences from the control. I, Mcl-1 levels in two cell lines of C3H mouse PRM left untreated, exposed to ionizing radiation or UV light (100 J/m²), or treated with cycloheximide are shown. J, Mcl-1 levels in C3H and B6 mouse bone marrow-derived proliferative macrophages left untreated, irradiated, or treated with cycloheximide are shown.
the mitochondrial transmembrane potential (Fig. 2, A and C). Irradiation disrupted mitochondrial transmembrane potential in C3H mouse PRM, and the disruption was suppressed by treatment with MG-132 but not z-VAD (Fig. 2D). Similarly, the radiation-induced release of cytochrome c into the cytosolic fraction in C3H mouse PRM was suppressed by MG-132 but not z-VAD (Fig. 2E). These results indicate that the mitochondria take part in radiation-induced apoptosis in C3H mouse PRM and that the proteasome inhibitor but not z-VAD acts directly on the mitochondria or upstream of them. A pan-caspase inhibitor, z-VAD, is capable of preventing apoptosis without protecting against cell death in many circumstances. Actually, irradiated C3H mouse PRM showed necrotic cell death (propidium iodide-positive) after overnight culture even in the presence of z-VAD (data not shown). Radiation-induced disruption of mitochondrial transmembrane potential that is not suppressed by z-VAD might result in cell death in C3H mouse PRM.

Depletion of Mcl-1 protein in C3H mouse PRM by irradiation

Many antiapoptotic proteins acting on mitochondria or upstream of mitochondria have been identified, but the antiapoptotic Bcl-2 family has been recognized as pivotal and studied extensively (38, 39). Therefore, Western blotting was performed to examine whether the amounts of Bcl-2 family proteins were altered by irradiation in C3H mouse PRM. As shown in Fig. 3, A–C, gamma radiation, H2O2 treatment for 15 min, or cycloheximide (a translation inhibitor) treatment for 4 h elicited apoptosis in C3H mouse PRM dose-dependently. A plateau level of apoptosis (70–80%) was achieved by irradiation at 40 Gy, by H2O2 at 4 × 10−5 M, or by cycloheximide at 4 × 10−5 M. C3H mouse PRM treated to induce a plateau level of apoptosis were collected after 4 h of culture and subjected to a Western blot analysis (Fig. 3D). Irradiation or cycloheximide treatment markedly eliminated Mcl-1 but not Bcl-2, Bcl-XL, Bax, A1, or cIAP1. Hydrogen peroxide treatment had no effect on the protein expression examined.

The radiation-induced depletion of Mcl-1 protein in C3H mouse PRM was dependent on the dose (Fig. 4A). On the other hand, Mcl-1 protein in B6 mouse PRM was not significantly affected by irradiation of up to 100 Gy. MG-132 treatment, but not z-VAD treatment, effectively suppressed the radiation-induced depletion of Mcl-1 (Fig. 4, B and C), whereas both treatments suppressed the radiation-induced apoptosis (Fig. 4D). The disappearance of Mcl-1 protein in C3H mouse PRM started 2–3 h after irradiation (Fig. 4E) and preceded the apoptosis (Fig. 4F).

Effect of irradiation on Mcl-1 mRNA expression

The effect of irradiation on gene expression was studied in C3H and B6 mouse PRM by quantitative PCR using transcript-specific
primers (Fig. 5). The level of Bax gene expression elevated and reached a plateau at a dose of 12.5 Gy in B6 mouse PRM but continued to increase at higher doses in C3H mouse PRM. That of Bcl-2 gene expression decreased with dosage in B6 mouse PRM but increased slightly rather than decreased in C3H mouse PRM. Irradiation markedly enhanced the Mcl-1 gene expression in C3H mouse PRM but not in B6 mouse PRM.

**Inhibition of protein synthesis by irradiation in C3H mouse PRM**

Radiation-induced depletion of Mcl-1 protein could be caused by accelerated protein degradation or the inhibition of protein synthesis. As an experiment, to measure the rate of protein synthesis, newly synthesized Mcl-1 was labeled with [35S]cysteine/methionine in the presence of MG-132 to inhibit protein degradation and was detected by autoradiography. As shown in Fig. 6, A–D, Mcl-1 protein synthesis was markedly suppressed by irradiation. In another experiment, the effect of irradiation on the rate at which Mcl-1 protein was degraded was measured by carrying out a Western blot analysis of C3H mouse PRM cultured in the presence of cycloheximide, where the disappearance of Mcl-1 was purely due to protein degradation. Fig. 6, E and F, shows that the rate of degradation was the same with or without irradiation. The effect of irradiation on other proteins known to have a rapid turnover was investigated. The level of GATA2, but not Flip, was decreased in C3H mouse PRM exposed to irradiation, although the decrease was not as remarkable as for Mcl-1 (Fig. 6G). The effect of irradiation on global protein synthesis was also examined. Irradiation suppressed dose-dependently the global protein synthesis in C3H mouse PRM but not in B6 mouse PRM (Fig. 6H). The effect of irradiation on Mcl-1 protein was studied in other cells. Mcl-1 protein in C3H mouse MEF was completely depleted by treatment with cycloheximide or exposure to UV light but not by ionizing radiation up to 150 Gy (Fig. 6I). Mcl-1 protein was decreased by a high dose of irradiation in bone marrow-derived proliferative macrophages of C3H mice but not of B6 mice, although the effect of irradiation was slight compared with that in C3H mouse PRM (Fig. 6J).

**Induction of apoptosis by the down-regulation of Mcl-1 with siRNA or antisense oligonucleotide**

To verify whether the radiation-induced apoptosis in C3H mouse PRM was attributable to the depletion of Mcl-1 protein, Mcl-1 expression was down-regulated with siRNA or antisense oligonucleotide. The Mcl-1 gene expression in PRM transfected with Mcl-1 antisense oligonucleotide decreased to ~50–60% of that in mock-transfected PRM (Fig. 7A). On the other hand, Mcl-1 siRNA treatment eliminated 75% of Mcl-1 gene expression, whereas the control siRNA had no effect. The amount of Mcl-1 protein in the PRM treated with antisense oligonucleotides or siRNA was also diminished to an extent similar to the reduction of Mcl-1 mRNA expression (Fig. 7B). Apoptosis was observed under a fluorescence microscope. Fig. 7, C and E, shows Alexa488-positive cells, indicating that a significant amount of sense or antisense oligonucleotide was taken up by the cells. Hoechst 33342-stained nuclei in Alexa 488-positive cells were observed. PRM transfected with sense oligonucleotide had a normal shaped nucleus (Fig. 7D), whereas fragmented and condensed nuclei were observed in some PRM transfected with antisense oligonucleotide (Fig. 7F). PRM transfected with control or Mcl-1 siRNA were stained with acridine orange and propidium iodide. PRM transfected with control siRNA were normal (Fig. 7G), whereas apoptosis was frequently observed in PRM transfected with Mcl-1 siRNA (Fig. 7H). Marked apoptosis was elicited in PRM transfected with Mcl-1 siRNA, whereas Mcl-1 antisense oligonucleotides only marginally induced apoptosis (Fig. 7I). Mcl-1 siRNA also induced apoptosis in B6 mouse PRM to the same extent as that in C3H mouse PRM (Fig. 7J).

**Discussion**

In this study, we found that apoptosis was induced shortly after the depletion of Mcl-1 in PRM, terminally differentiated tissue macrophages, and that irradiation elicited the depletion of Mcl-1 through translational arrest in C3H mouse PRM in a mouse strain-specific manner. Mcl-1 is a well-characterized antiapoptotic member of the Bcl-2 family that has recently been implicated in the pathogenesis of various myeloid neoplasms (40–42). Mcl-1 has also been shown to be essential for the homeostasis of early hematopoietic progenitors and for the development and maintenance of B and T lymphocytes (43, 44). The disappearance of Mcl-1 caused by transfection with antisense oligonucleotide is both necessary and sufficient to induce apoptosis in multiple myeloma cell lines (45). Therefore, the induction of apoptosis in PRM due to the depletion of Mcl-1 is consistent with previous studies that proved that Mcl-1 is critical to cells of the hematopoietic lineage for development and survival. On the other hand, the ionizing radiation-elicited translational arrest observed only in C3H mouse PRM, not in B6 PRM, is quite a novel finding. Mcl-1 siRNA elicited apoptosis in only 30% of cells 8 h after transfection, whereas irradiation elicited apoptosis in 70–80% of cells in C3H mouse PRM 4 h after irradiation. This difference might correspond to the extent to which Mcl-1 was depleted. Irradiation markedly eliminated Mcl-1 protein (to <10% of the control), whereas 20–30% of Mcl-1 was still expressed after siRNA treatment. Alternatively, it is also possible that other proteins related to apoptosis might be affected by irradiation. Unlike in PRM, the depletion of Mcl-1 in C3H mouse MEF and bone marrow-derived proliferative macrophages did not lead to immediate cell death (data not shown). The dependence on Mcl-1 for cell survival or time course from the depletion of Mcl-1 to the occurrence of cell death might vary depending on the cell type.

Quantitative PCR revealed that the radiation-induced depletion of Mcl-1 protein in C3H mouse PRM is not attributable to Mcl-1 mRNA expression because the expression was markedly increased by irradiation rather than decreased. Bax and Bcl-2 gene expression is enhanced and suppressed by genotoxic agents through a TP53-mediated pathway, respectively (46, 47). In B6 mouse PRM, the levels of Bax and Bcl-2 gene expression increased and decreased with dosage, respectively. Therefore, the expression pattern of these genes in B6 mouse PRM is consistent with previous reports, suggesting that the radiation-induced alteration in the expression of these genes in B6 mouse PRM originates from radiation-induced DNA damage. Mcl-1 gene expression was also reported to be enhanced by DNA-damaging agents (48). However, the expression was only slightly enhanced by irradiation in B6 mouse PRM, suggesting that DNA damage was largely not responsible for Mcl-1 gene expression in mouse PRM. On the other hand, the expression patterns of the Bax, Bcl-2, and Mcl-1 genes in irradiated C3H mouse PRM were quite different from those in B6 mouse PRM. Irradiation might regulate the expression of the Bax, Bcl-2, and Mcl-1 genes in C3H mouse PRM through both a DNA damage-triggered pathway and other signaling pathways that were not elicited in B6 mouse PRM.

Nijhawan et al. (49) reported that genotoxic stress induced by UV irradiation, gamma irradiation, or etoposide treatment efficiently induced the elimination of Mcl-1 in HeLa cells. UV light treatment is known to trigger the ubiquitination and subsequent degradation of RNA polymerase II (50) and the GCN2-mediated...
phosphorylation of eIF2α (51) to arrest transcription and translation, respectively. Therefore, it is highly possible that UV light-induced DNA damage triggers transcriptional and translational arrest, resulting in the elimination of Mcl-1 (49). Considering our previous report that ionizing radiation-induced DNA damage and TP53-, ATM-, or DNA-protein kinase-mediated cellular responses occurring downstream thereof were not involved in radiation-induced apoptosis in C3H mouse PRM (16), the radiation-induced elimination of Mcl-1 in C3H mouse PRM seems not to be mediated by DNA damage, TP53, ATM, or DNA-PK. In contrast to UV exposure, ionizing radiation did not arrest transcription in C3H mouse PRM. Furthermore, UV elicited depletion of Mcl-1 and apoptosis to the same extent in B6 mouse PRM as in C3H mouse PRM (data not shown), whereas ionizing radiation induced apoptosis and loss of Mcl-1 through translational arrest only in C3H mouse PRM. Therefore, Mcl-1’s elimination by ionizing radiation might be mediated through a pathway different from that elicited by UV light.

In this study, both ionizing radiation and treatment with hydrogen peroxide caused apoptosis in C3H mouse PRM, whereas the elimination of Mcl-1 was induced only by ionizing radiation. This means that the ionizing radiation-induced apoptosis in C3H mouse macrophages is not mediated by hydrogen peroxide, which is consistent with our recent report of the selective involvement of superoxide anion, but not other reactive oxygen species, in ionizing radiation-induced apoptosis in C3H mouse PRM (30). Therefore, the strain difference in ionizing radiation-induced apoptosis in mouse PRM might be attributable to a factor located upstream of the translational machinery and downstream of superoxide. Further study is necessary to elucidate the molecular mechanism involved.

Disclosures
The authors have no financial conflict of interest.

References


