Ionizing Radiation and Chemotherapeutic Drugs Induce Apoptosis in Lymphocytes in the Absence of Fas or FADD/MORT1 Signaling: Implications for Cancer Therapy

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Abstract

Ionizing radiation and cytotoxic drugs used in the treatment of cancer induce apoptosis in many cell types, including tumor cells. It has been reported that tumor cells treated with anticancer drugs increase surface expression of Fas ligand (FasL) and are killed by autocrine or paracrine apoptosis signaling through Fas (Friesen, C., I. Herr, P.H. Krammer, and K.-M. Debatin. 1996. *Nat. Med.* 2:574–577). We show that lymphocytes that cannot be killed by FasL, such as those from Fas-deficient *lpr* mice or transgenic mice expressing a dominant negative mutant of Fas-associated death domain protein (FADD/MORT1), are as sensitive as normal lymphocytes to killing by gamma radiation or the cytotoxic drugs cis-platin, doxorubicin, and etoposide. In contrast, p53 deficiency or constitutive expression of Bcl-2 markedly increased the resistance of lymphocytes to gamma radiation or anticancer drugs but had no effect on killing by FasL. Consistent with these observations, *lpr* and wild-type T cells both had a reduced capacity for mitogen-induced proliferation after drug treatment, whereas *bcl-2* transgenic or p53-deficient T cells retained significant clonogenic potential. These results demonstrate that apoptosis induced by ionizing radiation or anticancer drugs requires p53 and is regulated by the Bcl-2 protein family but does not require signals transduced by Fas and FADD/MORT1.

Key words: Fas/APO-1/CD95 • death receptors • Bcl-2 • p53 • caspases

Introduction

Fas (also known as CD95 or APO-1) belongs to a subset of the TNFR family, whose members have a cytoplasmic 'death domain.' When Fas is engaged by multimeric Fas ligand (L), its death domain binds to the death domain in the cytoplasmic adaptor protein FADD/MORT1 (Fas-associated death domain protein). A further homotypic interaction occurs between the 'death effector domain' in FADD/MORT1 and a death effector domain in procaspase-8. The close proximity of caspase-8 zymogens facilitates their autocatalytic activation, and this appears to initiate the proteolytic cascade that leads to apoptosis (1). It has been proposed that Fas signaling plays a critical role in drug-induced apoptosis, stemming from the observation that neutralization of FasL prevented doxorubicin-induced apoptosis in T cell leukemia lines (2). Subsequent reports have differed as to whether impaired Fas signaling in tumor cell lines blocks (3–5) or has no effect (6–14) on drug-induced apoptosis. As mutations affecting the cell death machinery can contribute to tumor formation

(15–18), these discrepancies may have arisen because the transformed cell lines studied harbor unknown mutations in genes that regulate cell death. To avoid such complications, we sought evidence of a role for Fas and FADD/MORT1 in gamma radiation– and drug-induced apoptosis using non-transformed cells. We used lymphocyte populations from genetically modified mice to determine if nontransformed cells that are resistant to FasL-induced apoptosis are also refractory to cytotoxic anticancer treatments.

Materials and Methods

Mice. All experiments with mice were approved by the Royal Melbourne Hospital Research Foundation Animal Ethics Committee. *lck*[pr]-FADD-DN (dominant negative) strain 64 transgenic mice (19), Eμ-*bcl*-2-36 and Eμ-*bcl*-2-25 transgenic mice (20), Fas-deficient mutant *lpr* mice (21), and *p53*^{−/−} mice (22) have been described previously. All animals had a C57BL/6J genetic background. *bcl*-2 transgenic T cells were isolated from Eμ-*bcl*-2-25 mice, whereas *bcl*-2 transgenic B cells were isolated from Eμ-*bcl*-2-36 mice. Spleen B cells and lymph node T cells were purified by negative cell sorting in a FACSII™ or FACStar[PLUS]™ sorter (Becton Dickinson). FITC-negative, propidium iodide (PI)-negative cells were selected after surface staining with a cocktail of FITC-con-

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jugated mAbs. MI/70 anti–Mac-1, 8C5 anti–Gr-1, Ter119 anti–erythroid cell surface marker, RA3-6B2 anti-B220, 5.1 anti-IgM, and 11-26C anti-IgD antibodies were used to isolate T cells. MI/70 anti–Mac-1, 8C5 anti–Gr-1, Ter119 antierythroid cell surface marker, H129.19.6.8 anti-CD4, YTS 169 anti-CD8, and T24.31.2 anti–Thy-1 antibodies were used to isolate B cells.

Cell Survival Experiments. Lymphocytes were cultured in the high-glucose version of DME supplemented with 13 μM folic acid, 250 μM l-asparagine, 50 μM 2-ME, and 10% FCS (Biosciences; starting concentration 10^5 cells/ml). B cells were stimulated with 20 μg/ml LPS, 20 μg/ml F(ab′)$_2$ goat anti–mouse IgM (Jackson ImmunoResearch), 100 U/ml IL-2, and 100 U/ml IL-4 plus 100 U/ml IL-5. T cells were stimulated with immobilized KT3 rat anti–mouse CD3 and 37N51 hamster anti–mouse CD28 antibodies plus 100 U/ml IL-2. Tissue culture plates were coated in PBS that contained 10 μg/ml of each antibody. Resting and activated cells were treated with gamma radiation (2.5–10 Gy from a ^{60}Co source), 5–500 ng/ml doxorubicin (David Bull Labs.), 0.1–10 μg/ml etoposide, and 0.1–10 μg/ml cis-platin (Della West Pty Ltd.) or were cultured on Neuro-2A.FasL or Neuro-2A.neo cell monolayers (23). Activated cells were maintained in cytokines during treatment to minimize death due to growth factor withdrawal. Cell viability was determined by flow cytometric analysis of PI-stained cells in a FACScan™ (Becton Dickinson). To correct for doxorubicin fluorescence at higher doses, living and dead cells were also discriminated on the basis of their forward and side light-scattering properties.

To determine clonogenic survival, purified T cells were incubated for 8 h at 37°C in medium that contained 0, 0.1, 1, or 5 μg/ml cis-platin or etoposide. Cells were then washed free of drug and plated at limiting dilution with 2 ng/ml PMA and 0.1 μg/ml ionomycin plus 100 U/ml IL-2 in flat-bottomed 96-well plates. Three dilutions (1, 5, 10, 50, or 100 cells/well) were prepared for each treatment, and 96 wells were prepared for each dilution. Wells were scored microscopically as positive or negative for T cell growth after 7-d incubation at 37°C. The surviving fraction (*S*) after each treatment was calculated according to the equation:

$$S = [(\ln x_i)/y_i] \cdot [y_u/(\ln x_u)],$$

where *x* is the fraction of wells negative for cell growth and *y* is the number of cells initially plated per well for treated (i) and untreated (u) cells (24). In other words, comparisons were made of the slopes of graphs plotting ln (fraction of wells negative for growth) versus the number of cells plated per well.

Results and Discussion

We have previously shown that T cells from Fas-deficient *lpr* mice (21) and FADD-DN transgenic mice that express a dominant interfering mutant of FADD/MORT1 (19) are highly resistant to apoptosis induced by cross-linked soluble human FasL (19). Resistance was also observed when we cultured the cells with Neuro-2A neuroblastoma cells stably transfected with a mouse FasL expression construct (Fig. 1, A and B). These Neuro-2A.FasL cells express membrane-anchored FasL because they lack the matrix metalloproteinase responsible for FasL cleavage from the cell surface (23). In contrast, wild-type thymocytes and mature T cells underwent extensive apoptosis when cultured with Neuro-2A.FasL cells (Fig. 1, A and B). FasL triggered this apopto-

sis, because it was not observed in lymphocytes cultured with Neuro-2A.neo cells transfected with vector DNA (data not shown). Similarly, wild-type T cells activated in culture for 3 d with cross-linking antibodies to CD3 and CD28 or wild-type B cells activated with LPS plus anti-IgM antibody for 3 d were sensitive to FasL killing, whereas their *lpr* counterparts were resistant (Fig. 1, D and E). Wild-type resting B cells were more resistant than activated B cells to FasL killing (Fig. 1 C), presumably due to very low surface expression of Fas (data not shown). The effect of FADD-DN on the sensitivity of B cells to killing by FasL could not be examined, because FADD-DN expression is limited to T cells in our transgenic mice. The proliferation defect of FADD-DN T cells prevented examination of activated FADD-DN T cells (19). As the anti-apoptotic protein Bcl-2 and the tumor suppressor p53 are known modulators of gamma radiation– and drug-induced apoptosis (22, 24–27), we included in our analysis lymphocytes from *bcl*-2 transgenic mice (20) and *p53*-deficient mice (22). Neither p53 deficiency nor constitutive expression of Bcl-2 protected lymphocytes from apoptosis induced by FasL (Fig. 1, A–E).

To test sensitivity to a range of anticancer treatments, lymphocytes were exposed in culture to ionizing radiation (2.5–10 Gy) or the cytotoxic drugs cis-platin (0.1–10 μg/ml), doxorubicin (50–500 ng/ml), and etoposide (0.1–10 μg/ml). Cell survival was determined after 24 and 48 h. Apoptosis in cultures of wild-type, *lpr*, or FADD-DN transgenic lymphocytes occurred in a similar dose-dependent manner (Fig. 1), and a comparison of the kinetics of cell killing revealed no difference between the three genotypes (Fig. 2). In contrast, thymocytes and resting mature B and T cells from $p53^{-/-}$ or *bcl*-2 transgenic mice were resistant to these apoptotic stimuli. For example, 2 d after a 10-Gy dose of gamma radiation or continuous culture in 1 μg/ml etoposide, >70% of all wild-type, *lpr*, and FADD-DN transgenic thymocytes were dead, whereas apoptosis in cultures of similarly treated $p53^{-/-}$ or *bcl*-2 transgenic thymocytes did not exceed 20% (Fig. 2 A). Interestingly, enforced Bcl-2 expression or p53 deficiency had relatively little impact on the death of gamma-irradiated or drug-treated cycling cells (Fig. 1, D–E, and Fig. 2, D–E).

Short-term survival assays may only reflect differences in the rate of cell killing. For cancer treatment, the critical determinant of success is whether or not cells retain the capacity to proliferate in the long term. Therefore, we examined the capacity of drug-treated T cells for mitogen-induced proliferation. Resting mature T cells from wild-type, *lpr*, *bcl*-2 transgenic, and $p53^{-/-}$ mice were cultured in varying concentrations of etoposide or cis-platin (0, 0.1, 1, and 5 μg/ml) for 8 h and then plated at limiting dilution in 96-well microtiter plates containing PMA and ionomycin plus recombinant IL-2. Plates were scored microscopically for T cell proliferation 7 d later. The results of these experiments are shown in Fig. 3. Clonogenic survival of wild-type cells decreased as the dose of drug increased. There was on average a 260-fold drop in the frequency of clonogenic cells after culture in 5 μg/ml cis-platin (Fig. 3 A) and a 12-fold drop after culture in 5 μg/ml etoposide (Fig. 3 B). *lpr* T cells
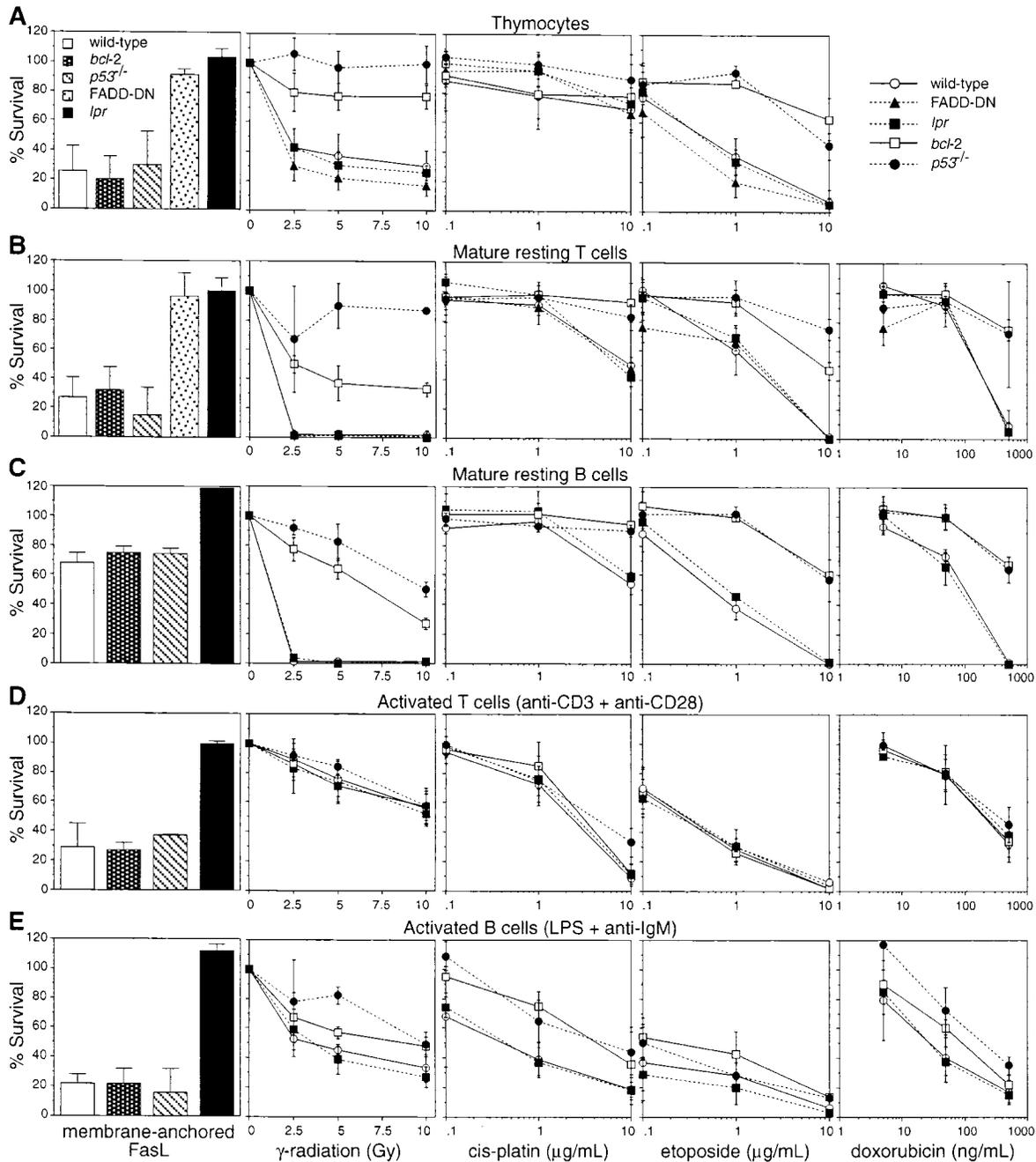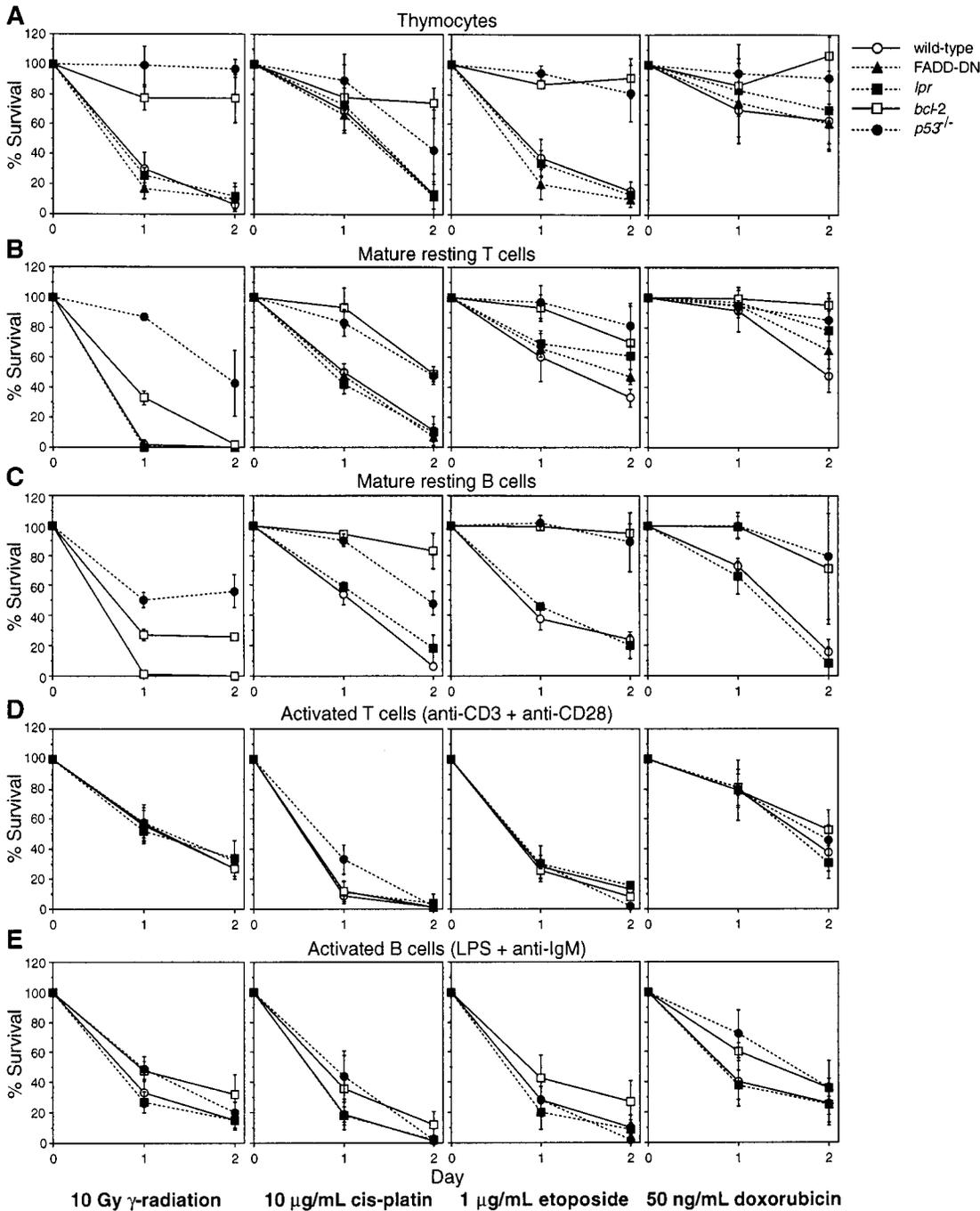
Figure 1. Gamma radiation and cytotoxic drugs can kill lymphocytes that are resistant to FasL. Thymocytes (A), mature T cells (B and D), and mature B cells (C and E) were isolated from wild-type, *bcl*-2 transgenic, FADD-DN transgenic, *lpr*, or *p53*$^{-/-}$ mice. Resting cells (A–C) or cells that had been activated for 3 d with mitogens (D and E) were cultured with Neuro-2A.FasL cells, subjected to gamma irradiation (2.5–10 Gy), or cultured in the continuous presence of cytotoxic drugs (0.1–10 μg/ml cis-platin, 0.1–10 μg/ml etoposide, or 5–500 ng/ml doxorubicin). Cell survival after 24 h was determined by flow cytometric analysis of PI-stained cells and is shown as a percentage of survival in culture medium without treatment. Lymphocytes cultured with control Neuro-2A cells were indistinguishable from lymphocytes cultured in medium alone. Data points represent the mean ± SD for greater than three mice of each genotype.

treated with cis-platin or etoposide exhibited a similar drop in clonogenic survival (Fig. 3). In comparison, enforced Bcl-2 expression or loss of p53 markedly enhanced clonogenic survival after exposure to cis-platin and etoposide. After treatment with 5 μg/ml cis-platin, clonogenic survival of *bcl*-2 transgenic and *p53*$^{-/-}$ T cells was reduced 16- and

8-fold, respectively (Fig. 3 A), and after treatment with 5 μg/ml etoposide, cells of both genotypes showed only a 1.8-fold reduction in clonogenic survival (Fig. 3 B).

We conclude from our findings that signals transduced by Fas and/or FADD/MORT1 are dispensable for ionizing radiation– and cytotoxic drug–induced apoptosis in lympho-

Figure 2. Lymphocytes lacking Fas or FADD/MORT1 function are normally sensitive to killing by gamma irradiation or cytotoxic drugs. Thymocytes (A), resting T (B) and B cells (C), and activated T (D) and B cells (E) from wild-type (○), *bcl-2* transgenic (□), FADD-DN transgenic (▲), *lpr* (■), or *p53*$^{-/-}$ (●) mice were subjected to gamma radiation or cytotoxic drugs as in Fig. 1. Cell survival was determined after 24 and 48 h by flow cytometric analysis of PI-stained cells and is shown as a percentage of that in culture medium without treatment. Data points represent the mean ± SD for greater than three mice of each genotype.

cytes. Our results show that in resting lymphocytes, these anticancer treatments trigger apoptosis signaling pathways that can be regulated by Bcl-2 and p53. Significantly, neither of these proteins influenced Fas death signaling (Fig. 1). These results and those of others (27, 28) directly contradict a recent study suggesting that Bcl-2 confers drug resistance via inhibition of nuclear factor of activated T lymphocytes (NFAT)-induced FasL expression (3). Increased Fas expression in response to anticancer drugs was reported to be dependent on p53, but the issue of whether induction of the Fas gene is essential for p53-induced apoptosis was not addressed (29). Our data from lymphocytes, together
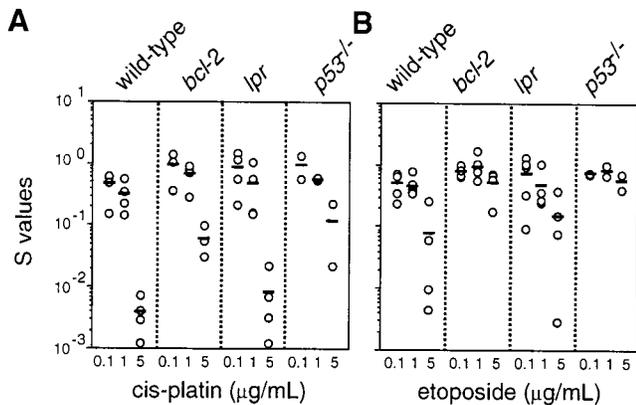
Figure 3. T cells treated with etoposide or cis-platin retain more clonogenic potential if they lack p53 or express Bcl-2, but not if they lack Fas. Resting T cells from wild-type, *bcl-2* transgenic, *lpr*, or $p53^{-/-}$ mice were cultured for 8 h in medium or were treated with 0.1, 1, and 5 μg/ml cis-platin (A) or etoposide (B). Cells were then washed and plated at limiting dilution in 96-well microtiter plates in the presence of PMA and ionomycin plus IL-2. Wells were scored for T cell growth after 7 d, and the precursor frequency of clonogenic T cells was determined as described in Materials and Methods. Each circle (○) represents one independent experiment. Horizontal bars represent the mean *S* value calculated from two to four independent experiments.

with the finding that FADD/MORT1- (30) or caspase-8–deficient (31) mouse embryo fibroblasts are resistant to killing by FasL but normally sensitive to drug-induced apoptosis, indicates that Fas is not a common mediator of cell killing by cytotoxic agents. By contrast, certain mouse cell types lacking Apaf-1 or caspase-9 are resistant to both p53-induced apoptosis (32) and killing by ionizing radiation or cytotoxic drugs (33–36). These observations support a model in which ionizing radiation and cytotoxic drugs trigger apoptosis via p53 and Apaf-1–mediated activation of caspase-9, whereas apoptosis signaling by death receptors such as Fas is dispensable (Fig. 4). Low levels of apoptosis in $p53^{-/-}$ resting lymphocytes after drug treatment may be due to p53-related proteins such as p73. Recent studies implicate
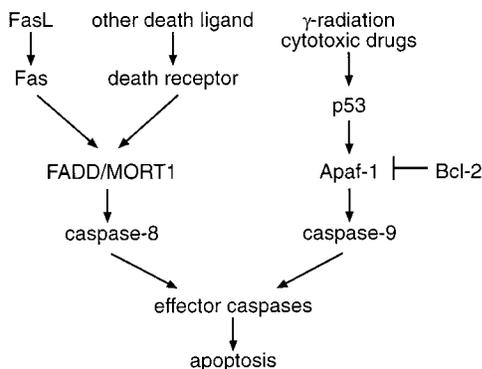
Figure 4. Model for apoptosis signaling by ionizing radiation and cytotoxic drugs. Fas and related death receptors that utilize the cytoplasmic adaptor protein FADD/MORT1 to activate caspase-8 zymogens are dispensable for apoptosis induced by ionizing radiation and cytotoxic drugs. In contrast, loss of p53, Apaf-1, or caspase-9 and expression of a *bcl-2* transgene can protect certain cells from these stimuli.

phosphorylation of p73 by the tyrosine kinase c-Abl as a crucial event in apoptosis induced by gamma radiation or cis-platin (37–39). However, the mechanism by which p73 triggers apoptosis remains to be established. Failure of enforced Bcl-2 expression or loss of p53 to perturb the death of activated B and T cells treated with ionizing radiation or cytotoxic drugs (Figs. 1 and 2) may reflect reproductive/necrotic cell death (40). Thus, even though apoptosis signaling is disabled in these cells, their capacity for continued cell growth is limited by irreparable DNA damage.

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