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Immunology

Tumor dormancy and cell signaling: Anti-μ-induced apoptosis in human B-lymphoma cells is not caused by an APO-1–APO-1 ligand interaction*

(B-cell lymphoma/membrane immunoglobulin/apoptosis)

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ABSTRACT **Signal transduction initiated by crosslinking of antigen-specific receptors on T- and B-lymphoma cells induces apoptosis. In T-lymphoma cells, such crosslinking results in upregulation of the APO-1 ligand, which then interacts with induced or constitutively expressed APO-1, thereby triggering apoptosis. Here we show that crosslinking the membrane immunoglobulin on human lymphoma cells (Daudi) (that constitutively express APO-1) does not induce synthesis of APO-1 ligand. Further, a noncytotoxic fragment of anti-APO-1 antibody that blocks T-cell-receptor-mediated apoptosis in T-lymphoma cells does not block anti-μ-induced apoptosis. Hence, in B-lymphoma cells, apoptosis induced by signaling via membrane IgM is not mediated by the APO-1 ligand.**

Apoptosis and cell cycle arrest are observed in B-lymphoma cells when their surface IgM is crosslinked. These signaling effects are of interest because they appear to be critical mechanisms for inducing tumor dormancy in mice that have either actively produced anti-idiotypic antibody or have been provided with anti-IgM antibody passively (1–3). In addition, other B-cell reactive antibodies that induce cell cycle arrest and/or apoptosis have been implicated as very effective anti-tumor agents in human lymphoma–SCID models (4, 5).

The APO-1 (Fas/CD95) cell surface receptor is a member of the nerve growth factor/tumor necrosis factor receptor superfamily that signals apoptosis (for review, see ref. 6). Activated T lymphocytes from MRL-*lpr/lpr* mutant mice express a reduced number of APO-1 receptors (APO-1) due to the presence of an insertional element in the APO-1 gene and are defective in T-cell-receptor (TCR)-mediated apoptosis in mature peripheral T cells (7–9). Recent evidence supports a mechanism whereby TCR-induced apoptosis is dependent on APO-1–APO-1 ligand interactions. Thus, crosslinking TCRs results in transient upregulation of APO-1 ligand in T-lymphoma cells. Apoptosis induced by TCR crosslinking is markedly inhibited by either anti-APO-1 $F(ab')_2$ fragments (which are not cytotoxic) or soluble APO-1-Fc (10–12). These results indicate that in T-lymphoma cells, apoptosis induced by TCR activation results from the induction of APO-1 ligand and its interaction with APO-1 resulting in the activation of the APO-1 signaling pathway.

In the present experiments, we addressed the question of whether apoptosis induced by membrane immunoglobulin (mIg) activation on a human lymphoma cell line (Daudi) follows a similar pathway. In contrast to the results using T cells and T-cell lymphomas, apoptosis in Daudi cells initiated by mIg activation does not appear to involve the APO-1 ligand.

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MATERIALS AND METHODS

Antibodies. Affinity-purified goat anti-human IgM (μ-chain specific) was purchased from Chemicon Int. (Temecula, CA). Goat anti-ovalbumin was produced and affinity-purified (13). Mouse anti-CD3 (64.1) and MOPC21 were purified from the supernatant of hybridomas purchased from the American Type Culture Collection (Rockville, MD). Anti-APO-1 $F(ab')_2$ and control $F(ab')_2$ (murine IgG3) were prepared as described (14). Biotinylated DX2 mouse IgG1 antibody specific for human APO-1 receptor and phycoerythrin (PE)-conjugated streptavidin were obtained from Pharmingen (San Diego, CA).

Activation of Cells Through Antigen Receptors. Human Burkitt B-cell lymphoma (Daudi) and T-cell lymphoma (Jurkat) were used. They were maintained *in vitro* in RPMI 1640 medium supplemented with 2% (wt/vol) L-glutamine and 10% (vol/vol) fetal bovine serum. Approximately 1.5×10^6 Daudi or Jurkat cells in 1 ml of medium were plated in wells of a Costar 12-well plate. Daudi cells were activated in the presence of goat anti-μ antibody (35 μg/ml). For Jurkat cells, the wells were precoated overnight at 37°C with either mouse anti-CD3 or control MOPC21. Excess antibody was removed and the wells washed once with phosphate-buffered saline (PBS) before the cells were added. After various times of incubation at 37°C, the cells were harvested, washed once in Eagle's salt solution, and analyzed as described below.

Flow Cytometric Measurement of Plasma Membrane Integrity, DNA Strand Breaks, and APO-1 Expression. For staining of permeable cells, 5 μl of propidium iodide solution (0.5 mg/ml in PBS) was added to half of each sample and incubated on ice for 5 min before addition of 1 ml of PBS. For determination of total DNA content, the other half of the sample was incubated on ice in 1 ml of 0.005% propidium iodide/0.1% sodium citrate/0.03% Nonidet P-40 for 30 min before acquisition on a FACScan system (Beckton Dickinson, San Jose, CA) equipped with a 488-nm argon-ion laser and a band-pass filter of 585 nm.

Detection of DNA strand breaks by terminal deoxynucleotidyltransferase-mediated dUTP nick-end-labeling (TUNEL) assay was performed with the reagents and the protocol included in a kit manufacturered by Boehringer-Mannheim (Mannheim, Germany). Briefly, antibody-treated cells were washed once in PBS and fixed in 100 μl of freshly prepared paraformaldehyde solution [4% (wt/vol) in PBS] for 30 min at room temperature. The cells were then permeabilized in 100 μl of 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice (4°C). After washing, the cells were resuspended

Abbreviations: TCR, T-cell receptor; mIg, membrane immunoglobulin; PE, phycoerythrin.

*This is paper no. 4 in a series. Paper no. 3 is ref. 18.

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in a mixture containing either terminal deoxynucleotidyltransferase from calf thymus and fluorescein isothiocyanate-conjugated dUTP or the later alone as a negative control. After incubation at 37°C for 1 h, the samples were washed twice in PBS and analyzed by flow cytometry.

Flow cytometric detection of APO-1 expression was performed by using primary labeling with biotinylated DX2 (anti-APO-1) and secondary detection with PE-conjugated streptavidin.

Reverse Transcriptase-Coupled PCR. Total RNA was isolated by using a guanidinium thiocyanate method (15) from Daudi or Jurkat cells activated as described above and lysed in 500 μl of solution D (4 M guanidinium thiocyanate/25 mM sodium citrate, pH 7.0/0.5% sarkosyl/0.1 M 2-mercaptoethanol). Complementary DNA was synthesized by using 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and random hexamer primers (16). Detection of APO-1 ligand mRNA was performed under standard conditions with *Taq* polymerase (Perkin Elmer), annealing at 55°C, 33 amplification cycles, and primers with the following sequences: 5′-TGATGCTGTGTGCATCTGGC and 5′-AGGCACAGTTCTTCCCTGTC. Analysis of GAPDH mRNA has been described (16). Amplification products electrophoresed on 2% agarose gels were visualized by staining with SYBR green (Molecular Probes) and analyzed by fluoroimaging with IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Kinetics of Apoptosis on B and T Cells Induced by Crosslinking Their Antigen Receptors. Activation of antigen receptors by antibody-mediated crosslinking on B- or T-lymphoma cells leads to cell death and cell cycle arrest (for review, see ref. 17). The kinetics of cell death as determined by loss of plasma membrane integrity and the presence of DNA strand breaks in cells was determined for B-lymphoma cells (Daudi) and T lymphoma cells (Jurkat). Distinct peaks representing cells with permeable membranes or DNA strand breaks can be seen in this representative flow cytometry analysis of mIg-activated Daudi cells (Fig. 1 *A* and *B*) at 24 h. Fig. 1*C* shows that Daudi cells with permeable membranes were detected by 4 h but an increase in cells with DNA strand breaks was not observed until 12–24 h after activation. In Jurkat cells, a significant increase in both parameters was detected by 8 h after crosslinking CD3 (Fig. 1*D*) and their kinetics were similar.

APO-1 Receptor Expression on B- and T-Lymphoma Cells. Both Daudi and Jurkat cells displayed APO-1 protein expression on the cell surface. Jurkat cells expressed 5-fold more APO-1 than Daudi (Fig. 2). No significant difference in the APO-1 expression in activated vs. nonactivated Daudi or Jurkat cells was observed at various times up to 24 h (data not shown).

Expression of APO-1 Ligand mRNA in Daudi and Jurkat Cells After Their Activation. The expression of APO-1 ligand mRNA was examined in antigen receptor-activated Daudi and Jurkat cells by PCR. The expression of APO-1 ligand mRNA was readily detected in Jurkat cells at 1 h of activation; the most intense band was seen at 4 h (Fig. 3); by 12 h, APO-1 ligand mRNA was not detected. In contrast, APO-1 ligand mRNA was not detected after antigen receptor activation in Daudi. Since DNA breaks were only observed in Daudi cells after 12 h of activation, we considered the possibility that a late induction of APO-1 ligand expression (between 12 and 24 h after anti-μ treatment) might be responsible for the activation of a classical apoptotic pathway. However, APO-1 ligand mRNA was not detected in activated Daudi cells 12, 14, 16, 18, 20, 22, and 24 h after anti-μ treatment (data not shown).

Induction of apoptosis in T cells requires that the anti-TCR antibody be immobilized on plastic; soluble antibody is not effective. In contrast, soluble polyclonal anti-mIg is effective at inducing apoptosis in Daudi cells without immobilization on plastic. We were concerned that the structure of the plate-coated anti-CD3 antibody might play a role in induction of APO-1 ligand. However, in experiments in which anti-mIg antibodies were coated on plates and effective at inducing apoptosis in Daudi cells, no induction of APO-1 ligand mRNA was detected (data not shown).

$F(ab')_2$ Anti-APO-1 Can Block Apoptosis in Activated Jurkat but Not in Activated Daudi Cells. PCR was used to measure APO-1 ligand mRNA levels in the experiment described above. Although PCR can be a very sensitive assay, it is difficult to exclude the possibility that a small amount of ligand is expressed that is below our level of detection; nor does it evaluate protein expression. To address these possibilities, anti-APO-1 $F(ab')_2$ was added to the medium of anti-μ-treated Daudi cells to block a putative APO-1 ligand–APO-1 interaction. As mentioned above, such treatment of Jurkat and other T cells markedly inhibits apoptosis in activated T cells (10). If Daudi cells are killed by low levels of ligand expression, then anti-APO-1 $F(ab')_2$ should prevent apoptosis.

Anti-APO-1 $F(ab')_2$ was highly effective at blocking a substantial portion of apoptotic cell death in activated Jurkat cells, with maximal effects detected with as little as 10 ng/ml with no further increase up to 1000 ng/ml (Fig. 4). In contrast, even at 1000 ng/ml, $F(ab')_2$ anti-APO-1 had no effect on the induction of cell death or loss of DNA in Daudi.

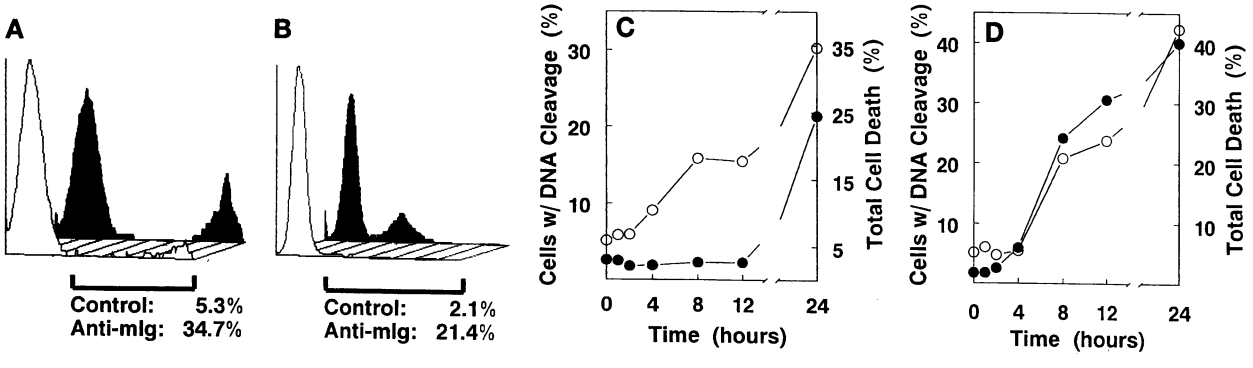

FIG. 1. Kinetics of DNA cleavage and cell death induced by antigen receptor activation in Daudi and Jurkat cells. Daudi cells were treated with either goat anti-μ (shaded peaks) or goat anti-ovalbumin (control; open peaks), harvested at 24 h, and analyzed for membrane permeability (*A*) or DNA strand breaks (*B*). The percentages of positive cells (see marker) in control and anti-immunoglobulin-treated samples are indicated. Daudi (*C*) and Jurkat (*D*) cells were treated with either anti-μ or anti-CD3, harvested (data not shown), and assessed for DNA strand breaks (solid symbols) or membrane permeability (open symbols). Samples treated with a control antibody at various times after antibody addition gave results similar to those for untreated cells (0 h) (two experiments done with similar results).

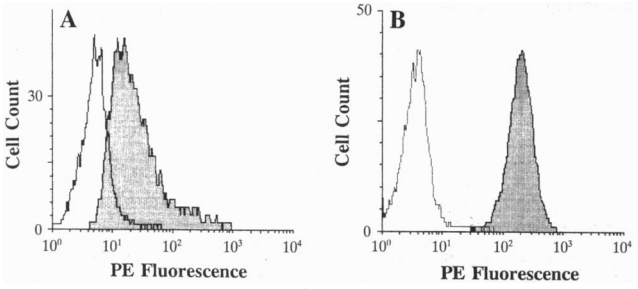

FIG. 2. Expression of APO-1 on activated B and T lymphomas. Daudi (*A*) and Jurkat (*B*) cells were plated in the presence of anti-IgM or anti-CD3, respectively, harvested at 4 h, and stained for APO-1 receptor expression. The histograms of APO-1-stained cells are shaded. The control stained cells are represented as open histograms (two experiments done with similar results).

DISCUSSION

The major findings to emerge from this study are that, although associated with apoptosis induction, treatment of human lymphoma cells (Daudi) with anti-μ (*i*) did not result in synthesis of APO-1 ligand as detected by PCR and (*ii*) simultaneous treatment with anti-μ and a blocking anti-APO-1 antibody did not alter the kinetics or extent of cell death.

The present studies indicate, therefore, that there are different signals leading to apoptosis after ligation of antigen-specific receptors on B cells compared to T cells. As summarized above, the evidence for an APO-1–APO-1 ligand interaction causing apoptosis in T cells appears conclusive. However, for B cells, the failure to inhibit apoptosis by a "blocking" anti-APO-1 antibody indicates that APO-1–APO-1 ligand interactions are not involved in the signaling events leading to apoptosis under the experimental conditions employed. This is probably due to the lack of APO-1 ligand induction. To ensure that results using B-lymphoma cells were not due to technical problems, we carried out parallel experiments in Jurkat lymphoma cells. Our results confirm earlier reports (10–12); i.e., APO-1 ligand expression was readily detectable by PCR 1–8 h after TCR ligation and reached a peak level at 4 h. Also, addition of anti-APO-1 $F(ab')_2$ to the activated cultures of Jurkat cells decreased apoptosis 60–75%.

Although the APO-1 ligand is not involved, the question remains whether APO-1 expression plays a role in the induction of apoptosis by anti-mIg in Daudi cells. Perhaps, crosslinking of the mIg complex results in its association with APO-1 with resultant productive oligomerization of APO-1 and the subsequent delivery of a death signal without the need of its ligand. It is also possible that another member of the APO-1 family with its ligand is responsible for IgM-mediated apoptosis. These putative mechanisms (and variants of them) may also be available to T lymphocytes since the suppression by anti-APO-1 $F(ab')_2$ was not complete in the published studies (10) or in our results (Fig. 4), even at high concentrations of blocking reagent. Thus, there could be a number of mechanisms for cell death in antigen-receptor-activated T and B lymphomas: (*i*) APO-1–APO-1 ligand interaction in T cells; (*ii*) APO-1 on B cells interacting with APO-1 ligand on or secreted by T cells (ref. 19; for review, see ref. 20); (*iii*) APO-1 activation after antigen–receptor crosslinking in B cells and, possibly, T cells, perhaps through a direct association with the antigen receptor itself; and (*iv*) an APO-independent death pathway in B and perhaps T cells.

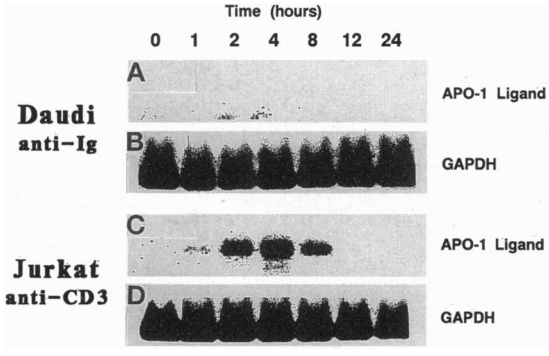

FIG. 3. Antigen receptor activation of Jurkat but not Daudi cells induces transient APO-1 ligand mRNA expression. Daudi or Jurkat cells were treated with either anti-μ or anti-CD3, respectively. At the indicated times, the cells were harvested and total RNA was isolated. mRNA levels of APO-1 ligand (*A* and *C*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*B* and *D*) were determined by reverse transcriptase-coupled PCR. Expression in samples treated with control antibody was similar to that in untreated cells (data not shown) (two experiments done with similar results).

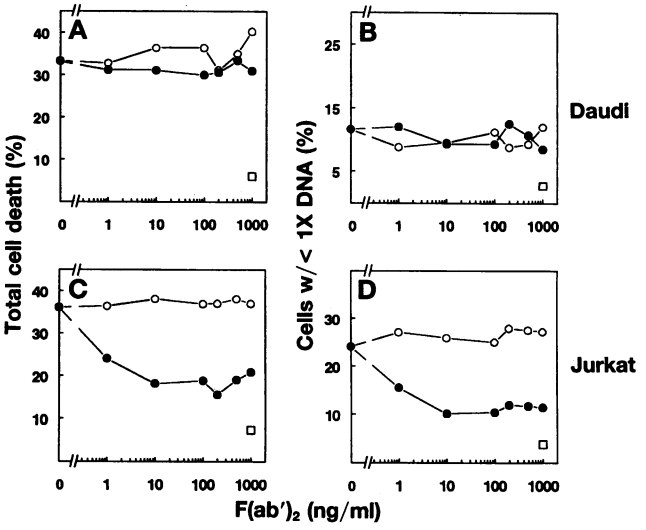

FIG. 4. $F(ab')_2$ anti-APO-1 blocks apoptosis induced by antigen receptor activation in Jurkat but not Daudi cells. Daudi or Jurkat cells were treated for 24 h with either anti-μ (*A* and *B*) or anti-CD3 (*C* and *D*) antibodies in the presence of anti-APO-1 $F(ab')_2$ (solid symbols) or control $F(ab')_2$ (open symbols). Cells were then harvested and stained for membrane permeability (*A* and *C*) or DNA content (*B* and *D*). Analysis of cells treated with control antibody MOPC21 and anti-APO-1 $F(ab')_2$ (1 μg/ml) is also indicated (open squares) (three experiments done with similar results).

The cell-signaling machinery for T and B cells have considerable structural and operational similarities. Thus, the antigen-specific receptor of each cell type has associated accessory molecules that are necessary for signaling. After crosslinking of the antigen receptor, protein tyrosine kinases phosphorylate intracellular proteins with resultant activation of a variety of second messengers and signaling pathways leading to either replication, differentiation, anergy, or death. Thus, *a priori*, we would have predicted that crosslinking the antigen-specific receptor on B cells, as on T cells, would also induce the upregulation and secretion of the APO-1 ligand resulting in apoptosis by suicide or fratricide. However, the present studies indicate that apoptosis mediated through membrane IgM can occur in the absence of detectable APO-1 ligand. Why should B and T cells differ in such a fundamental mechanism? One speculation that would place our results in the framework of the normal immune response would be as follows: The B-cell-antigen-specific receptors can be readily saturated and extensively crosslinked by circulating self antigens. If this were to result in coligation of APO-1 or another molecule in the APO-1 family, the coligated molecules could thereby be activated and directly signal apoptosis, resulting in deletion of

the cells (tolerance). In the case of T cells, the antigen is presented only in the form of a major histocompatibility complex–peptide fragment complex that may be much less effective in allowing self antigens to extensively crosslink the TCR. Thus, even in anti-CD3-treated T cells, it was necessary to immobilize the antibody to obtain significant apoptosis. Hence, for deletion of self-reactive T cells, it might be desirable to have an amplifying mechanism, i.e., secretion of the APO-1 ligand, which would increase sensitivity for induction of tolerance.

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