Cytoplasmic μ Heavy Chain Confers Sensitivity to Dexamethasone-induced Apoptosis in Early B-lineage Acute Lymphoblastic Leukemia

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Abstract

Most childhood acute lymphoblastic leukemia (ALL) arises from early B-lineage cells, and response to steroid treatment is critical to successful ALL therapy. To investigate the effect of the pre-B cell receptor (pre-BCR) complex on the response of leukemic cells to steroids, cytoplasmic μ protein (cyto μ) was transfected into cyto μ-, steroid-resistant early B cell lines. The presence of cyto μ and the assembled pre-BCR complex conferred sensitivity to dexamethasone-induced apoptosis. Both intrinsic and extrinsic apoptosis pathways are involved in this cell death. However, if the transfected cyto μ protein is unable to assemble the pre-BCR complex, the cells remain resistant to dexamethasone. These findings suggest a role for the pre-BCR complex in the response of ALL cells to treatment and provide insight into the mechanism of steroid response in the treatment of pre-B ALL.

Introduction

Approximately 75% of childhood ALL results from transformation events that occur in early B-lineage cells (1). Advances in understanding the stages of B cell ontogeny and the signaling pathways driving B cell development offer the opportunity to better characterize the biology of ALL and the mechanisms of treatment response. The key control point during normal B cell development comes at the transition from the pro-B through the pre-B stage. Cytoplasmic expression of intact IgM heavy chain protein (cyto μ) and assembly of the pre-BCR complex defines this stage of development (2, 3). The pre-BCR complex consists of μ heavy chain, surrogate light chain, and the Iga/β heterodimer. Iga/β serves as the signal transduction unit of the complex (3). Apoptotic signals as well as growth and survival signals work together to maintain B-lymphocyte homeostasis, delete the vast majority of B cells that fail to form an intact antigen receptor, and result in a functional humoral immune system (2). Because the pre-BCR complex is an important checkpoint at this stage of B cell development, we hypothesized that signaling through the pre-BCR complex would influence the response of early B-lineage cells to chemotherapy.

Materials and Methods

Cell Lines. Two early B-lineage cell lines were used in these studies. NFS-70 (American Type Culture Collection, Manassas, VA) is a pro-B cell murine lymphoblast line (B220/CD45R− CD5− CD32+ cyto μ+; Ref. 4). ret02/1 is also a pro-B murine lymphoblast line (B220/CD45R− CD34+ cyto μ+; Ref. 5). Jurkat cells (human T-cell leukemia line; American Type Culture Collection) were used as a control cell line for the apoptosis assays. The cells were maintained in RPMI 1640 with L-glutamine (Invitrogen Corp., Carlsbad, CA), 10 mM HEPES, 1 mM sodium pyruvate, 100 μM non-essential amino acids, penicillin 100 units/ml, streptomycin 100 μg/ml, and 10% FCS (C10 medium).

Transfection with Human μ Constructs. The ret02/1 and NFS-70 parent cell lines were transfected by electroporation (3) with a human μ construct containing the rearranged variable region of S107, a phosphohydroxy-specific murine IgM, and the human IgM constant region. These constructs have been described previously (3, 6). The cDNA sequence encoded either wild-type μ heavy chain or a mutant form with a 2-amino acid transmembrane region substitution (Y587/S588→V/V) referred to as Δμ. This substitution prevents the association of the Iγo/β heterodimer to the pre-BCR complex, thus eliminating the Δμ signal transduction (3, 6). The NFS-70 cell line was cotransfected with a plasmid containing the S107κ coding sequence and the neomycin resistance gene (neo). The ret02/1 cells were cotransfected with a neo-containing plasmid (pcDNA 3.1; Invitrogen) only. The presence of the transfected cDNA was verified by PCR for heavy and light chain-specific sequences. Using forward primer 5′-AAGGTGGGGCCTAGGGAT-3′ and reverse primer 5′-CACCTACAGGCAAGAGA-3′, we were able to amplify a 300-bp fragment in the transmembrane coding region of μ heavy chain.

Transfection of μ or Δμ was confirmed by sequencing this PCR product that included the relevant portion of the transmembrane coding sequence. Translation of μ protein was verified by flow cytometry as well as immunoblot.

Western Blot and Immunoprecipitation. Equal numbers of cells were lysed with 1% Triton X-100 (Sigma, St. Louis, MO) plus protease inhibitors. To confirm equal total protein in the lysates before immunoprecipitation, protein concentrations of lysates were quantitated using the Bradford technique. Transfected μ protein was immunoprecipitated with rabbit anti-human IgM antibody (Jackson ImmunoResearch, West Grove PA) and protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz CA). Murine Bcl-2 protein was immunoprecipitated with anti-Bcl-2 antibody-conjugated agarose beads (Santa Cruz Biotechnology). Murine Bax protein was immunoprecipitated by rabbit anti-Bax IgG (Upstate Biotechnology, Lake Placid, NY) and protein G agarose beads (Invitrogen). Specific proteins were detected on polyvinylidene difluoride membranes using horseradish peroxidase-conjugated anti-human IgM (Jackson ImmunoResearch) or horseradish peroxidase-conjugated anti-Bcl-2 antibody (Santa Cruz Biotechnology) antibodies and developed with enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). The protein bands were quantitated from the film by densitometry using NIH Image (Scion Corporation, Frederick, MD).

Flow-cytometric Analyses. Surface IgM was detected in permeabilized cells using FITC-conjugated anti-human IgM and anti-mouse IgM polyclonal antibodies (Jackson ImmunoResearch). Cytoplasmic μ was detected in permeabilized cells using the same antibodies. FITC-conjugated IgG of the same species was used to measure nonspecific labeling. Viable FITC-Annexin V and paraformaldehyde-fixed TUNEL-labeled cells were also analyzed by flow cytometry.
Fluorescence intensity of labeled cells was measured using a FACScan cytometer (BD Biosciences, Franklin Lakes, NJ).

**Apoptosis Assays.** Viable cells were counted by trypan blue and plated at 0.5–1 × 10^5 cells/ml in C10 medium. The cells were then incubated with 0.1–10 μM dexamethasone (Sigma) or 1–2 μg/ml anti-fas antibody (R&D Systems, Minneapolis, MN) for 12–65 h. For the apoptosis inhibition assays, the cells were incubated with dexamethasone or anti-fas antibody and with 50 μM pancaspase inhibitor z-VAD-fmk (Calbiochem, San Diego, CA). Levels of exposed phosphatidylserine on viable cells were measured using the ApoAlert Annexin V detection kit (Clontech, Palo Alto, CA). Cells were incubated with FITC-conjugated Annexin V, and log fluorescence intensity was analyzed by flow cytometry.

DNA nicks created by endonucleases during initiation of apoptosis were detected by TUNEL assay (ApopTag Pro-Long Detection Kit; Roche Molecular Biochemicals, Indianapolis, IN). A positive control (apoptotic U937 cells) was also provided in the kit. The DNA was labeled with FITC-dUTP and detected by flow cytometry.

**Detection of Activated Caspases.** CaspaTag 8 (benzyloxycarbonyl leucylglutamylhistidylaspartic acid fluoromethyl ketone [FAM-LDH-fmk]; Serologicals Corp., Purchase, NY) is a carboxyfluorescein derivative of the inhibitor of caspase 8. CaspaTag 9 (carboxyfluorescein benzyloxycarbonyl leucylglutamylhistidylaspartic acid fluoromethyl ketone [FAM LEHD-fmk]; Intergen, Purchase NY) is a carboxyfluorescein derivative of the inhibitor of caspase 9. Lysylated CaspaTag 8 and 9 were reconstituted in DMSO at 150× and diluted to 30× with PBS just before use. The cells were incubated with CaspaTag 8 or 9 and then analyzed immediately by flow cytometry.

**Statistics.** STATA 7.0 (Stata Corporation, College Park, TX) was used for all statistical analysis. Paired and unpaired t tests with unequal variances were used to compare apoptotic indices within and between cell lines. The rate of change in apoptosis over time and area under the dose-response curve were estimated by STATA functions. These values were compared between cell lines with t tests with unequal variances. All Ps were two sided and 0.05 was used as the level of significance.

**Results**

**Cytoplasmic μ Heavy Chain Transfected into Early B-lineage ALL Cell Lines Confers Sensitivity to dex-induced Apoptosis.** To examine the role of the μ heavy chain protein in the response of pre-B cells to steroids, we transfected cytoμ-negative, murine pro-B cell lines with a μ construct that directs the synthesis of a membrane-bound form of μ protein. Western analysis verified the presence of μ protein in the transfected cells, and there was no detectable expression of IgM on the cell surface (not shown). However, μ protein was detected intracellularly after transfected cells were permeabilized (not shown). To assess the requirement for the assembly of the complete pre-BCR signaling complex, we transfected the same pro-B cell lines with the signal-impaired Δμ construct, which does not assemble the pre-BCR complex (3). The presence of the appropriate construct (μ versus Δμ) was verified by sequencing of a PCR product that amplified the coding sequence of the transmembrane region.

We then treated our transfected and parent cell lines with 1 μM dex for 48–60 h and labeled them with Annexin V-FITC or by TUNEL, or detected fragmented DNA ladders to identify cells undergoing apoptosis. Representative flow histograms of Annexin V assays are shown in Fig. 1. A and B. The untreated ret02/1 parent cells and the μ- and Δμ-transfected cells were not apoptotic and showed lack of Annexin V labeling. However, expression of μ in the context of an intact pre-B cell receptor complex (ret/μ) conferred sensitivity to dex. In Fig. 1A, 92% of μ-transfected cells treated with 1 μM dex for 60 h were apoptotic as shown by labeling with Annexin V. However, expression of μ protein without assembly of the pre-BCR complex (ret/Δμ) did not change the sensitivity of these pro-B cell lines to dex, and the lack of apoptotic response to dex was identical to that seen in

![Fig. 1](https://cancerres.aacrjournals.org/figure/1/)

**Fig. 1.** Transfected cyto μ heavy chain confers sensitivity to dex-induced apoptosis in early B cell lines. Two different parent early B cell lines and μ- or Δμ-transfected cell lines were treated with 1 μM dex for 60 h and then labeled with Annexin V. Flow-cytometric histograms are shown: A. dex-treated ret02/1 parent pro-B cells, μ-transfected (ret/μ) ret cells, and Δμ-transfected (ret/Δμ) ret cells; B. dex-treated NFS-70 parent cells, μ-transfected (NFS/μ) NFS-70 cells, and Δμ-transfected (NFS/Δμ) NFS-70 cells. ret02/1, ret/μ, and ret/Δμ cells (C) treated with 10 μM dex and analyzed at multiple time points (D) were treated with various concentrations of dex and analyzed after 65 h of treatment. Annexin V labeling was measured by flow cytometry.
the parent cell line (Fig. 1A). Treatment with anti-fas antibody had no effect on the ret02/1, ret/μ, and ret/Δμ cells (not shown).

To confirm the effect of pre-BCR expression on dex sensitivity in early B cell lines, we repeated these experiments using the pro-B cell line NFS-70. Similar results were seen with NFS-70 parent and transfected NFS-70 cells treated with 1 μM dex for 60 h (Fig. 1B). With expression of intact μ (NFS/μ), dex-resistant NFS-70 cells became apoptotic in response to dex. The parent NFS-70 and NFS/Δμ cells showed minimal apoptotic response to dex.

Time course and dose-response curves for dex apoptosis assays performed in triplicate are shown in Fig. 1. C and D, respectively. ret02/1, ret/Δμ, and ret/μ cells were treated with dex at concentrations of 0.1, 1, and 10 μM dex and evaluated after 24, 48, and 65 h of treatment. The time course for 10 μM dex treatment is shown in Fig. 1C. ret/μ-transfected cells showed significantly greater apoptosis in response to dex over time (versus ret02/1, \( P = 0.00001 \); versus ret/Δμ, \( P = 0.00002 \)). ret02/1 parent cells showed almost no response to dex treatment over time. Like the parent cells, cells transfected with signal-impaired μ (Δμ) also had almost no response to dex up to 65 h of treatment. The dose-response curve at 65 h showed that the ret/μ cells were sensitive to dex doses as low as 0.1 μM, and this apoptotic response was significant (Fig. 1D; versus ret02/1, \( P < 0.0001 \); versus ret/Δμ, \( P = 0.0002 \)). After 24 h of dex treatment, the parent and Δμ cells showed essentially no (3–7%) apoptotic response with escalating doses of dex. Even at this earlier time point, dex caused cell death in ret/μ cells in a dose-dependent fashion, with apoptosis increasing by 20% with 0.1 μM dex and increasing by 30% with 10 μM dex (not shown). A similar response was seen at 48 h of dex treatment, where we observed a 28% increase in apoptosis with 0.1 μM and a 54% increase with 10 μM dex (not shown).

After treating ret02/1 parent and μ- and Δμ-transfected cells with 1 μM dex for 48 h, the characteristic apoptotic DNA ladder was only detected in ret/μ cells treated with dex (Fig. 2A). DNA from the dex-treated parent and Δμ-transfected cells was unchanged from the DNA of the untreated controls. When apoptosis was measured by TUNEL (Fig. 2B), 92% of μ-transfected cells were apoptotic after treatment with 1 μM dex for 48 h. No appreciable TUNEL response was seen in parent or Δμ cells treated with dex (not shown).

Pancaspase Inhibitor Partially Blocks dex-induced Apoptosis in Cell Lines Transfected with Cytoplasmic μ Protein. To investigate possible mechanisms of dex-induced cell death in μ-transfected early B cells, we explored the caspase pathways. z-VAD-fmk is an irreversible pancaspase inhibitor that binds to and inhibits activated caspases-1 through -9. μ-transfected cell lines treated with dex and z-VAD-fmk for 60 h showed 41% inhibition of apoptosis as shown by TUNEL, compared with treatment with dex alone (Fig. 2B). There was no effect of the pancaspase inhibitor on the parent cell lines or on the Δμ-transfected cells. As a control experiment, inhibition of apoptosis was also seen in Jurkat cells treated with anti-fas antibody and z-VAD-fmk (not shown).

Activated Caspases 8 and 9 Are Involved in dex-induced Apoptosis. Caspases 8 and 9 are key initiators in extrinsic and intrinsic pathways of apoptosis, respectively, although there can be cross-talk and amplification between the two pathways (7). Using carboxyfluorescein-labeled specific caspase inhibitors that bind to active caspases 8 and 9 (CaspaTag 8 and 9), both activated caspases 8 and 9 were detected in μ-transfected cells treated with dex. Untreated cells showed <12% of cells expressing activated caspase 9 (Fig. 3A). Activated caspase 8 was also low in these untreated cells (not shown).

After treatment with dex, 67% of μ-transfected ret cells had detectable levels of activated caspase 8 enzyme and 63% had activated caspase 9 (Fig. 3, B and C). Dex-treated ret02/1 parent cells and ret/Δμ-transfected cells showed minimal activation of caspase 8 or 9 (Fig. 3, D–I). A positive control was established using Jurkat cells treated with anti-fas antibody. Jurkat cells are known to activate caspase 8 and 9 and undergo programmed cell death after ligation of fas (8), and >50% of anti-fas antibody-treated Jurkat cells showed the presence of active caspases 8 and 9 (not shown).

Bcl-2 Protein Is Down-regulated but Bax Levels Remain Unchanged during dex-induced Apoptosis. To assess further the apoptotic effects of dex, Bcl-2 and Bax protein levels were evaluated by immunoprecipitation and Western blot in untreated and dex-treated cells. ret and Δμ cells showed no change in Bcl-2 protein levels after treatment with dex (Fig. 4A). However, there was a 2-fold decrease in Bcl-2 protein in μ-transfected cells after 60 h of treatment with 1 μM dex (Fig. 4A), as shown by immunoblot. Total cellular Bax levels remained unchanged in the μ-transfected, apoptotic cells after treatment with dex (Fig. 4B).

Discussion

Early B-lineage ("pre-B") cell ALL is the most common immunophenotype in childhood ALL and is known to respond well to chemotherapy (1). Steroids are a major part of both induction and maintenance therapy for ALL, and the initial response of ALL to steroid treatment is an established prognostic variable (9, 10). We demonstrate three significant findings which establish a role for pre-BCR complex-mediated signaling in the apoptotic response of early B cell ALL lines to steroid treatment. (a) Steroid-resistant, cyto μ-negative early B cell lines transfected with μ protein become sensitive to dex-induced cell death in both a dose- and time-dependent manner. (b) μ, in the absence of the pre-BCR signaling complex, does not confer apoptotic sensitivity to steroid treatment. These findings indicate that both cyto μ- and Igα/β-mediated signaling are involved in facilitating...
the apoptotic response to dex. (c) Both intrinsic and extrinsic pathways of apoptotic signaling are involved in this model of dex-induced apoptosis in early B-lineage lymphoblasts. Caspases 8 and 9 are activated in this apoptotic pathway as a result of dex treatment. Other factors independent of the caspases must also be involved because caspase inhibition does not fully reverse the apoptotic response.

Here, we show that treating cyto-μ-transfected cells with dex results in activation of both caspases 8 and 9. Treatment with anti-fas antibodies had no effect on these cells. Early B-lineage cells are not known to have tumor necrosis factor family receptors with death domains on their cell surface. The pathway(s) by which caspase 8 might be activated in cyto-μ-transfected cells is not clear. However, intracellular mechanisms bridging extrinsic and intrinsic apoptotic pathways do exist (7, 11).

Expression of Bcl-2 family members in acute lymphoblastic leukemias has been described (12–14). Because Bcl-2 promotes cell survival, one might expect high levels of Bcl-2 to be associated with poor response to therapy and poorer outcome. However, one study looked at 338 children with ALL and found that, in general, high levels of Bcl-2 expression did not predict slow early response, failure to achieve remission, or poor event-free survival (13). Another study suggested that Bcl-2 may protect pre-B cells from glucocorticoid-induced apoptosis, even in the face of c-myc repression (15). In our model, Bcl-2 protein was down-regulated in μ-transfected cells that underwent apoptosis after treatment with dex, but other proapoptotic mechanisms are most likely also involved. In our apoptotic cells, there was not an increase in Bax protein expression; however, we cannot exclude that Bax has translocated to the mitochondrion.

Glucocorticoid-associated new protein expression may result in the activation of tyrosine kinases that can transmit signals linking the pre-BCR complex to the apoptotic machinery, or the presence of the pre-BCR complex may provide a signaling milieu in which the dex response is possible. B cell receptor (BCR)-mediated apoptosis has been described in immature and mature B cells (16). Signaling events after BCR ligation include phosphorylation of the immunoreceptor tyrosine-based activation motifs in the Igα/Igβ heterodimer that initiate downstream events leading to activation or apoptosis (17). Proto-oncogene c-Myc is reported to be enhanced after BCR ligation resulting in mitochondrial dysfunction and apoptosis (16). Our data do not suggest that actual ligation of the pre-BCR is required for the apoptotic signaling in pre-B cells in response to dex. The pre-BCR may indeed not need a ligand, as its purpose in B cell development is to indicate that an intact cyto-μ protein capable of assembling the BCR complex has been expressed. Additional studies will be required to determine the mechanism(s) by which cyto-μ and the pre-BCR complex participate in the apoptotic pathway.

The effect of cyto-μ on event-free survival was examined in the Pediatric Oncology Group 8602 study. In this study, the presence of cyto-μ in the diagnostic lymphoblasts was determined, and patients were stratified according to “pre-B” (cyto-μ+ ) versus “early pre-B” (cyto-μ−) phenotype. There was no difference in outcome between the pre-B and early pre-B patients, either as a whole or when stratified by risk group and treatment (18). Early response to induction therapy was not reported for these patients, so that detecting an association between rapid early response and cyto-μ expression was not possible, but would be interesting, given the findings of our study. This similarity in outcome between patients with early pre-B and pre-B phenotypes differed from earlier observations based on less intensive treatment regimens (19). A recent study in pediatric T-cell ALL reported that in vitro dex-induced apoptosis of primary lymphoblasts correlated with a good in vivo early response to initial therapy by the day-15 bone marrow blast percentage (20). It is clear that other factors also play a role in therapy response because cyto-μ+ ALL accounts for only 20–30% of pre-B ALL (21), and >90% of children with ALL attain complete remission after induction therapy (which includes other drugs in addition to steroids).

In summary, expression and assembly of cyto-μ with the pre-BCR complex is known to be a central regulatory checkpoint in normal B cell development. In the ALL cell lines studied here, the presence of cyto-μ and an intact pre-BCR complex confers sensitivity to steroid-induced apoptosis, and this apoptotic signaling involves intrinsic and extrinsic death pathways. Regulation of caspases and Bcl-2 protein plays a role in this cell death signaling. Although patients with cyto-μ+ pre-B ALL are a minority of all patients with B-lineage ALL, our data suggest that cyto-μ+ lymphoblasts may be more responsive to steroids, leading to the hypothesis that these patients may respond
better to the steroid component of their treatment during induction chemotherapy. More importantly, the role of cyto μ and pre-BCR complex signaling in pre-B ALL steroid responsiveness will allow further mechanistic analyses of the response to steroids in the pre-B cell signaling milieu.

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References

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