Eosinophils are potent inflammatory cells involved in allergic reactions. Inhibition of apoptosis of purified eosinophils by certain cytokines has been previously shown to be an important mechanism causing tissue eosinophilia. To elucidate the role of Bcl-2 family members in the inhibition of eosinophil apoptosis, we examined the expression of the known anti-apoptotic genes Bcl-2, Bcl-x\(_L\), and A1, as well as Bax and Bcl-x\(_S\), which promote apoptosis in other systems. We show herein that freshly isolated human eosinophils express significant amounts of Bcl-x\(_L\) and Bax, but only little or no Bcl-2, Bcl-x\(_S\), or A1. As assessed by reverse transcription-polymerase chain reaction, immunoblotting, flow cytometry, and immunocytochemistry, we show that spontaneous eosinophil apoptosis is associated with a decrease in Bcl-x\(_L\). mRNA and protein levels. In contrast, stimulation of the cells with granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-5 (IL-5) results in maintenance or upregulation of Bcl-x\(_L\) mRNA and protein levels. Moreover, Bcl-2 protein is not induced by GM-CSF or IL-5 in purified eosinophils. Bcl-2 protein is also not expressed in tissue eosinophils as assessed by immunohistochemistry using two different eosinophilic tissue models. Furthermore, Bcl-x\(_L\) antisense but not scrambled phosphorothioate oligodeoxynucleotides can partially block the cytokine-mediated rescue of apoptotic death in these cells. These data suggest that Bcl-x\(_L\) acts as an anti-apoptotic molecule in eosinophils.

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MATERIALS AND METHODS

Antibodies. Anti-Bcl-2 monoclonal antibody (MoAb), control IgG1 MoAb, swine anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary IgG antibody, and control rabbit IgG were from Dako (Zurich, Switzerland). FITC-conjugated anti-Bcl-2 MoAb was from Ancell Corp (Bayport, MN). Polyclonal rabbit antibodies against Bcl-x and Bax were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Polyclonal rabbit anti-Bcl-x antibody was from Calbiochem-Novabiochem Corp (San Diego, CA). Goat anti-rabbit and anti-mouse horseradish peroxidase (HRP)-labeled secondary antibodies were obtained from Amersham International (Bucks, UK). FITC-conjugated control IgG1 MoAb was from Coulter (Hialeah, FL). Anti–IL-3 and anti–GM-CSF MoAbs were purchased from Genzyme (Cambridge, MA). Anti–IL-5 MoAb (5A5) was a kind gift from Dr J. Tavernier (University of Gent, Gent, Belgium). Anti-eosinophil cationic protein (ECP) MoAb (EG1) was from Pharmacia (Uppsala, Sweden). Anti-CD16 MoAb microbeads were from Miltenyi Biotec (Bergisch Gladbach, Germany).
Eosinophil purification and cell cultures. Eosinophils were purified from patients with atopic dermatitis and healthy normal individuals as previously described.11-14 Eosinophils were cultured at 1 \times 10^6/mL (expression experiments) or 0.5 to 10^6/mL (antisense experiments) for the indicated times using complete culture medium (RPMI 1640 supplemented with 10% fetal calf serum [FCS]). GM-CSF was a kind gift from Dr T. Hartung (University of Konstanz, Konstanz, Germany). IL-5 was obtained from Genzyme. The final cytokine concentrations were 25 ng/mL. Phosphorothioate oligodeoxynucleotides were synthesized by Genset S.A. (Paris, France). Sequences used were as follows: antisense Bcl-xL, 5'-TGT ATC TCT TCT GGG AAA GC-3'; and scrambled Bcl-xL, 5'-TAA GGG ATG CGA TTT GTT-3'. These antisense molecules were selected from a panel of different oligodeoxynucleotides as previously described.15 The oligodeoxynucleotides were given to purified eosinophils that had been cultured for 20 hours in complete culture medium (at this time, the cells did not express detectable Bcl-xL protein levels), and were delivered in the form of complexes with the cationic lipid DOTAP (Boehringer Mannheim, Mannheim, Germany). Equal volumes of oligodeoxynucleotide (6 µmol/L) and DOTAP (0.2 mmol/L) were mixed and allowed to complex for 10 minutes at room temperature. The final oligodeoxynucleotide concentrations were 0.45 µmol/L. The initial phase (first 4 hours) of incubation with the oligodeoxynucleotides was performed in medium without serum to increase the uptake of these molecules by the eosinophils. Cells were then cultured again in complete culture medium in the presence or absence of GM-CSF or IL-5 for another 24 hours (apoptosis experiments were 0.45 µmol/L). The initial phase (first 4 hours) of incubation with the oligodeoxynucleotides was performed in medium without serum to increase the uptake of these molecules by the eosinophils. Cells were then cultured again in complete culture medium in the presence or absence of GM-CSF or IL-5 for another 24 hours (apoptosis assay) or 36 hours (cell death assay). Therefore, cell viability was determined after the eosinophils had been in culture for a total of 60 hours.

Reverse transcription-polymerase chain reaction (RT-PCR). mRNA expression of Bcl-2 family members was studied using Southern blot analysis linked to RT-PCR.11,12 Primers for Bcl-2 (5'-ACA ACA AGC CTT GTC GAT TCG ACG TTT TGC C-3'), Bcl-xL (5'-GGG ATT CTT GGA CAA TGG ACT GGT TGA-3' and 5'-CCC AAG CTG ATG TAG TTC CTG TGA-3'), Bax (5'-GGA ATT CTT CCA CAA GAT GAC GGA GGA-3' and 5'-GAA GAT GCC AGA CGA TCA TCA ATG GGA-3'), and A1 (5'-GGG ATT CTT CCT GGA CAA TGG ACT GGT TGA-3' and 5'-CA ACA GCA GGA GG-3') amplifications were synthesized (Bcl-2: HSC Biotechnology Service Centre, Toronto, Ontario, Canada; Bcl-xL and Bax: R & D Systems, Abingdon, UK; A1: Microsys, Balgach, Switzerland) according to previously published sequences.15,16 For positive controls, PCR amplifications were performed at the same time using cDNAs from HL-60 (Bcl-2, Bcl-xL, and Bax) and Jurkat (A1) cells. All data are representative of eight independent experiments.

Immunofluorescence. Purified eosinophils (0.1 \times 10^6) were stained with FITC-conjugated anti-Bcl-2 or control MoAb for 15 minutes at room temperature after a 45-minute permeabilization of the cell membrane with Permeafix (Ortho Diagnostic Systems, Raritan, NJ). To determine Bcl-xL and Bax expression, cells were initially incubated with 10 µg/mL anti-Bcl-xL, anti-Bcl-xS, anti-Bax, or control rabbit antibody for 15 minutes at room temperature, washed, and then incubated with FITC-conjugated purified swine anti-rabbit IgG antibody for 15 minutes at room temperature. Directly or indirectly stained cells were washed, fixed in phosphate-buffered saline (PBS)-buffered 2% paraformaldehyde solution. Eosinophils were analyzed by flow cytometry in an EPICS XL (Coulter).

Immunocytochemistry. Bcl-xL, Bcl-xS, and Bax protein expression was also investigated by immunocytochemistry using a commercial kit (Histostain SP kit; Zymed Laboratories, San Francisco, CA) according to the manufacturer’s instructions. Briefly, cytopsins were prepared from 0.1 \times 10^6 purified eosinophils. Slides were fixed in freshly made and filtered PBS-buffered 4% paraformaldehyde solution for 20 hours at room temperature in the dark. After washing with H2O and PBS, slides were incubated with Peroxo-Block (Zymed Laboratories) for 10 minutes to quench endogenous peroxidase activity. After blocking with nonimmune serum, 1 µg/mL primary antibody was added for 1 hour. This incubation was followed by addition of biotinylated secondary antibody and streptavidin-peroxidase conjugate. Bound peroxidase was detected by addition of substrate chromogen mixture, followed by hematoxylin counterstaining. Slides were mounted and examined under a Zeiss Axioscope microscope (Oberkochen, Germany) at a magnification of \times 1,000.

For Bcl-2 staining, the alkaline phosphatase-anti-alkaline phosphatase (APAAP) method was used.11 Staining was performed with a commercial kit (Dako) according to the manufacturer’s instructions.

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Immunoprecipitation, gel electrophoresis, and immunoblotting. Immunoprecipitation, gel electrophoresis, and immunoblotting were performed as previously described, using anti-Bcl-x antibody.

Immunohistochemistry. Immunohistochemistry was performed using formalin-fixed and paraffin-embedded tissues. Tissues were examined under a Zeiss Axioskope microscope at a magnification of ×400 or ×1,000.

Determination of eosinophil death and apoptosis. Cell death was assessed by uptake of 1 µmol/L ethidium bromide and flow cytometric analysis. The percentages of apoptotic cells were calculated as the numbers of cells with distinct dual-labeled fluorescence (red and green) over total cell number.

RESULTS AND DISCUSSION

Bcl-xL, but not Bcl-xS, is significantly expressed by eosinophils. We first measured mRNA levels by RT-PCR in freshly isolated as well as cultured eosinophils in the presence or absence of the survival factors GM-CSF or IL-5. Because the PCR primers used bind to sequences shared by Bcl-xL and Bcl-xS, this technique allowed simultaneous identification of both Bcl-x mRNAs. As shown in Fig 1, freshly purified eosinophils expressed Bcl-xL but not Bcl-xS mRNA. The expression of Bcl-xL appeared to increase after GM-CSF stimulation of the eosinophils in vitro but rapidly decreased when eosinophils were cultured without cytokine support (Fig 1). To better quantify the expression of Bcl-xL mRNA in response to eosinophil hemato poietins, RT-PCR was performed using different numbers of cycles. These experiments clearly showed that Bcl-xL mRNA expression in eosinophils is downregulated in the absence and upregulated in the presence of eosinophil survival factors such as GM-CSF (Fig 2D) or IL-5 (not presented).

To determine whether the expression of Bcl-xL and Bcl-xS mRNAs correlates with the expression of their proteins, we performed immunocytochemistry, flow cytometry, and immunoblotting following immunoprecipitation using anti-Bcl-x (reacts with both Bcl-xL and Bcl-xS proteins) and specific anti-Bcl-xS antibodies. As shown in Fig 2A and B, and Fig 3A, freshly purified blood and tissue eosinophils expressed Bcl-xL protein. Moreover, levels of Bcl-xL protein were maintained or increased in GM-CSF–stimulated eosinophils. In contrast, levels of Bcl-x protein decreased under conditions of cytokine withdrawal in vitro (Fig 2A through C). Immunoblotting studies after anti-Bcl-x immunoprecipitation showed that the Bcl-xL expression observed by immunocytochemistry and flow cytometry was totally caused by Bcl-xL expression, because no protein from the Bcl-xS splice form was detected (Fig 2C). In agreement with these data and recently published work,30 Bcl-xL expression was also not seen using a specific anti–Bcl-xL antibody as assessed by immunocytochemistry (Fig 2E) and flow cytometry (Fig 2F). Together, these results suggest that expression of Bcl-xL transcripts correlates with the patterns of Bcl-xL protein expression in both freshly isolated or in vitro cultured eosino-
philis. Moreover, Bcl-x function does not appear to be regulated at the level of splicing in eosinophils.

**Bax is expressed at high levels by eosinophils.** As shown in Fig 1, Bax mRNA was highly expressed in freshly isolated eosinophils. In addition, Bax mRNA levels remained unchanged when eosinophils were cultured in the presence or absence of GM-CSF (Fig 1) or IL-5 (not presented).

To determine Bax protein expression, an anti-Bax antibody was used. This antibody was able to stain tissue and purified blood eosinophils (Fig 3A, and Fig 4A and B). Moreover, we had no evidence for a change in Bax protein expression after in vitro cell cultures of eosinophils in the presence or absence of cytokines with anti-apoptotic properties (Fig 4A and B).

**Bcl-2 and A1 are expressed at very low levels by eosinophils.** We again used RT-PCR to determine Bcl-2 and A1 mRNA levels. As shown in Fig 1, freshly isolated as well as cultured eosinophils in the presence or absence of GM-CSF from normal control individuals and allergic patients do not express Bcl-2 and A1 mRNA. Note that positive signals for Bcl-2 and A1 were only obtained when RT-PCR with a higher number of cycles (>25) was performed.

To determine whether eosinophils express Bcl-2 protein under conditions where they are exposed to eosinophil hematopoietins, we investigated tissues with significant eosinophil infiltration using immunohistochemistry. We have previously shown that delayed eosinophil apoptosis occurs in nasal polyp tissues. Although high levels of IL-5 were present in these tissues, Bcl-2 expression was not observed in eosinophils (Fig 3A). Moreover, we had the chance to investigate eosinophil-infiltrated tissue from a patient with bladder cancer. Immunohistochemical examination of this tissue showed that the eosinophil hematopoietins IL-3, IL-5, and GM-CSF were highly expressed, especially by the cancer cells (Fig 3B). Again, the eosinophils under these pathologic conditions did not appear to express significant amounts of Bcl-2 protein (Fig 3B). Furthermore, we used immunocytochemistry and flow cytometric analysis to measure Bcl-2 protein levels in purified blood eosinophils. We did not observe immunoreactive Bcl-2 in resting or GM-CSF–stimulated eosinophils (not presented). Together, these results show that Bcl-2 and A1 are either not present or are present at very low levels in human eosinophils.

**Role for Bcl-xL in the regulation of eosinophil apoptosis by cytokines.** Bcl-xL is an anti-apoptotic protein that regulates apoptosis in many cellular systems, perhaps by regulating the electrical and osmotic homeostasis of mitochondria. Although the expression of Bcl-xL in mature eosinophils might be little compared with umbilical cord blood–derived eosinophils as assessed by immunoblotting, our in vivo and in vitro expression studies suggested that Bcl-xL might be a good candidate involved in the anti-apoptotic pathway mediated by cytokines in eosinophils. To test this hypothesis, we determined the effect of decreasing the levels of Bcl-xL expression. Eosinophils that had been cultured for 20 hours did not express detectable levels of Bcl-xL protein (Fig 2B). Exposure of these eosinophils to an optimal dose of phosphorothioate-derivatized Bcl-xL antisense oligodeoxynucleotides for another 28 hours clearly inhibited by approximately 34% the ability of IL-5 or GM-CSF to prevent eosinophil death (Fig 5B). The effect of the antisense molecules was highly significant (t-test, P < .001). We also investigated whether the observed cell death was apoptosis. Using analysis of the eosinophil morphology, we observed much more pyknosis of the nucleus and cell shrinkage in cells that had been treated with antisense Bcl-xL oligodeoxynucleotides (Fig 5C). Quantitative analysis (counting of 300 cells) showed similar results as observed in the cell death experiments: The antisense molecules reduced the anti-apoptotic effect of IL-5 in average by 33% (not presented). Furthermore, no differences were observed between eosinophils from normal control individuals and allergic patients.

These data suggest that Bcl-xL is functionally active within the intracellular anti-apoptotic pathway mediated by cytokines such as IL-5 or GM-CSF in eosinophils. However, the inhibitory effect of Bcl-xL antisense oligodeoxynucleotides on GM-CSF– or IL-5–mediated delay of eosinophil apoptosis was only partial. This could be due in part to the fact that, although the antisense molecules significantly blocked Bcl-xL protein synthesis, some Bcl-xL expression was still induced by IL-5. It is possible that these relatively little Bcl-xL levels were enough to promote GM-CSF or IL-5 responses in the majority of the eosinophils. Another explanation for the incomplete block of cytokine-mediated inhibition of eosinophil apoptosis by Bcl-xL antisense oligodeoxynucleotides would be the involvement of other, as yet unidentified, gene products. Therefore, further definition of the genetic control of eosinophil apoptosis is required.

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Role for Bcl-xL in Delayed Eosinophil Apoptosis Mediated by Granulocyte-Macrophage Colony-Stimulating Factor and Interleukin-5

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