Crucial Role of Apoptosis in the Resolution of Experimental Autoimmune Anterior Uveitis

PURPOSE. Experimental autoimmune anterior uveitis (EAAU) serves as an animal model of human idiopathic anterior uveitis. This study was undertaken to investigate the role of apoptosis in the resolution of EAAU.

METHODS. EAAU was induced in Lewis rats by bovine melanin-associated antigen (MAA). Animals were killed at different time points during EAAU, and apoptosis of the inflammatory cells within the eye was monitored.

RESULTS. Flow cytometry, TUNEL staining, and light microscopy demonstrated that CD11b/c+ and CD4+ T cells undergo apoptosis during EAAU. Electron microscopic analysis demonstrated that the macrophages remove these apoptotic infiltrating cells from the eye by phagocytosis. Caspase-3 levels peaked during the resolution of EAAU, and the upregulation of caspase-8 and -9 preceded that of caspase-3, suggesting that both extrinsic and intrinsic pathways of apoptosis are involved. There was an inverse relationship between the expression of proapoptotic protein Bax and antiapoptotic protein Bcl-2 during EAAU. Cytochrome c was present in the cytoplasm of the infiltrating cells undergoing apoptosis.

CONCLUSIONS. These results demonstrate that extrinsic and intrinsic pathways of apoptosis are involved in the resolution of EAAU. They further suggest that apoptosis followed by phagocytosis plays a critical role in the clearance of infiltrating cells from eyes with uveitis and leads to the resolution of EAAU. (Invest Ophtalmol Vis Sci. 2007;48:5091–5100) DOI:10.1167/iovs.07-0651

Diabetic anterior uveitis (AU) is the most common form of intraocular inflammation in humans.1–2 Experimental autoimmune anterior uveitis (EAAU) is an organ-specific immune disease of the eye that serves as an animal model of idiopathic human AU.3–5 EAAU is characterized histologically by lymphocytic infiltration in the iris, ciliary body, and anterior chamber. EAAU resolves spontaneously, and after the inflammation clears, the structures of the eye remain intact. The present study was undertaken to study the role of apoptosis in the resolution of EAAU.

Apoptosis is a noninflammatory mode of cell death compared with necrosis6 because apoptotic cells are eliminated from the site of inflammation through phagocytosis.7,8 There are two major apoptotic pathways, namely the extrinsic pathway (death receptor pathway) and the intrinsic pathway (the mitochondrial pathway).9 The extrinsic pathway is initiated by the death receptor ligands, including FasL, and results in the activation of caspase-8.10 The intrinsic pathway is initiated by death signals originating inside the cells—including DNA damage, oxidative stress, and starvation—that disrupt the mitochondrial membrane potential.11 As a result of this disruption, several proapoptotic proteins, such as cytochrome c, are released in the cytoplasm, leading to the activation of caspase-9. Both pathways result in the activation of caspase-3, -6, and -7, which leads to eventual cell death.

Apoptosis plays an important role in several diseases,12 including uveitis. In the past it has been shown that infiltrating CD4+ T cells undergo apoptosis during EAAU.13 Fas and FasL have been shown to be upregulated in the eyes during the course of EAAU, suggesting that the extrinsic pathway of apoptosis may be involved in EAAU. However, the role of the intrinsic pathway of apoptosis and the clearance of apoptotic cells from the eye with EAAU has not been explored. In the present study, we investigated the involvement of intrinsic and extrinsic pathways of apoptosis in the death of the infiltrating cells and the mechanism of clearance of these apoptotic cells from eyes with EAAU.

MATERIALS AND METHODS

Animals

Pathogen-free male Lewis rats (5–6 weeks old) were obtained from Harlan Sprague-Dawley. This study was approved by the Institutional Animal Care and Use Committee of the University of Arkansas for Medical Sciences (UAMS), and all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antibodies

Anti-caspase-8 (rabbit polyclonal antibody) and anti-caspase-9 (rabbit polyclonal antibody) were purchased from Biovision (Mountain View, CA). Anti-CD4-APC, anti-CD8-PerCP, and anti-CD11b/c-APC were purchased from BD Biosciences (San Jose, CA). Anti-CD8-APC was purchased from Biologend (San Diego, CA), and anti-cleaved-caspase-3 (Asp175, SA1, rabbit monoclonal antibody [mAb]), rabbit anti-Bcl-2, rabbit anti-Bax, and anti-cytocrome c (rabbit polyclonal antibody) were from Cell Signaling Technology (Danvers, MA). Anti-COX complex IV subunit I (MSD04) was purchased from Mitosciences Inc (Eugene, OR). Monoclonal β-actin (mouse IgG1), FITC labeled goat anti-rabbit IgG, and Cy3-conjugated sheep anti-mouse IgG were purchased from Sigma-Aldrich.

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Supported in part by National Institutes of Health Grants EY13335, EY014623, and EY016205; Research to Prevent Blindness; and the Pat and Willard Walker Eye Research Center, Jones Eye Institute, University of Arkansas for Medical Sciences.

Submitted for publication June 1, 2007; revised June 29, 2007; accepted August 22, 2007.

Disclosure: P. Jha, None; B. Matta, None; V. Lyzogubov, None; R. Tytarenko, None; P.S. Bora, None; N.S. Bora, None

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Induction and Evaluation of EAAU

Melanin-associated antigen (MAA) was purified from bovine iris and ciliary body as previously described by us.3,4 Male Lewis rats were immunized with stable emulsion containing soluble MAA emulsified in CFA (Sigma-Aldrich, St. Louis, MO) using a single-dose induction protocol in the hind footpad as previously described by us.3,4 Purified pertussis toxin (Sigma-Aldrich) was used as an additional adjuvant. EAAU was graded in a masked fashion daily between days 7 and 30 after injection according to criteria previously reported.3

Sample Collection

Anesthetized animals were perfused through the heart with 200 mL sterile pyrogen-free saline. Eyes were then immediately enucleated. Intraocular tissue from each eye was prepared using a previously described method.6 The intraocular tissues, which consisted of uvea, retina, lens, aqueous humor, and vitreous, were used in total RNA and protein extraction for RT-PCR and Western blotting, respectively.

RT-PCR Analysis

Total RNA (0.1 μg) from pooled intraocular content, isolated from Lewis rats killed at different time points (three rats per time point) during EAAU, was used to detect the mRNA levels of β-actin, caspase-8, and caspase-9 by semi-quantitative RT-PCR with a synthesis kit and DNA polymerase (iScript cDNA and iTaq; Bio-Rad, Hercules, CA). Total RNA was prepared using the SV total RNA isolation kit (Promega, Madison, WI). These kits were used according to the manufacturer’s specifications. Primers for rat proteins were synthesized at Integrated DNA Technologies (Coralville, IA). Primer sequences and predicted sizes of amplified cDNA were as follows: β-actin, 5'-GGTGGAGACCTTCACAACCC-3' (forward) and 5'-GTGGGACTCTCTGCTGAAATGGCT-3' (reverse) (318 bp); caspase-8, 5'-TGGTCTCGTGTACTTTAGGACT-3' (forward) and 5'-TGCGGAGATAAGCCTGCTTC-3' (reverse) (827 bp); and caspase-9, 5'-TCCCTGGACACTAAGCTTAAT-3' (forward) and 5'-AGAGGAATGAGGCGACCTCAA-3' (reverse) (686 bp).

All reactions were normalized for β-actin expression. The negative controls consisted of the omission of RNA template or reverse transcriptase from the reaction mixture. PCR products were analyzed on a 2% agarose gel and quantitated by densitometry (Quantity One 4.2.0; Bio-Rad). These experiments were repeated three times with similar results.

Western Blot Analysis

Intraocular content, prepared as described, was pooled separately for each time point (three eyes per time point). The samples were subjected to SDS-PAGE. After SDS-PAGE on a 12% linear slab gel, separated proteins were transferred to a polyvinylidine difluoride membrane using a semidry electrophoretic transfer cell (Trans-Blot, Bio-Rad). Blots were stained at room temperature with 1:1000 dilution of one of the following antibodies: rabbit anti- rat caspase-3, rabbit anti-Bcl-2, rabbit anti-Bax, rabbit anti-caspase-8, rabbit anti-caspase-9, or anti-β-actin. After washing and incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody, blots were developed using the enhanced chemiluminescence Western blotting detection system (ECL Plus; Amersham Biosciences, Piscataway, NJ). Quantification of proteins was accomplished by analyzing the intensity of the bands (Quantity One 4.2.0; Bio-Rad). These experiments were repeated three times with similar results.

Histology

Whole rat eyes were fixed overnight at 4°C in 2.5% glutaraldehyde (Polysciences, Inc., Warrington, PA) buffered with 0.05 M sodium cacodylate, pH 7.4 (Polysciences, Inc.). After fixation the eyes were dissected, and the anterior part of the eye, including the cornea and uvea, were isolated and fixed in 1% osmium tetroxide (Polysciences, Inc.) for transmission electron microscopy (TEM). Semithin sections (1 μm) were stained with hematoxylin (Gomori Chrome Alum Hematoxylin; Fisher, Fair Lawn, NJ), which stains nuclei of cells in gray and black colors and pyknotic nuclei and apoptotic bodies in dark gray or black.10 The other set of eyes was fixed with neutral-buffered 10% formalin solution (Sigma-Aldrich) and was embedded in paraffin for hematoxylin and eosin (H&E) staining. Sections were examined under a microscope (Axiioskop; Carl Zeiss Meditec, Inc., Thornwood, NY).

TUNEL

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay (Biovision Inc., Mountain View, CA) was used to demonstrate the apoptotic cells. After deparaffinization the sections of whole eye, slides were treated with proteinase K (20 μg/mL) in 10 mM Tris-HCl (pH 8.0) for 20 minutes at 37°C, followed by washing with PBS. The slides were incubated with the TUNEL reaction mixture in a humidified chamber at 37°C for 1 hour. Negative control slides were treated identically, but the enzyme solution was omitted. Then the slides were incubated with biotin-Texas red conjugate for 30 minutes at room temperature. For detecting TUNEL macrophages, CD4+ and CD8+ cells, the sections were treated with proteinase K and incubated with FITC-conjugated rabbit anti-rat macrophage antibody (1:200; Cedarside, ON, Canada); mouse anti-rat CD8 alpha chain monoclonal antibody (1:50; Chemicon, Temecula, CA.), and mouse anti-rat CD4 monoclonal antibody (1:50; Chemicon) at 4°C overnight. After washing with PBS, the slides were incubated separately with goat anti-mouse IgG (H+L) FITC-conjugate (Invitrogen, Carlsbad, CA) for 2 hours at 25°C. This was followed by TUNEL staining, as mentioned. The sections were covered by antifade reagent with DAPI (Invitrogen) and were examined by fluorescence microscopy (Carl Zeiss Meditec).

Detection of Apoptosis Using Flow Cytometry

Lewis rats immunized with MAA were killed at different time points (three rats per time point) after immunization. The eyes (six eyes per time point) were harvested. Each eye was washed in PBS separately, fragmented, and incubated with 0.6 U/mL dispase (Gibco) in PBS (Ca2+ and Mg2+ free) at 37°C for 15 minutes. Cells were mixed and filtered through a 100-μm nylon cell strainer (BD Falcon; BD Biosciences). The suspensions were centrifuged, and the pellet was resuspended in staining buffer (BD Biosciences) (10,000 events/sample) was performed on FACScan (BD Biosciences), according to manufacturer’s instructions. Flow cytometric analysis (10,000 events/sample) was performed on FACSscan (BD Biosciences), and data were analyzed in cytometry software (Win MDI 2.8; Windows Multiple Document Interface for Flow Cytometry). Similar results were obtained from three separate experiments.

Immunofluorescence Staining

We looked for cytochrome c release in the cytoplasm by performing double-immunofluorescence staining for cytochrome c and COX (Complex IV) subunit I (marker for mitochondria). Paraffin sections were deparaffinized, rehydrated, and incubated in antigen retrieval buffer (100 mM Tris, 5% [wt/vol] urea, pH 9.5) at 95°C for 20 minutes. The slides were washed with PBS and permeabilized by 0.1% Triton X-100 in PBS. After blocking the slides with 10% goat serum, the following reactions were carried out: overnight at 4°C with polyclonal rabbit anti-cytochrome c antibody (Cell Signaling Technology, Danvers, MA) at a dilution of 1:200; 1 hour with AF488 conjugated goat anti-rabbit antibody (Invitrogen) at a dilution of 1:400; 2 hours at room temperature with mouse anti–COX (Complex IV) subunit I (Mitosciences Inc., Eugene, OR) at a dilution of 1:200; 1 hour at room temperature with...
biotin-conjugated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA) at dilution of 1:400; and 1 hour at room temperature with Texas red–labeled streptavidin (Vector Laboratories) at a dilution of 1:400. In separate sets of experiments, the slides were incubated with anti–caspase-8 (rabbit polyclonal antibody), anti–caspase-9 (rabbit polyclonal antibody), or anti–cleaved-caspase-3 (Asp175, 5A1, rabbit mAb) overnight at 4°C (at dilution of 1:100). After staining with the secondary antibody, the sections were covered with medium with DAPI (ProLong Gold Mounting Medium; Invitrogen). Excitation wavelengths for AF488, Texas red, and DAPI fluorescence were 495 nm, 510 nm, and 360 nm, respectively. The slides were examined by fluorescence microscopy (Carl Zeiss Meditec).

Statistical Analysis
The data are expressed as the mean ± SD. Data were analyzed and compared using Student’s t-test, and differences were considered statistically significant with *P < 0.05.*

RESULTS
EAAU was arbitrarily divided into four clinical stages: onset (day 14, clinical score 1–2); peak (day 19, clinical score 3–4); during resolution (day 23, clinical score 1–2), and after resolution (day 30, clinical score 0). The following studies were performed to explore the role of apoptosis in the resolution of EAAU.

Identification and Clearance of Apoptotic Cells during EAAU

Flow Cytometric Analysis. Eyes (six eyes per time point) were harvested at the specified time points, and flow cytometry was performed to determine the progression of apoptosis and to identify the types of infiltrating leukocytes undergoing apoptosis within the eyes. The proportion of apoptotic cells was calculated as the percentage of total cells analyzed for every sample. Data were expressed as mean ± SD. Representative flow cytometry density plots of cells harvested from rat eyes at different time points during EAAU are shown in Figure 1. Of the total number of cells, approximately 23% of the cells (23.4% ± 2.2%) underwent apoptosis in the eyes of naive Lewis rats (Fig. 1A). The percentage of apoptotic cells increased (42.2% ± 4.4%) in the eyes at day 14 (onset of EAAU) after immunization (Fig. 1A). At the peak of EAAU (day 19), the percentage of apoptotic cells within the eyes of MAA-immunized rats further increased (52.4% ± 1.5%). This was followed by a decrease on day 23 (38.4% ± 3.9%). The lower percentage of apoptotic cells at day 23 may be attributed to the decrease in the number of inflammatory cells in the anterior chamber at this time point. The percentage of apoptotic cells further declined (25% ± 1%) on day 30 (resolution of EAAU) to a level which similar to

![Flow cytometric analysis of apoptosis during EAAU.](image-url)
that found in naive animals. Most of the apoptotic cells were at the early stage at day 14 (31% as gated R2 in Fig. 1A) and day 19 (37% as gated R2 in Fig. 1A), whereas most of the cells were late apoptotic at day 23 (33% gated as R3 and R4, as shown in Fig. 1A).

Next we investigated the kinetics of apoptosis in different subsets of infiltrating cells. Few apoptotic CD4-h T cells (8.75% ± 1.6%) were observed on day 14 (Fig. 1B). Our results indicated a significant relative increase in the percentage of apoptotic CD4-h T cells in the eye of rats with EAAU at day 19 (20.6% ± 3.5%; P < 0.05 compared with day 14), which was then reduced to 5.3% ± 1% by day 23. Few apoptotic CD4-h cells, similar to naive (0.55% ± 0.2%), could be detected on day 30 (0.45% ± 0.2%). Interestingly, low numbers of CD8-h T cells were detected at days 14, 19, and 25 during EAAU, and these cells were never annexin V-positive, indicating that they did not undergo apoptosis. Next we investigated the apoptosis of CD11b/c-h cells. Few apoptotic CD11b/c-h cells (1.8% ± 0.2%) could be detected in the naive eyes (Fig. 1D). However, compared with naive rats, the numbers of apoptotic CD11b/c-h cells (15.8% ± 3.2%) increased significantly (P < 0.05) at day 14. The number of apoptotic CD11b/c-h cells further increased (P < 0.05 compared with naive) on day 19 (21.75% ± 2.8%) and then decreased on day 23 (11.1% ± 2.7%). The proportion of apoptotic CD11b/c-h cells returned to basal levels on day 30 (similar to naive eyes). These results indicated that the apoptosis of the infiltrating CD4-h T cells and CD11b/c-h cells actively took place during the course of EAAU.

**Histologic Analysis and TUNEL Staining.** Progression of inflammation during EAAU was monitored by histologic analysis using H&E staining of paraffin-sectioned eyes of Lewis rats obtained at different times during EAAU. No cellular infiltration in the anterior chamber (AC), iris (I), or ciliary body (CB) could be observed in the eyes of naive rats (Fig. 2A). Few inflammatory cells could be detected in the ciliary body (Fig. 2B, arrows) on day 14. Heavy infiltration in the anterior chamber, ciliary body, and iris was observed on day 19 (Fig. 2C), and the number of infiltrating cells declined on day 23 (Fig. 2D). EAAU was completely resolved, and all infiltrating cells were cleared from the anterior chamber on day 30 (Fig. 2E).

Analysis of H&E-stained sections of rat eyes with EAAU at a higher magnification revealed the presence of apoptotic bodies (Fig. 3A, arrow) and large phagocyte-like cells (Fig. 3A, φ) on days 14, 19 (data not shown), and 23 (Fig. 3A) in the anterior segment. The presence of apoptotic cells within the anterior chamber of the eye during EAAU was further confirmed using TUNEL staining (Figs. 3B, 3D, 3E, 3G, 3H). Strong fluorescence (Fig. 3D) could be detected in the positive control slides (provided by Biovision Inc.). Few TUNEL-h cells could be detected on day 14 (onset of EAAU; Fig. 3E). However, the proportion of apoptotic cells increased on day 19 (peak of EAAU; Fig. 3G) and day 23 (during the resolution of EAAU; Fig. 3H). No TUNEL-h cells could be detected after the resolution of EAAU on day 30 (data not shown). No fluorescence was observed in the negative control (without primary antibody; Fig. 3B).

To confirm the results obtained by flow cytometry, paraffin sections obtained from the animals with EAAU were double stained for TUNEL and for CD4-h and CD8-h cells (Figs. 3C, 3F, 3D). Few CD4-h cells were observed on day 14 (data not shown). Similar to the results of flow cytometric analysis, the numbers of TUNEL-h cells (red fluorescence) and CD4-h cells (green fluorescence) were very high on day 19 (data not shown) and day 23 (Fig. 3F). Few CD8-h cells could be detected on days 14, 19 (data not shown), and 23 (Fig. 3D). Similar to the results obtained from the flow cytometric analysis, no CD8-h cells were TUNEL-h, indicating that the CD8-h cells did not undergo apoptosis (Fig. 3I). No fluorescence was observed in the negative control (without primary antibody; Fig. 3C).

**Morphologic Analysis of Apoptotic Cells.** Morphologic analysis using light microscopy was performed by staining of the semithin sections of eyes (harvested at day 23) with hematoxylin (Gomori Chrome Alum Hematoxylin; Fisher). Viable or nonapoptotic cells with well-preserved cytoplasm and nucleus were now present in the anterior segment (Fig. 4A, arrows). Some cells in the anterior segment displayed condensed chromatin, fragmented or nonfragmented nuclei, and preserved cytoplasm organelles. These cells were considered to be in early stages of apoptosis (Fig. 4A, asterisks). Several cells displayed vacuolations and nuclear translocation to the cellular periphery because of extreme cytoplasmic vacuolation (Fig. 4B, φ). Macrophage-like phagocytic cells were also detected in the anterior segment (Figs. 4A, 4B, φ). In these cells, several ingested apoptotic bodies (seen as dark, condensed chromatin material; Fig. 4B, arrows) could be observed, indicating that phagocytosis of apoptotic bodies is one of the mechanisms for removing cells from the anterior segment of the eye. In some apoptotic cells, plasma membranes were in close apposition to those of an adjacent macrophages (Figs. 4A, 4B, μ). Phagocytic macrophages (Figs. 4C, φ) were observed interacting with apoptotic lymphocytes (Fig. 3C, asterisk) by formation of pseudopodia (Fig. 4C, arrows).

These results were further confirmed by TEM analysis (Fig. 4). During the resolution of EAAU (day 23), several cells with partially condensed nuclei and intact cellular membranes were detected (Fig. 4D, arrows). Apoptotic cells were surrounded by young (Fig. 4D, Y φ) and mature (Fig. 4D, M φ) macrophages, indicating that they were ready to be engulfed by these macrophages. Mature macrophages could be distinguished from young macrophages by the presence of vacuoles and apoptotic bodies. Most of the cells in the advanced stages of apoptosis (Fig. 4E, asterisk) were ingested by macrophages (Fig. 4E, φ). Apoptotic cells inside the macrophage formed a tight-fitting phagosome (Figs. 4E–I). A double-membrane structure formed by the phagosomal and the apoptotic body membrane was observed after the engulfment of apoptotic cells by macrophages (Fig. 4G).

Several macrophages were also identified that exhibited signs of engulfment of cell material and apoptotic bodies in advanced stages of apoptosis, including secondary necrosis in the phagosomes (Figs. 4F, 4H, 4I). Apoptotic bodies inside phagosomes underwent secondary degeneration and dissolution of internal and external membranes. They could be distinguished from necrotic cells because they contained recognizable remains of condensed nuclei (Figs. 4F, 4I). No secondary necrosis of apoptotic cells was observed outside the macrophages. The presence of macrophages in the anterior segment was confirmed by immunofluorescence staining using antibody specific for macrophages (Figs. 4J–L).

Taken together our results clearly demonstrated that apoptotic infiltrating cells were engulfed by macrophages and thus were cleared from the anterior chamber during the resolution of EAAU.

**Mechanism of Apoptosis during EAAU**

The molecular mechanism of inflammatory cell apoptosis during EAAU was investigated by studying the involvement of caspase-dependent pathways of apoptosis.

**Time-Dependent Activation of Caspase-3 during EAAU.** Given that caspase-3-dependent apoptosis has been shown to play a role in various models of autoimmunity and that caspase-3 is the major effector molecule of caspase-dependent apoptosis,14 we first studied the levels of activated caspase-3 (caspase-3) in the anterior chamber of the eye during EAAU.
caspase-3 within the eye during EAAU by semiquantitative Western blot analysis. Protein bands corresponding to cleaved fragment of active caspase-3 (17 kDa) were identified in normal eyes and in the eyes of MAA-injected animals killed at different time points (Fig. 5A). Densitometric analysis of the protein bands demonstrated that the levels of active caspase-3 protein increased dramatically on days 14 and 19, peaked on day 23, and returned to basal levels on day 30 (Figs. 5A, 5B).

Paraffin sections of rat eyes with EAAU were stained for cleaved caspase-3 using immunofluorescence to further confirm caspase-3 activation during EAAU (Figs. 5C–E). Few caspase-3 fluorescent cells were detected in the anterior segment at day 14 (data not shown). In contrast, strong fluorescence indicating the activation of caspase-3 could be detected within the infiltrating cells in the anterior segment of the eye on day 19 (Fig. 5D). The number of fluorescent cells in the anterior segment increased on day 23 (Fig. 5E). No fluorescence could be detected on day 30 (data not shown) or in the negative control (without primary antibody; Fig. 5C). These results suggested that caspase-dependent pathways of apoptosis are involved in the resolution of EAAU.

Caspase-8 and Caspase-9 Expression and Activation during EAAU.

We next studied the expression and activation of caspase-8 and -9 during EAAU to explore the involvement of extrinsic and intrinsic pathways of apoptosis in the resolution of EAAU. The expression of caspase-8 and -9 mRNA was studied using semiquantitative RT-PCR analysis of total RNA isolated from the eyes of MAA-immunized animals killed at different time points. Low constitutive levels of caspase-8 (827 bp) and caspase-9 (686 bp) tran
scripts were detected in naive animals (Fig. 6A). In MAA-sensitized animals, a moderate increase in caspase-8 mRNA was observed at day 14 after immunization compared with naive animals (Figs. 6A, 6B). Caspase-8 transcripts further increased at the peak (day 19) of EAAU and remained elevated during the resolution (day 23) of the disease (Figs. 6A, 6B). Caspase-9 mRNA also increased during EAAU.

**FIGURE 4.** Morphologic analysis of apoptotic cells. (A-C) Photomicrographs from light microscopy illustrating different stages of apoptosis and phagocytosis of apoptotic cells in the anterior chamber (AC) and iris (I) during the resolution of EAAU. (A) Apoptotic lymphocyte (*) showing nuclear condensation and a macrophage (φ) containing apoptotic bodies (→) in the cytoplasm are closely associated. (B) In areas of intense inflammation and infiltration, most macrophages (φ) were abundantly packed with multiple large phagosomes. Many phagocytes packed with apoptotic bodies (arrows) can be seen near blood vessel (V) in the iris. (C) A phagocyte (φ) engulfing a lymphocyte (*) with a pseudopodia-like formation (arrow). (D-I) Electron micrograph of rat eye with EAAU on day 23. (D) Apoptotic cell (arrow) with evidence of condensation of the cytoplasm and electron-dense nucleus and surrounded by a mature (Mφ) and a young macrophage (Yφ). (E) Apoptotic cell engulfed by a macrophage. Cytoplasm (arrow) and nucleus (N) of the macrophage (φ) engulfing the apoptotic cell (*) can be seen. (F) Apoptotic cell (arrow) with evidence of secondary necrosis inside a phagosome. An adjacent macrophage (φ) containing multiple, spherical apoptotic bodies and vacuoles can also be seen. (G) Lymphocyte at early stage of apoptosis completely engulfed inside a macrophage phagosome. Arrows: phagosomal membrane containing a complete apoptotic cell with condensed nucleus (N) and a structurally intact cytoplasm. (H) Partially degraded apoptotic body (arrow) with remnant-condensed nucleus showing characteristic half-moon structure, which lies within a phagosome. (I) Apoptotic cell showing signs of secondary necrosis (arrow) inside a phagosome. A large condensed apoptotic body (N) with compressed nucleus and cytoplasm can also be seen. (J-L) Immunofluorescence staining for macrophages in the anterior chamber of eye on day 23 of EAAU. (J) Strong green fluorescence represents macrophages. (K) Double immunostaining was performed for TUNEL* cells and macrophages 23. Red fluorescence represents TUNEL* cells, and the green fluorescence depicts macrophages. Arrows: cells that stained for TUNEL and macrophage marker. (L) Negative control (without primary antibodies). I, iris; AC, anterior chamber. Original magnification: (C) ×100; (D-F) ×5000; (G-I) ×20,000; (J-L) ×40. Scale bar: (D-F) 50 µm; (G-I) 10 µm.

**FIGURE 5.** Activation of caspase-3 during EAAU. Representative semi-quantitative Western blot (A) of cleaved caspase-3 proteins in the eye of MAA-immunized Lewis rats during various stages of EAAU. Results of densitometric analysis are expressed as the ratios of the intensity of the caspase-3 to those of β-actin protein bands (B). Data are representative of three different experiments. (C-E) Immunofluorescence staining of paraffin sections prepared from the eye. Sections were stained with mAbs against cleaved caspase-3. Strong fluorescence for activated caspase-3 was observed in the eye of rats with EAAU on days 19 (D) and 23 (E). No fluorescence was observed in negative control (C). Each micrograph is representative of three separate experiments. Original magnification, ×100.
Caspase-9 transcript levels further increased at the onset of EAAU (day 14) and remained elevated at the peak (day 19) and during the resolution (day 23) of EAAU (Figs. 6A, 6B). Interestingly, a further increase caspase-9 mRNA level was observed after the resolution of EAAU (Figs. 6A, 6B). No bands were detected in the controls without RNA (data not shown) or reverse transcriptase (data not shown).

We then investigated changes in the protein levels of cleaved caspase-8 and -9 in the eye during EAAU. Figure 6C shows the Western blot of active (cleaved) caspase-8 (26 kDa) and -9 proteins (25 kDa) at different time points during EAAU. In MAA-sensitized animals, moderate increases in cleaved caspase-8 and -9 proteins were observed on day 14 after immunization compared with naive animals (Figs. 6C, 6D). Cleaved caspase-8 and -9 drastically increased at the peak (day 19) of EAAU. Cleaved caspase-8 and -9 levels decreased during resolution (day 23) of the disease (Figs. 6C, 6D). Interestingly, protein levels of these enzymes were lower than those observed in naive rats after EAAU resolution (day 30; Figs. 6C, 6D).

The presence of cleaved caspase-8 and -9 within the infiltrating cells was further confirmed using immunofluorescence staining. Figure 6E shows the presence of cleaved caspase-8 (Ga) and green fluorescence for caspase-9 (Gb) cells in the anterior segments of rat eyes on day 19, confirming the observation of Western blot analysis. No fluorescence was detected in the negative control sections that were stained with respective secondary antibodies alone (Ge, Gf). Nuclei in all the figures (blue, DAPI). Data are representative of three separate experiments. Original magnification, ×63.
8–dependent extrinsic pathway has already been reported in EAU.17

**Bax and Bcl-2 Levels during EAAU.** Protein bands corresponding to Bax (20 kDa) were identified in normal eyes and in the eyes of MAA-injected animals killed at different times during EAAU (Fig. 7A). Densitometric analysis (Fig. 7B) showed that on day 10 after immunization, Bax levels were similar to those in naive animals, increased sharply on day 12, peaked on day 14 after immunization, and declined on days 23 and 30 after MAA injection.

To determine whether Bcl-2, a key regulator of intrinsic pathway, was also involved in apoptosis during EAAU, we determined its expression in the eyes of rats during the course of EAAU with the use of Western blot analysis. Protein bands corresponding to Bcl-2 (28 kDa) were identified in normal eyes and in the eyes of MAA-injected animals killed at different times (Fig. 7A). Densitometry analysis of protein bands showed that Bcl-2 levels decreased on days 10, 12, and 14 after immunization and that these levels were lower than those detected in naive rats at these times (Fig. 7B). Bcl-2 levels increased sharply on day 19, increased moderately on day 23, and declined slightly on day 30 (Fig. 7B).

**Cytochrome c Release in Cytoplasm.** Cytochrome c released from the mitochondrion to the cytosol triggers caspase-9 cleavage in the apoptosome. Therefore, we evaluated cytochrome c release in the cytoplasm using immunofluorescence staining of paraffin sections of the eyes of Lewis rats killed at the peak of EAAU (day 19). Sections were incubated with the antibody against cytochrome c and COX (complex IV) subunit I (used as a marker for mitochondria). Cell nuclei were stained with DAPI. Microscopic images demonstrated that cytochrome c colocalized (orange fluorescence) with mitochondria (red fluorescence) within the infiltrating cells (Figs. 7D, 7E). Cytochrome c (green fluorescence, shown by arrows) was released in the cytoplasm in some infiltrating cells at the peak of the disease, suggesting that cytochrome c release from mitochondria plays an important role in the activation of caspase-9 in EAAU. Higher magnification of the slides further revealed that some of the infiltrating cells in which cytochrome c was diffused to the cytoplasm show the typical morphology of apoptotic bodies (Fig. 7E). These cells (Fig. 7E, shown inside white squares) had condensed nuclear material localized at the periphery of the cells. Negative control slides that were stained with secondary antibodies only did not show any fluorescence (Fig. 7C).

**DISCUSSION**

EAU is an animal model that closely resembles human idiopathic anterior uveitis.3–4 In the present study, we used an EAU animal model to investigate the role of apoptosis in the clearance of infiltrating cells from the eye during the resolution of idiopathic anterior uveitis. Our results demonstrated for the first time that CD4⁺ T cells and CD11b/c⁺ cells undergo apoptosis and that these cells are cleared from the anterior chamber through phagocytosis by macrophages. Our data further demonstrated that intrinsic and extrinsic pathways of apoptosis are involved in the clearance of the infiltrating cells from eyes with EAAU.

Apoptosis plays a role in the resolution of inflammation in different animal models of uveitis.15,17–20 Yu et al.19 have shown that apoptosis of CD4⁺ T cells occurs during the resolution of EAAU and that FasL expression increases during EAAU. However, the exact mechanism of apoptosis and the clearance of apoptotic cells from the eye were not explored in this report. Our results demonstrated that apoptosis of the infiltrating cells began as early as onset (day 14) of EAAU. Our results also show that CD11b/c⁺ and CD4⁺ cells undergo apoptosis. Furthermore, at the onset of EAAU, the proportion of apoptotic CD11b/c⁺ cells undergoing apoptosis was more than apoptotic CD4⁻ T cells (P < 0.05). On the basis of these results, we hypothesize that CD11b/c⁺ cells undergoing apoptosis at early stages of EAAU may represent the antigen-presenting cells required for sustained activation of CD4⁺ T-cell population. Because these cells undergo apoptosis at a very early stage, they may no longer be available at the later stages of EAAU, which may be the reason for the lack of T-cell activation during the later stages of EAAU and may lead to the resolution of disease. We are working on this hypothesis. Interestingly, we found that CD8⁺ T cells do not undergo apoptosis throughout the course of EAAU, possibly indicating a regulatory function for these CD8⁺ T cells. Other investigators21,22 have demonstrated a regulatory role for CD8⁺ T cells in autoimmune diseases.

On histopathologic examination, we observed several apoptotic cells in the anterior segment of rat eyes with EAAU. To confirm this observation, we performed TUNEL staining. TUNEL⁺ cells were present in the anterior segment of the eyes of animals with EAAU as early as onset (day 14) of EAAU, and their numbers increased at the peak and during the resolution of disease. TEM analysis confirmed and validated the involvement of apoptosis and the removal of apoptotic cells by phagocytosis in EAAU and suggested that the apoptotic cells were ingested by macrophages. The secondary necrosis detected by TEM was confined to the phagosomes inside the macrophages; we could not detect any significant secondary necrosis of the infiltrating cells in the anterior segment. The presence of macrophages detected during TEM analysis was confirmed by immunofluorescence staining for macrophages. This is an important observation and may represent the mechanism by which the destruction of ocular tissue as a result of necrosis during intraocular inflammation is avoided. Macrophages are scavengers of cellular material and debris.23 Phagocytosis of apoptotic cells by macrophage does not activate the macrophages and has been reported to clear the apoptotic infiltrating cells during various inflammatory conditions.24–26

Activated caspases lead to cell death by two distinct caspase-dependent pathways, the extrinsic (death receptor) pathway and the intrinsic (mitochondria mediated) pathway.27 Identifying apoptotic pathways during EAAU is important because this gives insight into the exact mechanisms of cell death and identifies potential targets for therapeutic interventions. We observed that caspase-8 and -9 are expressed constitutively in the eyes of naive rats and that their expression is elevated during EAAU. We identified increased levels of cleaved fragments of caspase-8 and caspase-9 within the eye at the onset, the peak, and during the resolution of EAAU. These results clearly demonstrated that extrinsic and intrinsic pathways of apoptosis were involved in the resolution of EAAU. The activation of caspases-8 and -9 have also been implicated in other ocular inflammatory conditions, such as glaucoma28 and retinal degeneration.29

The active forms of caspase-8 and caspase-9 cleave and activate the downstream effector caspase-3.27 The activation of caspase-3 in the eyes of Lewis rats with EAAU was also investigated in this study. Our data demonstrated that caspase-3 activation occurs as early as EAAU onset. Caspase-3 activation that increased at the peak remained elevated during the resolution of EAAU. Caspase-8–mediated intrinsic pathway of apoptosis can be activated by death receptors or proapoptotic proteins such as Bax. Recently, it was reported that caspase-8 activation after the engagement of Fas or TNFR1 receptors can
activate BID, which can activate the intrinsic pathway. In the present study we did not investigate the role of BID cleavage; hence, we are unable to comment on the involvement of death receptor-mediated activation of the intrinsic pathway. Previously, we have reported that nitric oxide (NO) may play an important role in pathogenesis of EAU, as it does in the induction of the mitochondrial pathway of apoptosis. The role of Bax and Bcl-2 in apoptosis has been widely investigated in numerous animal models, including experimental glomerulonephritis and carrageenan-induced pleurisy. Our data demonstrated that Bax was upregulated at an early stage (day 12) of EAAU and that, at this stage, the Bcl-2 level was very low (lower than naive). We speculate, therefore, that this disrupts the balance of proapoptotic/antiapoptotic protein and results in the activation of intrinsic pathway of apoptosis at the onset of the disease. The upregulation of Bcl-2 to the normal level at the later stage of the disease (day 19) suggests that Bcl-2 counteracts the proapoptotic stimuli provided by Bax. This may be necessary to protect the ocular tissue from excessive apoptosis and may help inflamed ocular tissue return to the normal state, as in the naive healthy eye.

Bax translocation from cytoplasm to mitochondria is an important step for the release of cytochrome c from mitochondria to cytoplasm. We did not investigate Bax translocation in our present study; however, we have shown the translocation of cytochrome c from mitochondria to cytoplasm using immunofluorescence staining. As mentioned earlier, Bax level is increased during EAAU. Based on our findings, we can conclude that Bax is upregulated and translocated to mitochondria, leading to the release of cytochrome c in the cytoplasm. The reduced levels of Bcl-2 further facilitate the release of cytochrome c. Once cytochrome c is released in the cytoplasm, it cleaves and activates caspase-9, leading to the intrinsic pathway of apoptosis.

Overall, our results suggest that the apoptosis of infiltrating cells and their phagocytosis by macrophages is responsible for the resolution of inflammation in EAAU. Our results clearly demonstrate that extrinsic and intrinsic pathways of apoptosis are involved in the resolution of EAAU. This may represent an important mechanism for the protection of ocular tissue during intraocular inflammation.

References


8. Jha P, Sohn JH, Xu Q, Wang Y, Kaplan HJ, Bora PS, Bora NS. Suppression of complement regulatory proteins (CRPs) exacer-


