Kinetics of Apoptotic Cells in Experimental Autoimmune Uveoretinitis

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PURPOSE. To investigate the role of apoptosis in immunopathogenic mechanisms of experimental autoimmune uveoretinitis (EAU), the kinetics of apoptotic cells and expression of Fas and Fas ligand (FasL) in the eye with EAU were studied.

METHODS. Male inbred Lewis rats were immunized with S-antigen (40 µg/rat), and eyes were examined to detect apoptotic cells on days 1, 4, 8, and 10 post-immunization and days 0, 2, 4, 6, and 8 after the onset of EAU. The clinical and pathologic scores were used for estimating EAU. Apoptotic cells were analyzed by TdT-mediated dUTP nick-end labeling, electron microscopic and immunohistologic examinations, and agarose gel electrophoresis. The anti-rat Fas and anti-rat FasL antibodies were used to examine the expression of Fas and FasL.

RESULTS. Apoptotic cells were detected in the infiltrating cells in the aqueous humor, the vitreous body, the iris-ciliary body, and the retina. Apoptotic cells were observed as early as the day of EAU onset and reached a peak on day 2 after the disease onset. Fas and FasL were expressed on the infiltrating cells in the aqueous humor and the vitreous. FasL was expressed on resident cells of the ciliary body. The kinetics of the expression of FasL corresponded with the kinetics of apoptotic cells.

CONCLUSIONS. Fas–FasL-mediated apoptosis is considered to occur in the eye with EAU and plays a role in the immunopathogenic mechanisms to eliminate ocular infiltrating cells, thereby down-regulating the inflammatory processes. (Invest Ophthalmol Vis Sci. 2000;41:799–804)

EXPERIMENTAL AUTOIMMUNE UVEORETINITIS (EAU) is an organ-specific autoimmune disease of the eye induced by immunization with retinal-specific antigens. Although the precise immunopathogenic mechanisms of EAU are still controversial, the disease is considered to be mediated by T lymphocytes. CD4-positive T lymphocytes sensitized to retinal antigens are capable of transferring EAU in naive recipient, whereas CD8-positive T lymphocytes suppress the disease. CD4-positive lymphocytes are divided into two distinct types: type 1 T helper (Th1) cells and type 2 T helper (Th2) cells. Th1 cells produce interferon-γ and interleukin (IL)-2, whereas Th2 cells produce IL-4 and IL-10. Recent studies have shown that Th1 responses are responsible for the onset of EAU in rats, whereas Th2 responses downregulate the disease. However, it has not yet been clarified how ocular infiltrating cells are eliminated from the eye.

Apoptosis is a mechanism that causes programmed cell death. Although apoptosis can be induced by a variety of signals, Fas–Fas ligand (FasL) are the most important signals to cause apoptosis. Interaction between Fas-positive cells and FasL molecules causes signal transduction in the Fas-positive cells, resulting in apoptosis of the cells. Fas–FasL interaction and apoptosis may occur in eyes with EAU and participate in the mechanism to downregulate the disease, but no studies have been carried out to examine the apoptosis and expression of Fas and FasL in EAU. The present study was, therefore, aimed at investigating the role of apoptosis and Fas–FasL in EAU.

METHODS

Animals

Rats of the inbred Lewis strain were purchased from Seac Yoshitomi, Ltd. (Fukuoka, Japan). Male rats 8 to 12 weeks old were used in the study after being kept in laminar flow cages for 1 week before the experiments. These investigations conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunization

S-antigen was prepared from bovine retinas according to the methods described by Fujino et al. The antigen was emulsified (1:1, vol:vol) in complete Freund’s adjuvant (Difco, Detroit, MI), containing Mycobacterium tuberculosis H37 RA (Difco) at a concentration of 2.0 mg/ml. A total volume of 100 µl per rat, containing 40 µg S-antigen, was injected into one hind footpad.

Evaluation of EAU

Eyes of S-antigen–immunized rats were clinically examined with a slit-lamp microscope on days 1, 4, 8, and 10 post-immunization, and on days 0, 2, 4, 6, and 8 after the onset of
EAU. The onset of EAU was confirmed when fibrins were detected in the anterior chamber. The clinical score was graded into four categories from 0 (no inflammation) to 3+ (severe inflammation with hypopyon). Eyes were enucleated under general anesthesia with diethylether on the days described above, fixed with 2.5% glutaraldehyde 4% formaldehyde solution, and embedded in paraffin. Sections cut at 4 \( \mu m \) were stained with hematoxylin and eosin and examined with a light microscope. The EAU scores by histologic examinations were graded from 0 (no inflammation) to 4+ (full thickness retinal damage in at least 1/4 of the retina).

Terminal Deoxynucleotide Transferase–Mediated dUTP Nick End Labeling Methods

In situ detection of apoptotic cells was conducted by terminal deoxynucleotide transferase (TdT)–mediated dUTP nick end labeling (TUNEL) methods using a commercially available kit (Apop Tag In Situ Apoptosis Detection Kit; Oncor, Gaithersburg, MD), according to the manufacturer’s protocol. Briefly, eyes of S-antigen–immunized rats were fixed with a 2.5% glutaraldehyde 4% formaldehyde solution and embedded in paraffin. Sections cut at 4 \( \mu m \) were deparaffinized and stained with hematoxylin and eosin and examined with a light microscope. The EAU scores by histologic examinations were graded from 0 (no inflammation) to 4+ (full thickness retinal damage in at least 1/4 of the retina).

Electron Microscopic Examinations

A small aliquot of aqueous humor was centrifuged at 1500 rpm (380g) for 5 minutes, and the pellets were fixed with 2.5% glutaraldehyde for 1 hour at room temperature. The pellets were washed by cacodylate buffer to remove the excess of glutaraldehyde and postfixed with 1% OsO4 for 1 hour at 4°C. The pellets were washed by cacodylate buffer again, embedded in stable gels, centrifuged at 380g for 5 minutes, diced into 2-mm cubes, and dehydrated by ethanol (50%, 70%, 90%, and 100%). The cubes were immersed in 100% ethanol with epoxy resin (1:1, vol:vol) for 12 hours at room temperature. They were then embedded in 100% epoxy resin for 6 hours at room temperature and for 30 hours at 60°C. The cubes were cut in thin sections (80 nm), placed on grids to be stained with uranyl acetate and lead citrate, and studied by transmission electron microscope.

DNA Gel Electrophoresis

The fragmentation of the nucleus of infiltrating cells in the aqueous humor and the vitreous was determined by DNA gel electrophoresis using a Apoptosis Ladder Detection Kit (Wako, Osaka, Japan). A small aliquot of aqueous humor was collected from eyes of S-antigen–immunized rats using a 30-gauge needle. A total of \( 1 \times 10^6 \) cells was used for DNA gel electrophoresis according to the manufacturer’s protocol. Briefly, the cells were collected in a microcentrifuge tube by a brief centrifugation, discarding the supernatant. Enzyme reaction solution, RNase solution, enzyme activator solution, and protein digestion enzyme solution were added to the tube and incubated at 50°C for 30 minutes. DNA extraction solution was added to the tube and mixed well. Isopropanol was added and left at room temperature for 15 minutes. The sample was centrifuged at 10,000g at room temperature for 10 minutes and decanted. Seventy percent ethanol was added and centrifuged at 10,000g at room temperature for 5 minutes and decanted. DNA samples were

Figure 1. Photomicrographs showing apoptotic cells in the EAU eyes on day 2 after EAU onset: aqueous humor (A), ciliary body (B), vitreous (C), and retina (D). c, cornea; l, lens; pc, posterior chamber; ch, choroid. Apoptotic cells were stained in brown by TUNEL methods. Scale bar, 100 \( \mu m \).
were extracted. The samples were electrophoresed in a 1.5% agarose gel and visualized by SYBR green I.

**Detection of Fas-Positive Cells and FasL-Positive Cells**

The expression of Fas was examined by the streptavidin-biotin peroxidase method using rabbit anti-mouse Fas polyclonal antibodies (Wako) as the primary antibody. The expression of FasL was examined by the same technique as Fas, but rabbit anti-rat FasL polyclonal antibodies (Wako) were used as the primary antibody. Eyes were enucleated under general anesthesia with diethyl ether, fixed with 0.5% Zn acetate 0.5% ZnCl in Tris–Ca acetate buffer, and embedded in paraffin. Deparaffinized and rehydrated sections were immersed in 0.3% H2O2 in methanol to block endogenous peroxidase and were preincubated with 500 μg/ml goat IgG and 5% bovine serum albumin (BSA) in PBS for 1 hour at room temperature to block nonspecific binding of antibodies. The sections were reacted with the primary antibodies (1:200) diluted with 1% BSA in PBS overnight at 4°C. Polyoxyethylene lauryl ether (Brij35), a nonionic detergent, was used to reduce nonspecific binding of antibodies. After washing in PBS with 0.075% Brij35 three times for 15 minutes each time, the sections were incubated with biotinylated goat anti-rabbit IgG (Histofine SAB-PO [R]; Nichirei, Tokyo, Japan) for 2 hours at room temperature and washed as described above. Peroxidase-conjugated streptavidin was added, and the sections were washed again. The sites of peroxidase were visualized by DAB and counterstained by 0.5% methyl green. Ovaries and testes of naive healthy rats were used for positive control of Fas and FasL.

**RESULTS**

Clinical and histologic examinations revealed that all S-antigen-immunized rats developed EAU on day 11 post-immunization or thereafter. The clinical score of EAU reached a peak on day 2 after the EAU onset, and the histologic score reached a peak on day 6 (data not shown).

Immunohistologic examination by TUNEL performed on day 2 after EAU onset demonstrated that TUNEL-positive cells were present in the aqueous humor, ciliary body, vitreous and retina and that the number of TUNEL-positive cells were higher in the aqueous humor and the vitreous than in the ciliary body and the retina (Fig. 1). Electron microscopic examinations and
DNA gel electrophoresis were performed to confirm that the TUNEL-positive cells were apoptotic cells. Electron microscopic examinations revealed that cells with highly condensed and aggregated chromatin in the nuclei were present in the aqueous humor (Fig. 2A). The DNA ladder formation was clearly detected on day 2 after EAU onset using cells in the aqueous humor (Fig. 2B). After confirming that the TUNEL-positive cells were apoptotic cells, kinetics of apoptotic cells were examined by TUNEL methods. No apoptotic cells were detected before the onset of EAU. Apoptotic cells were detected on the day of EAU onset and reached a peak on day 2 after EAU onset. The proportions of apoptotic cells in the infiltrating cells on day 2 after EAU onset were 12.4% in the aqueous humor and 10.0% in the vitreous. Examples of kinetics of apoptotic cells in the aqueous humor and the vitreous are shown in Figure 2C.

To examine whether Fas–FasL-mediated apoptosis is involved in EAU, the expression of Fas and FasL in the aqueous humor, vitreous, and ciliary body was investigated by immunohistologic examinations. Expression of FasL was detected on cells in the aqueous humor, the vitreous, and the ciliary body (Fig. 3A), whereas expression of Fas was detected on cells in the aqueous humor and the vitreous but not in the ciliary body (Fig. 3B). The kinetics of FasL expression in the ciliary body revealed that the expression was detected only after EAU onset and was most remarkable on day 2 after EAU onset (Fig. 4).

DISCUSSION

The data recorded here demonstrated for the first time that apoptotic cells were present in the eye with EAU and that Fas and FasL were expressed in the eye with EAU. The apoptotic cells in EAU eyes were confirmed by three different methods (i.e., TUNEL methods, electron microscopic examinations, and DNA gel electrophoresis). The apoptotic cells were detected in the aqueous humor, vitreous, ciliary body, and retina. A kinetic study after S-antigen immunization showed that the apoptotic cells were found only after EAU onset and that they were most remarkable on day 2 after the disease onset. The kinetics of apoptotic cells in the eye with EAU were in accord with the disease activity. Apoptosis is considered to be a mechanism that causes programmed cell death and eliminates activated lymphocytes and malignant cells. Therefore, it is suggested that apoptosis might play a role in the mechanisms that regulate EAU by eliminating infiltrating cells in the eye. However, the maximum proportion of apoptotic cells in the ocular infiltrating cells was 12.4% in the aqueous humor and 10.0% in the vitreous at the peak time of clinical EAU. According to a time-course study of apoptosis, it occurs in a few hours after apoptosis-inducing signals and completes in 24 hours. The apoptotic cells will be eliminated by inflammatory cells soon after the occurrence of apoptosis. It is, therefore, assumed that the percentage of apoptotic cells in the ocular infiltrating cells described above would be underestimated because many of infiltrating cells that had undergone apoptosis and been eliminated from the eye were not detected by the present assay methods. This suggests that a part, but not all, of infiltrating cells in EAU eyes are eliminated by apoptosis. In fact, ocular tissue damages demonstrated by histologic examination still progressed after the peak time of apoptosis. Therefore, apoptosis occurs in the eye with EAU, but its contribution in the mechanisms to downregulate EAU might be limited.

Apoptosis can be mediated by a variety of signals. One of the most important signals to cause apoptosis is Fas–FasL interaction. Current dogma is that ocular resident cells express FasL and that infiltrating T cells expressing Fas interact with FasL and then go into apoptosis. To investigate whether the Fas–FasL-mediated apoptosis takes place in EAU, the expression of Fas and FasL was studied in the eye with EAU by immunohistopathologic examinations. Fas expression was abundantly detected on infiltrating cells in the eye with EAU. Although in the present study FasL expression was hardly detected on ocular resident cells of naive rats, previous studies demonstrate significant expression of FasL on naive ocular resident cells, such as corneal endothelium. Therefore, it is considered that FasL is expressed on naive ocular resident cells, although the intensity of its expression might be weak. In contrast to naive ocular resident cells, ocular infiltrating cells abundantly expressed FasL in the present study. The previous studies together with the present study indicate two possible mech-
anisms by which T cells in the eye with EAU go into apo-
poptosis: T cells go into apoptosis due to Fas–FasL interaction
with other T cells and FasL expression on resident ocular
cells is upregulated during inflammation followed by inter-
action with Fas-expressing T cells.

In conclusion, the present study suggests that Fas–FasL–
mediated apoptosis occurs in the eye with EAU and that it plays
a role in the immunopathogenic mechanisms to eliminate
ocular infiltrating cells and downregulate the inflammation
of EAU.

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