Visualization of Cell Death In Vivo during Murine Endotoxin-Induced Uveitis

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PURPOSE. To develop a technology to image cell death in the eye of a live mouse and to apply that technology to characterize the role of apoptosis and necrosis in the evolution of endotoxin-induced uveitis (EIU), a standard model of intraocular inflammation.

METHODS. To induce EIU, 250 ng Escherichia coli 055:B5 lipopolysaccharide was injected into the vitreous body of BALB/c mice. At 0, 6, 10, 16, 20, 24, 48, 72, and 96 hours and on day 7 after endotoxin injection, annexin V and propidium iodide were injected into the anterior chamber of these mice, and labeled cells were observed by using intravital epifluorescence video microscopy. Iris and corneal whole mounts isolated from labeled cells were observed by using intravital epifluorescence microscopy. TUNEL staining was performed on iris whole mounts taken from additional mice similarly injected with endotoxin, to confirm the in vivo results.

RESULTS. Uveitis was induced in all the mice that received an endotoxin injection. The percentages of annexin V–propidium–, annexin V–propidium–, and annexin V–propidium– cells in the iris tissues visible by intravital microscopy were comparable to those observed by TUNEL staining in vitro. In addition, intravital microscopy allowed observation of labeled cells in the aqueous humor and on the surface of the lens. Both the number and the pattern of labeled cells changed dramatically over time. The cells stained with annexin V had a variety of morphologies, including small and round, round with a lobulated or a kidney-shaped nucleus, dendriform, and irregular.

CONCLUSIONS. A technique was developed to image cell death in the anterior segment of the eye in vivo and used to demonstrate that the number and proportion of early apoptotic (annexin V–propidium–) and late apoptotic or necrotic (annexin V–propidium– and annexin V–propidium–) cells change over the course of EIU. A variety of inflammatory cells and resident cells undergo apoptosis, or possibly necrosis, which may contribute to the rapid resolution of EIU. This in vivo technique will be a valuable tool for future studies on the resolution of ocular inflammation. (Invest Ophthalmol Vis Sci. 2003;44:1993–1997) DOI:10.1167/iovs.02-05822

Uveitis is one of the leading causes of blindness in the world.1,2 A variety of animal models, including experimental autoimmune uveoretinitis (EAU)3,4 and endotoxin-induced uveitis (EIU)5,6 have been used for studying the pathogenesis of uveitis. There is, however, a substantial difference in the clinical course of human uveitis versus these animal models, despite the similarities in their clinical manifestations and pathologic changes. Human uveitis commonly presents as recurrent episodes or a chronic inflammation. In contrast, the intraocular inflammation induced with different challenges in animals typically manifests as an acute and self-limited course, although a recurrent uveitis has been induced in mice with endotoxin7–9 or in rats with bovine ocular melanin.10,11 This difference raises the possibility that different mechanisms may be involved in the ultimate resolution of human uveitis and its counterpart in the animal models. Nonetheless, episodic and transient acute uveitis must both involve a mechanism for reduction of inflammation.

Recent studies on apoptosis have opened a new avenue for investigating the mechanisms by which inflammatory diseases subside. Apoptosis, also known as programmed cell death, is involved physiologically in the control of development.12 It is considered to be an important mechanism for deletion of unwanted cells from the body, thereby maintaining the stability of microenvironments within the body.13–15 Apoptosis plays a major role in maintaining immune privilege within the eye.16 The role of apoptosis in uveitis has been studied in patients16–19 and in animal models of infection induced with retinal antigens (EAU)20–23 or bovine ocular melanin.24–26 These studies provided evidence that apoptosis is involved in the resolution of intraocular inflammation. In EIU, a model that may be especially relevant to human uveitis associated with ankylosing spondylitis or Crohn’s disease,4,27 a recent study revealed the presence of apoptosis of the infiltrating cells.25 EIU is distinctly different from EAU, in that it begins soon (within 4 to 6 hours) after challenge and is characterized by massive influx of neutrophils and monocytes into the anterior uvea and aqueous humor. This study, conducted in the rat, suggests that infiltrating mononuclear cells are highly susceptible to apoptosis.25 The reported relative resistance of neutrophils to apoptosis, and the rapid regression of EIU raise the question as to the presence of other mechanisms, in addition to apoptosis, involved in the resolution of this inflammation.

A variety of techniques have been developed to investigate the process of cellular apoptosis in different contexts.25,16,23,25 Most of these studies have been performed in vitro with tissue cross sections or whole mounts. Indeed, these studies have provided abundant data for understanding the process of apoptosis physiologically and pathologically. Visualization of the...
apoptotic cells in vivo should provide additional information about this process including its kinetics. Recently, Dumont et al.28 and Narula et al.29 have developed an in vivo technique using annexin V labeling to image apoptotic cells in the living heart. Annexin V is able to bind to phosphatidylserine, a membrane phospholipid translocated from the inner to the outer leaflet of the cell membrane when apoptosis is initiated. In contrast, propidium iodide is a dye that labels necrotic or late apoptotic cells with membranes that have increased permeability.30,31 Cells labeled with fluorescently tagged annexin V or propidium iodide can be visualized with a fluorescence microscope. Our research group and others have successfully established a method to image leukocyte migration in vivo in the BALB/c iris during EIU.32,33 Based on these studies, we have extended the technique to visualize apoptotic cells in the anterior segment of the eye after endotoxin injection by labeling with fluorescent annexin V and propidium iodide.

MATERIALS AND METHODS

Animals

Female BALB/c mice, 6 to 8 weeks of age and 20 to 26 g body weight, purchased from Jackson Laboratories (Bar Harbor, ME), were used in this study. The mice were fed standard laboratory chow and sterile water ad libitum. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Induction of Uveitis

The mice were anesthetized by inhalation of 1.7% isofluorane in oxygen. Injection of 2 μL saline containing 250 ng Escherichia coli 055:B5 lipopolysaccharide (List Biological Laboratories, Campbell, CA) posterior to the limbus and into the vitreous body induced uveitis, as established a method to image leukocyte migration in vivo in the BALB/c iris during EIU.32,33 Based on these studies, we have extended the technique to visualize apoptotic cells in the anterior segment of the eye after endotoxin injection by labeling with fluorescent annexin V and propidium iodide.

Visualization of Cell Death In Vivo with Intravital Fluorescence Microscopy

Equal volumes of annexin V (Alexafluor 488 conjugate, catalog no.A-13201; Molecular Probes, Eugene, OR) and propidium iodide (0.2 mg/mL, Molecular Probes) were mixed, and 2 μL of the mixture was injected into the anterior chamber of the mice 30 minutes before visualization by intravital microscopy, as described previously.24 Imaging of labeled cells was performed at 0, 6, 10, 16, 20, 24, 48, 72, and 96 hours and on day 7 after endotoxin injection. The microscopic images were captured by a color video camera (Kappa CF11 DSP) and recorded at 30 frames per second. Single and double-labeled cells were counted in the iris, and the percentages of cells labeled with annexin V and/or propidium iodide were calculated at different time points.

Observation of the Labeled Cells in the Iris and Corneal Wholesmounts

The mice were killed immediately after intravital microscopic imaging. The eyes were fixed in 4% paraformaldehyde at 4°C overnight and then divided behind the ciliary body into anterior and posterior parts. The iris was gently separated from the underlying sclera and limbus with a knife.24 The cornea was cut radially to flatten it. The iris and cornea wholesmounts were placed on slides, embedded in antifade mounting medium (Slow Fade; Molecular Probes) and subsequently covered. These tissue wholesmounts were viewed with standard epifluorescence microscopy. Percentages of single and double-labeled cells in the iris were determined. Confocal fluorescence microscopy with a confocal laser scanning microscope (J900; Leica, Deerfield, IL) and analysis system was used to analyze the details of staining and the morphologic features of the labeled cells in the iris wholesmounts.

TUNEL Staining on the Iris and Corneal Wholesmounts

Detection of apoptosis with a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit was performed according to the manufacturer’s instructions (Intergen, Purchase, NY), as described briefly. The iris and corneal wholesmounts were incubated with TdT enzyme and digoxigenin-conjugated dUTP at 37°C for 1.5 hours after sequentially treating with 95% and 70% ethanol and equilibration buffer. These tissue wholesmounts were then incubated in stop/wash buffer for 30 minutes and subsequently in working-strength rhodamine-conjugated anti-digoxigenin antibodies for 1.5 hours at room temperature. After a wash with phosphate-buffered saline, the tissue wholesmounts were embedded in antifade mounting medium and covered for observation.

Statistical Analysis

Because of the uneven distribution of the labeled cells and the difficulty in counting individual cells in the areas of clumping, we calculated the percentage of labeled cells in the iris in preference to counting the number of labeled cells per unit area. We analyzed 30 cells for the time points of 6, 10, 72, and 96 hours after endotoxin injection, and 50 cells for the time points of 16, 20, 24, and 48 hours after endotoxin injection. The Student’s t test was used to determine the statistical significance of differences in percentage of single and double-labeled cells between intravital microscopy and fluorescence microscopy at each time point; P < 0.05 was considered significant.

RESULTS

Anterior uveitis developed in all mice that received an intravitreal endotoxin injection, as evidenced by cells in the anterior chamber, keratic precipitates (KPs), iris hyperemia, miosis, posterior synechiae, a fibrinous pupillary membrane, and, occasionally, hypopyon. The inflammation was apparent by 6 hours after endotoxin injection and reached its peak at 16 to 24 hours. Generally, it subsided gradually after 24 hours and disappeared by day 6 or 7, although a slight increase in the inflammation was noted at 96 hours.

The results obtained with intravital microscopy were, by and large, in accordance with those obtained with wholemounts in vitro, except that labeled cells in the aqueous and on the surface of the lens were, of course, not observable in the dissected wholemounts. Both intravital microscopy and confocal microscopy revealed that annexin V labeled only the cell membrane, whereas propidium iodide stained the nucleus (Figs. 1A, 1B). As described later, the percentages of both single- and double-labeled cells in the iris changed during the course of EIU. No significant difference in these percentages was found when comparing the in vivo with the in vitro assay results at any time during EIU (Fig. 2). However, confocal microscopy, with its higher sensitivity and resolution, revealed more labeled cells and more morphologic details of these labeled cells.

Cells stained only with annexin V (annexin V+ propidium− cells) and those stained with both annexin V and propidium iodide (annexin V+ propidium+ cells) were occasionally observed in the normal iris but not in the aqueous, on the cornea, or on the surface of the lens. Six hours after endotoxin injection, an increased number of annexin V+ propidium− cells was observed in the iris (Fig. 1C) and on the endothelium of the cornea. Approximately 11% of these cells in the iris were double labeled (annexin V+ propidium+; Figs. 1C, 2). Most
cells adhering to the lens surface were stained with propidium iodide (annexin V<sup>+</sup> propidium<sup>+</sup> cells; Fig. 1D) although annexin V<sup>+</sup> propidium<sup>−</sup> cells and annexin V<sup>−</sup> propidium<sup>+</sup> cells were also noted.

As the EIU progressed, the number and percentage of labeled cells and the pattern of single and double-labeled cells changed rapidly and markedly (Fig. 2). Although we could not obtain precise cell counts, the number of labeled cells clearly increased for the first 24 hours after endotoxin injection and then gradually decreased. From 6 to 24 hours, the percentage of annexin V<sup>+</sup> propidium<sup>−</sup> cells decreased to approximately 10%, whereas the percentage of annexin V<sup>−</sup> propidium<sup>+</sup> cells (Figs 1E, 1F) increased to approximately 65%. During this time, annexin V<sup>−</sup> propidium<sup>+</sup> cells began to be seen in the iris (Figs. 1E, 1F, 1G), in the aqueous humor (Fig. 1H), and on the corneal endothelium (Fig. 1I). At 24 hours postendotoxin, approximately 25% of the labeled cells were propidium positive.

As EIU regressed with additional time, the percentages of annexin V<sup>−</sup> propidium<sup>−</sup> and annexin V<sup>−</sup> propidium<sup>+</sup> cells decreased, and the percentage of annexin V<sup>−</sup> propidium<sup>+</sup> cells increased. At the 96-hour time point, approximately 90% of the labeled cells were annexin V<sup>−</sup> propidium<sup>−</sup>. These cells were unevenly distributed in the iris, aqueous humor, and on the surface of the lens. They tended to accumulate in some areas, especially in the fibrous pupillary membrane (Fig. 1J). By 7 days, the inflammation had resolved and, as in normal eyes, labeled cells were seen only rarely.

To confirm that the cells labeled with annexin V and propidium iodide were apoptotic, we also performed TUNEL staining on iris wholemounts at the 24-hour time point. The pattern of TUNEL<sup>−</sup> cells was similar to that detected with fluorescence microscopy in iris wholemounts after staining in vivo with annexin V and propidium iodide (Figs. 1F, 1K).

The cells labeled with annexin V and/or propidium iodide had different morphologic appearances, such as small and round (Fig. 1B), round with a lobulated nucleus or a kidney-shaped nucleus (Fig. 1L), irregular (Fig. 1E) and dendriform (Figs. 1E, 1M, 1N). The labeled cells attached to the endothelium of the cornea, in the aqueous, and on the surface of the lens appeared to be round and slightly irregular (Figs. 1A, 1D, 1G). In the iris, labeled cells showed a typical dendriform appearance, in addition to the round and irregular forms (Figs. 1E, 1L, 1M, 1N).

**DISCUSSION**

Our study establishes a technique to visualize cell death in the anterior segment of the eye in living BALB/c mice. Labeling of necrotic or apoptotic cells was successfully performed with injection of fluorescently tagged annexin V and propidium}

**FIGURE 1.** Cells labeled with annexin V and/or propidium iodide in wholemounts and apoptotic cells identified with TUNEL staining in the ocular tissues or wholemounts after endotoxin injection into the vitreous body. Cell membranes were stained with annexin V and nuclei were stained with propidium iodide. (A) Intravital microscopy of the aqueous humor; (B) confocal microscopy of the iris. Annexin V<sup>−</sup> propidium<sup>−</sup> cells and annexin V<sup>−</sup> propidium<sup>+</sup> cells were noted in the iris (C, intravital microscopy) and annexin V<sup>−</sup> propidium<sup>−</sup> cells on the surface of the lens (D, intravital microscopy) at 6 hours. Different labeled cells are shown in the iris (E, F, epifluorescence microscopy; G, confocal microscopy), in the aqueous humor (H, intravital microscopy), and on the corneal endothelium (I, intravital microscopy) at 20 or 24 hours. Numerous annexin V<sup>−</sup> propidium<sup>−</sup> cells were present in a fibrous membrane (J, epifluorescence microscopy) at 96 hours. TUNEL staining in iris wholemounts (K) shows a result similar to that in epifluorescence microscopy (F). Labeled cells were identified in the iris as small round cells (B, arrow), cells with kidney-shaped nuclei (L, confocal microscopy), irregular cells (E), and dendriform cells (M, N, confocal microscopy). Magnification: (A, D–F, H, J, K) ×400; (C, I) ×200; scale bars: (B, M, N) 10 μm; (G) 50 μm; (L) 5 μm.
iodide into the anterior chamber. Cell necrosis was characterized by disruption of the plasma membrane, lytic degradation of cytoplasmic organelles, and nuclear staining with propidium iodide. In contrast, the apoptotic cells typically showed decreased volume, shrinkage and fragmentation of cytoplasm, condensed chromatin and DNA fragmentation, and apoptotic bodies.35-36 The apoptotic cells initially demonstrated membrane staining with annexin V and subsequently showed nuclear staining with propidium iodide. It is well established that late-stage apoptotic cells and necrotic cells are stained by propidium iodide.30,31 Intravital microscopy after intravitreal injection of these fluorescent labels made it possible to visualize the dead and dying inflammatory cells in the aqueous humor, an important indicator of anterior uveitis, without any manipulation of the aqueous humor and these cells. This technique also allowed us to omit a series of procedures used for in vitro experiments, such as fixation, sectioning, dissection, and sequential treatment with a number of reagents, thus providing simple and direct visualization of cell death. Furthermore, visualization of apoptotic cells in vivo can be repeated serially in the same animal.

We used this in vivo technique and a wholemount technique to study EIU and obtained comparable results with the two methods. We observed labeled cells in the iris and aqueous humor and on the corneal endothelium for up to 7 days after endotoxin injection. Changes occurred not only in the number of labeled cells, but also in their profile of staining for markers of apoptosis and necrosis. The number of labeled cells paralleled the process of the intraocular inflammation. Even at an early stage of inflammation, some infiltrating cells underwent apoptosis, indicating that these cells die soon after they migrate into the tissues. Numerous apoptotic cells labeled with annexin V and propidium were visualized at the peak of intraocular inflammation. The rapidly declining number of annexin V−propidium− cells and annexin V+propidium+ cells was closely correlated with decreased intraocular inflammation. At this time, the percentage of annexin V+propidium+ cells increased markedly. These results suggest that apoptosis of the infiltrating cells is an important mechanism involved in the regression of EIU. The previous in vitro study of apoptosis in EIU, which was conducted in the rat, also supports this conclusion.25

It is interesting to note that some of the cells attached to the endothelium of the cornea, clinically known as KPs, and those adherent to the anterior surface of the lens were propidium positive and annexin V negative during the early stage of EIU. In the iris, annexin V−propidium− cells were also observed after 20 hours and were a prominent feature at 48, 72, and 96 hours. We do not know whether the cells that stain only with propidium iodide were previously annexin V+propidium− cells. Therefore, the presence of annexin V−propidium− cells at a very early stage of EIU suggests that necrosis of infiltrating cells may also occur during this form of inflammation. These two possibilities have different implications for the pathologic course of the disease. In general, apoptotic cells cause neither disruption of tissue nor perpetuation of inflammation. In contrast, cell necrosis is always associated with damage to the surrounding tissue.30,35 The annexin V−propidium− cells present in the early stage may provide an explanation for the development of the KPs noted in EIU. It is well known that damage to the endothelium of the cornea is necessary for the formation of KPs.37 Therefore, it is possible that the alteration of the corneal endothelium that allows the inflammatory cells to deposit at this site is caused by necrosis of the infiltrating cells, as well as by other inflammatory mediators and cytokines.36 Necrosis of inflammatory cells may also account for the small second peak of intraocular inflammation that generally occurs at 72 and 96 hours after endotoxin injection in mice. The marked increase in the number and percentage of annexin V−propidium− cells was coincidentally present in this period.

In our results, different kinds of cells underwent apoptosis during EIU. Based on morphologic features, we characterize the apoptotic cells as monocytes, macrophages, neutrophils, lymphocytes, and resident dendriform cells. Our methods do not allow us to determine the proportion of apoptotic cells for any given leukocyte subpopulation. Previous work in rat EIU,25 as well as human acute anterior uveitis,17 suggests that certain cell types may be more susceptible to apoptosis than others.

In conclusion, the technique of visualizing apoptotic cells presented herein is a powerful tool for the study of cell death in the living animal. It is able to provide real-time images of the apoptotic and necrotic cells. Using this technique and the wholemount technique, we find that apoptosis and perhaps necrosis of infiltrating inflammatory cells and resident cells occurs in EIU and there is a dramatic change over time with regard to number and the profile of labeling. Our results suggest that apoptosis is an important mechanism to account for the rapid resolution of EIU. Intravital technology will be useful to study the role of apoptosis in intraocular inflammation with regard to issues including quantifying the role of specific proteins such as Fas/FasL or caspases in apoptosis, determining the effect of medications such as corticosteroids, identifying specific populations of dying cells, and contrasting apoptosis in different pathogenic models of anterior segment inflammation.
References