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Apotosis of infiltrating cells in experimental autoimmune uveoretinitis

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Keywords® experimental autoimmune uveoretinitis ® immunohistochemistry ® apoptosis ® wholemounts technique

Objective To investigate the cellular phenotype and apoptosis of infiltrating cells involved in experimental autoimmune uveoretinitis® EAU®

Methods Immunohistochemical staining and in situ apoptosis staining were performed using monoclonal antibodies to monocytes and macrophages® ED1® major histocompatibility complex® MHC® class- II® antigen® OX6® lymphocytes® R73® and TACS 1© Klenow kit on both ocular sections and wholemounts from Lewis rats after immunization with interphotoreceptor retinoid-binding protein® IRBP®

Results EAU was induced in 12 of 16 Lewis rats with a mean clinical inflammation score of 1.29 ± 0.7. Influx of monocytes® lymphocytes and MHC class II®-positive cells into the uvea and retina was noted after immunization with IRBP. Apoptosis of infiltrating cells was observed in the uvea and retina and more apoptotic cells were present in the iris and ciliary body compared with those in the choroid and retina.

Conclusion Apoptosis of infiltrating cells occurs at the early stage of EAU® which may greatly contribute to the rapid regression of the inflammation induced by IRBP.

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Uveitis is one of leading causes of blindness in the world.® Its pathogenesis has been an area of intensive study during the past decades. Recent studies on experimental autoimmune uveoretinitis® EAU® have contributed greatly to our understanding of how human uveitis occurs. EAU induced by retinal S-antigen® interphotoreceptor retinoid-binding protein® IRBP® or other antigens has clinical and pathological features in common with human uveitis.® However® there is a great difference between EAU and human uveitis regarding the duration of inflammation. The inflammation in animal model often resolves rapidly® in contrast® human uveitis tends to persist for a longer time. The reason that they have a different course is still not clear.

Recent studies reveal that apoptosis might be involved in autoimmune diseases such as multiple sclerosis® and Hashimoto thyroiditis.® Using TdT-mediated dUTP-biotin nick end labelling® Nakamura found that inflammatory cells in ocular tissue underwent apoptosis during EAU.® This result suggests that apoptosis in EAU might be an important factor in eliminating the inflammatory cells and in resolving the inflammation. In this study® using a wholemount technique® we demonstrated the presence of apoptosis of infiltrating cells at the early stages of EAU.

METHODS

Experimental protocol Eighteen inbred male Lewis rats® 6 to 8 weeks of age® 150 to 200 g of body weight® were used in the study. All procedures in this study complied with the ARVO Statement for the use of animals in ophthalmic and vision research. IRBP emulsified with equal volume of Freundï’s complete adjuvant was injected into each hind footpad of sixteen Lewis rats at a total dose of 50 ìg IRBP per animal. Two rats that did not receive an injection served as controls. Careful clinical examination with a slit-lamp microscope was carried out before and 10® 11® 12 and 13 days after immunization. Inflammation was evaluated according to the scoring system described previously by de Smet et al.® On day 13® all rats were perfused with cold phosphate-buffered saline® PBS® through the left ventricle to expel all blood and hematogenous substances from the capillary bed. Ocular frozen sections were prepared from 8 eyes® 8 rats® Ocular wholemounts

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were isolated and prepared from 8 eyes of the other 8 rats and were briefly described below.

Eyes obtained after perfusion were dissected into two parts—anterior and posterior behind the ciliary body. Lens and vitreous body were carefully removed from the posterior part. The retina was gently separated from the underlying choroid. The choroid–iris and ciliary body were isolated from the sclera. The retina–choroid–iris and ciliary body obtained in this manner were fixed in cold 100% ethanol for 5 minutes placed in PBS in a 24-well tissue culture dish and then stored at 4°C until use.

**Immunohistochemistry**

Immunohistochemistry was performed on both ocular sections and whomlaments using a standard ABC technique as described previously. The monoclonal antibodies used in this study were ED1 recognizing a cytoplasmic antigen in rat monocytes/macrophages and 90% of dendritic cells ED1 was kindly provided by Dr. C. D. Dijkstra Free University Amsterdam, recognizing rat MHC class II antigen, Sera Lab, Sussex, UK, recognizing alpha-beta receptor on T cells, Endogenous peroxidase activity was inactivated by incubating the ocular sections and whomlaments in 1% H2O2-PBS for 20 minutes. These samples were incubated with the first antibodies overnight at 4°C and with biotinylated sheep anti-mouse antibody Amersham Life Science or 1 hour at room temperature. Streptavidin-peroxidase complex (Dako Denmark) and 3-diaminobenzidine tetrahydrochloride Sigma were used to visualize color. Immunostained whomlaments were placed on gelatin-coated glass with the inner side facing up and the sections were embedded in Entellan.

**In situ apoptosis staining**

In situ apoptosis staining was performed on the ocular sections and whomlaments using TACS 1 Klenow′ DAB Kit Trevigen Inc., Gaithersburg MD USA The staining procedure was carried out according to kit instructions as described briefly. The ocular sections and whomlaments were treated with proteinase K solution for 5 minutes at room temperature to increase permeability of the cell membrane. Endogenous peroxidase activity was eliminated by immersing the specimens in 2% H2O2 for 5 minutes at room temperature. The specimens were placed in Klenow labeling buffer for 2 minutes to remove hydrogen peroxide. Subsequently they were incubated with Klenow labeling reaction mix in a humidity chamber in a 37°C incubator for 60 minutes. The labeling reaction was stopped by immersing them in stop buffer at room temperature for 5 minutes. The specimens were incubated with streptavidin-horseradish peroxidase for 10 minutes at room temperature and then visualized using DAB for 2 – 10 minutes until a satisfactory color appeared. Methyl green was used for counterstaining. The specimens were treated in turn with deionized water 95% ethanol 100% ethanol and Xylene and finally embedded in Entellen.

**RESULTS**

**Clinical observation**

Inflammation was noted with the slit-lamp microscope 11 days after immunization. On the 13th day inflammation was obvious in 12 rats that showed clouding of the red reflex exude in the aqueous humor posterior synechiae and hypopyon. Inflammation was 1.29 ± 0.7 in the 12 rats according to the clinical scoring system described by de Smet et al.

**Immunohistochemical changes**

Immunostaining on ocular tissue of normal Lewis rats revealed a network of ED1+ cells Fig. 1 and OX6+ cells in the iris and ciliary body and choroid whereas only ED1+ cells were noted in the retina. The distribution and density of these cells were the same as results previously described by us. No R73+ cell was observed in these ocular tissues.

Massive influx of ED1+ cells Fig. 2 and OX6+ cells and R73+ cells into the iris and ciliary body was observed in rats with clinical signs of inflammation. Increasing numbers of these cells were also noted in the choroid and retina of these rats. The results from the rats without obvious clinical signs of inflammation also showed an influx of these positive cells into ocular tissue although the change was severe less than what was seen in the rats with clinical signs of inflammation.

**Apoptotic cells in ocular tissues**

The result either from cular sections or from whomlaments revealed the absence of apoptotic cells in the normal ocular tissue. Apoptotic cells with brown staining were seen in all of the examined tissues in the rats immunized with IRBP. They were predominantly noted in the iris and ciliary body Figs. 3 and 8 Moreover most apoptotic cells were noted in the area where a severe inflammation was present. Morphologically the apoptotic cells showed a variety of appearances including small round cells large round cells irregular cells and dendriform cells although the first two kinds of cells were dominant.
Fig. 1. Immunohistochemical staining on wholemount of the iris of normal Lewis rat with monoclonal antibody ED1E. Original magnification × 250E©
Fig. 2. ED1E cells in the iris of Lewis rat after immunization with IRBE. WholemountE©Original magnification × 250E©
Fig. 3. No apoptotic cell in the iris of normal Lewis ratE©WholemountE©Original magnification × 250E©
Fig. 4. A lot of apoptotic cells in the iris of Lewis rat after immunization with IRBE. WholemountE©Original magnification × 250E©
Fig. 5. No apoptotic cell in the retina of normal Lewis ratE©WholemountE©Original magnification × 250E©
Fig. 6. A number of apoptotic cells in the retina of Lewis rat after immunization with IRBE. WholemountE©Original magnification × 250E©
Fig. 7. No apoptotic cell in the retina of normal Lewis ratE©SectionE©Original magnification × 100E©
Fig. 8. Few apoptotic cells in the retina of Lewis rat after immunization with IRBE. SectionE©Original magnification × 100E©

DISCUSSION

Our study revealed that monocytesE©lymphocytes and MHC class II cells are all involved in the intraocular inflammation induced with IRBP. Inflammation is more severe in the iris and ciliary body compared with the choroid and retinai which is consistent with our previous reports.10 It is very interesting to note that severe inflammation induced by IRBP is found in the anterior segment rather than in the posterior segment. Previous studies revealed that IRBP is distributed mainly in the retina.2 The question as to why IRBP induces a severe iridocyclitis remains unclear. However, this model has provided a useful tool for the generalized uveitis seen in humans such as Behçet’s disease and Vogt-Koyanagi-Harada SyndromeE©in which the immune response to retinal antigens has been thought to be implicated.11

One of interesting findings in this study is the influx of MHC class II positive cells into the iris-E©ciliary body-E©choroid and retina after immunization with IRBP. These cells have been found capable of presenting antigens to helper LEymphocytes-E©resulting in a subsequent autoimmune response.12 Previous studies have demonstrated the presence of a number of uveitogenic antigens in the retina such as retinal S-antigenE©IRBE©rhodopsin and phosducin.2 If the autoimmune response is elicited by the influx of MHC class II positive cells-E©it would seem unlikely that EAU induced with IRBP is a short-lived inflammation as shown previously.2 It is likely that ocular tissue has a regulatory network-E©especially in the retina-E©to downregulate this autoimmune response. MicrogliaE©i.e., the macrophages present in normal human retina-E©Müller cells and retinal pigment epithelium might be candidates for this network. Previous work revealed that macrophages in the lung might suppress dendritic cell-inducible antigen presentation.13 Müller cells have been shown to inhibit activation and proliferation of antigen-specific cells.14 Retinal pigment epithelium has been shown to secrete inhibitory mediators including prostaglandin E2 and nitric oxide-E©which are able to downregulate dendritic cell function and produce anti-inflammatory cytokines.15 In addition-E©it is likely that there are no costimulatory signals in the ocular tissues during EAU-E©which might also contribute to rapid regression of the inflammation.

Another important mechanism involved in the downregulation of the inflammation induced by IRBP might be apoptosis of the inflammatory cells. Apoptosis is a mechanism for the precise elimination of unwanted cells and is essential to the normal physiologic turnover of cells as well as in various pathological processes.16 It is characterized by condensation and margination of chromatin-E©structural disorganization of the nucleus and formation of cell fragments or so-called apoptosis bodies. Apoptosis of autoreactive lymphocytes has been considered an important mechanism for immune tolerance. Autoreactive lymphocytes which are not able to undergo apoptosis might be an essential cause of some autoimmune diseases.17 The present study reveals a number of inflammatory cells undergoing apoptosis during EAU. Apoptosis of infiltrating cells seen in the iris-E©ciliary body-E©choroid and retina seems to be one of the first events-E©which indicate the initial regression of EAU. It is not surprising to see more apoptotic cells in the iris and ciliary body because earlier and more severe inflammation of these tissues has been observed during EAU. It would be likely
that all kinds of inflammatory cells undergo apoptosis since they display morphological features in common with lymphocytes, monocytes, macrophages and MHC class II positive cells, as evidenced by immunohistochemical staining. However, it is not known how the signal which induces apoptosis occurs in the tissues in response to the insult of IRBP.

There is a great difference in the course of inflammation between human uveitis and EAU. In most patients with uveitis, the inflammation has a long and recurrent course. However, the inflammation in animals induced by either S-antigen or IRBP has a limited duration. Resistance to apoptosis of certain cells in patients with uveitis might explain the different course of human uveitis and EAU. Nakamura revealed a high resistance of T lymphocytes to apoptosis in Behçet’s disease. A recent study by Chan et al. suggests that apoptosis occurring in uveitis might limit ocular inflammation. The present study shows that apoptosis of infiltrating cells occurs at the early stage of EAU. These studies suggest that apoptosis of infiltrating cells may play an important role in controlling inflammation. A study on the induction of apoptosis in inflammatory cells in uveitis and the factors related to it might provide a new strategy for the treatment of human uveitis.

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REFERENCES