Pigment Epithelium–Derived Factor (PEDF) Attenuated Capsaicin-Induced Neurotrophic Keratouveitis

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Purpose. To reveal the influence of retrobulbar capsaicin treatment on rats' eyes and to test the protective effects of PEDF, a known neurotrophic and antiangiogenic substance, against neurotrophic keratouveitis.

Methods. A single retrobulbar injection of capsaicin (50 mg/kg) was performed in young rats, and the effect of subsequent retrobulbar injections of PEDF 3.2 or 6.4 μg was recorded. Tear fluid alterations were evaluated with the Schirmer test and corneal alterations with slit lamp biomicroscopy. Histopathologic alterations were studied with light and electron microscopy. The number of leukocytes (myeloid cells) in the anterior and posterior chambers, peripheral retina, and vitreous were quantitatively evaluated.

Results. Reduced tear secretion was found in capsaicin-treated rats compared with the control, but this effect was significantly attenuated by PEDF. Corneal ulceration developed and was followed by scar formation and neovascularization in the capsaicin-treated, and it was also significantly attenuated by PEDF treatment. Leukocyte infiltration of the anterior and posterior chambers, as well as the peripheral retina and vitreous, was also observed in capsaicin-treated eyes and was significantly reduced by PEDF treatment. The protective effects of PEDF were dose dependent for each parameter, even if the treatment was initiated at day 14 after the challenge.

Conclusions. PEDF accelerated the recovery of tear secretion and also prevented capsaicin-induced neurotrophic keratouveitis and peripheral vitreoretinal inflammation. These effects of PEDF, described herein for the first time, may have a clinical application in inflammatory and neovascular diseases of the eye. (*Invest Ophthalmol Vis Sci.* 2009;50:5173–5180) DOI: 10.1167/iovs.08-1852

Sensory innervation of the eye comes from the first branch of the trigeminal nerve, and it plays an essential role in the physiology and pathophysiology of both the anterior and posterior segments of the eye.[1,2] Sensory nerves regulate tear secretion either, through the sensory arc of reflex tearing[3] or directly through releasing neuropeptides from sensory nerve endings.[4] Both mechanisms are involved in the development of dry eye syndromes.[5–7] It is also well known that normal sensory innervation is essential for maintaining corneal integrity.[8] Corneal nerve damage in animals results in abnormalities of the corneal epithelium, such as an increase in permeability, a decrease in cell proliferation, changes in cellular phenotype, and delayed wound healing.[9,10] Various types of corneal injury[11] or disease in humans, including trauma, herpetic keratitis,[12] and diabetic keratopathy[13] result in the development of neurotrophic keratouveitis, which is often accompanied by persistent corneal epithelial defects or trophic ulcer.[14] Sensory nerves also contribute to the regulation of uveal blood flow and to the regulation of ocular hydrodynamics. Thus, they are involved in the development of ocular hypertension, a common risk factor for glaucomatous damage to the optic nerve.[15,16] Although the retina has no sensory innervations, substance P (SP)– and calcitonin gene-related peptide (CGRP)–containing amacrine cells and ganglion cells have been observed in the retina in various species including humans.[17–19] Furthermore, several experimental studies have suggested that these neuropeptides are involved in some retinal diseases.[20–22] Recently, increase in SP and CGRP immunoreactivity was found in the retina after electric stimulation of the trigeminal ganglion.[23] However, the mechanism by which these neuropeptides are generated in the retina has yet to be elucidated.

Capsaicin (CAP), a neurotoxic substance for polymodal C sensory nerve fibers, is widely used in experiments, either for sensory nerve stimulation or sensory nerve damage, depending on the dose applied.[24] Animals exposed to CAP exhibit various corneal lesions, including a reduction in the number of corneal nerve fibers, and disintegration of epithelial cells.[25,26] In addition, changes in the physiology of the ocular surface, such as a reduction in tear fluid secretion, impairment of corneal epithelial barrier function, and a delay in corneal epithelial wound healing, are apparent in such animals.[27] These CAP-induced changes thus appear to be similar to those characteristic of neurotrophic keratouveitis in humans.[28] Degeneration of amacrine and ganglion cells has also been observed after CAP treatment.[29]

Pigment epithelium–derived factor (PEDF) has been isolated from the retinal pigment epithelium, and it has also been found in the vitreous and the cornea.[30] It belongs to the serine protease inhibitor family and is present at high levels in these ocular tissues. PEDF was originally identified as a neurotrophic factor.[31] Subsequently, it was found to have potent antiangiogenic activity.[32,33] It has been shown that PEDF is essential for maintaining the avascularity of the cornea and vitreous.[34,35]

The purpose of our study was to reveal the influence of PEDF on CAP-induced neurotrophic keratouveitis. We present, for the first time, evidence that retrobulbar administration of PEDF may attenuate the effect of CAP on tear secretion, keratouveitis, and the retina.

Methods

Experimental Model

One hundred forty-four 4-week-old Sprague-Dawley rats, weighing approximately 200 g and of both sexes, maintained in standard

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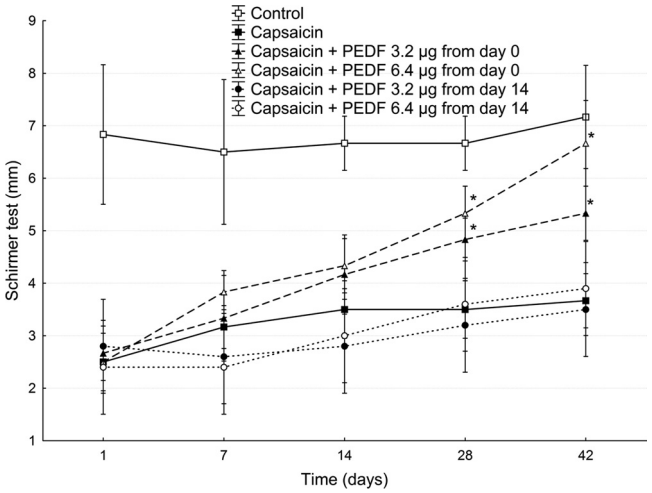

FIGURE 1. Tear flow histogram shows that PEDF improved tear secretion in a dose-dependent manner (*$P < 0.001$, compared with the CAP group at the given time points; $n = 6$).

laboratory conditions at 22°C temperature, 60% humidity, and a 12-hour light/dark cycle, were divided into six groups of 24 rats each. Care and treatment of the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rats of the first group (CAP) were given a single retrobulbar injection of CAP 50 mg/kg (Sigma-Aldrich, St. Louis, MO) in a procedure described elsewhere.[36] Rats in the second (CAP+PEDF 3.2 from day 0) and third (CAP+PEDF 6.4 from 0) groups received the same injection of CAP plus daily retrobulbar injections of 3.2 or 6.4 μg/kg PEDF starting from day 0 (BioProducts MD, Middletown, MD), respectively. Rats in the fourth (CAP+PEDF 3.2 from day 14) and fifth (CAP+PEDF 6.4 from day14) groups also received the same CAP plus daily retrobulbar injection of 3.2 or 6.4 μg/kg PEDF starting at day 14 after CAP challenge. The sixth (control) group (C) received the vehicle solution alone. One randomly selected eye of each rat was used, and the untreated fellow eye was used to evaluate eventual systemic effects. PEDF treatment was repeated daily until the end of the study.

Tear Secretion

Tear fluid secretion was measured without topical anesthesia by a modified version of the Schirmer test. Schirmer strips cut to dimensions of 20 × 1 mm were inserted below the lower eyelid of the test animal for 1 minute. The wet length of the strip was measured to an accuracy of ±0.5 mm. The Schirmer test was performed at day 1 (before any treatment) and at days 7, 14, 28, and 42 after CAP injection.

Light Microscopy

Six rats from each group were killed with carbon dioxide at days 7, 14, 28, and 42. The eyes were enucleated and fixed in Karnovsky solution for 48 hours, dehydrated, and embedded in paraffin, and the 6-μm-thick sections were colored with H&E and Masson trichrome staining for light microscopy. In addition, myeloid leukocytes labeled with chloroacetate-esterase were counted in the H&E-stained sections under light microscopy. Chloroacetate-esterase specifically identifies cells of the granulocyte lineage, from the early promyelocyte stage to mature neutrophils. The number of leukocytes was counted in the anterior chamber, posterior chamber, peripheral retina, and peripheral vitreous at the same microscopic magnification.

Transmission Electron Microscopy

Small pieces (1 × 1 mm) of the cornea were dissected and fixed in buffered 2% glutaraldehyde for 2 hours, postfixed in 2% osmiumtetroxide for another 2 hours, dehydrated, and embedded in Araldite. Ultrathin sections were cut with an ultramicrotome (Reichert, Vienna, Austria), contrasted with uranyl-acetate and lead-citrate, and studied with an electron microscope (109 EM; Carl Zeiss Meditec, GmbH, Oberkochen, Germany).

Scanning Electron Microscopy

After prefixation, tissue samples were oriented, and the exposed surface was coated with gold-carbon vapor and examined with an electron microscope equipped with a high-resolution scanning device used for photography (EM Asid JEM-100B; JEOL, Tokyo, Japan).

Statistical Analysis

Data are expressed as the mean ± SD. Statistical analysis was performed with repeated-measures ANOVA using the Dunnett multiple-comparison test for results in the three groups. After Bonferroni adjustment, the significance level was set at $P < 0.01$.

RESULTS

Tear Secretion

Tear fluid secretion was evaluated by Schirmer test (Fig. 1). CAP treatment resulted in a statistically significant decrease in tear secretion at days 1 (2.50 ± 0.55), 7 (3.17 ± 0.41), 14

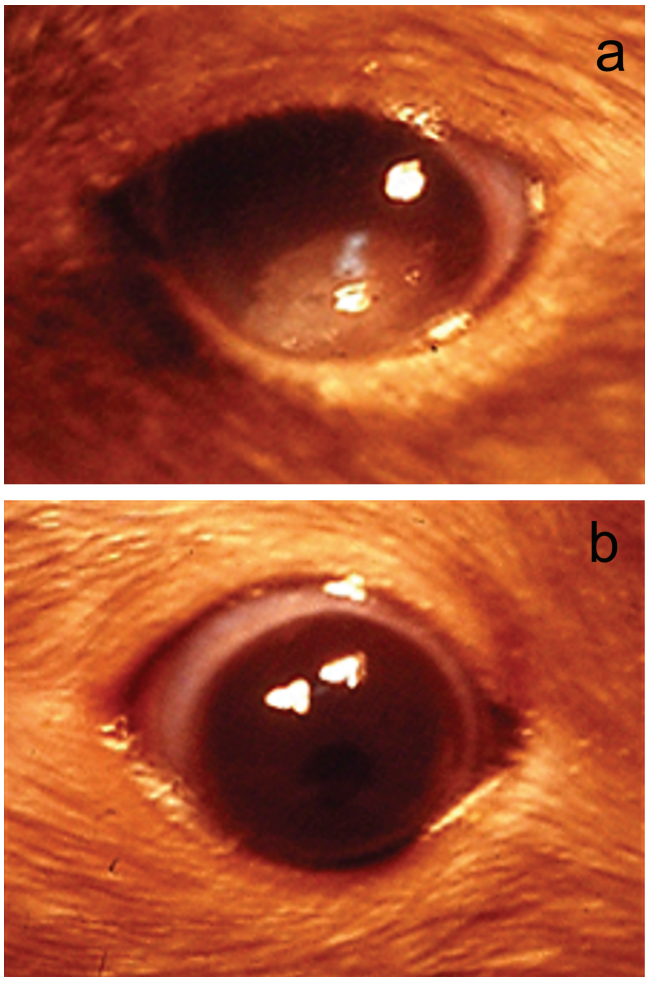

FIGURE 2. Clinical appearance of CAP-induced keratouveitis (**a**) shows a large corneal scar with neovascularization and thickening of the stroma. (**b**) After treatment with CAP+PEDF 6.4, showing a transparent cornea at day 42.

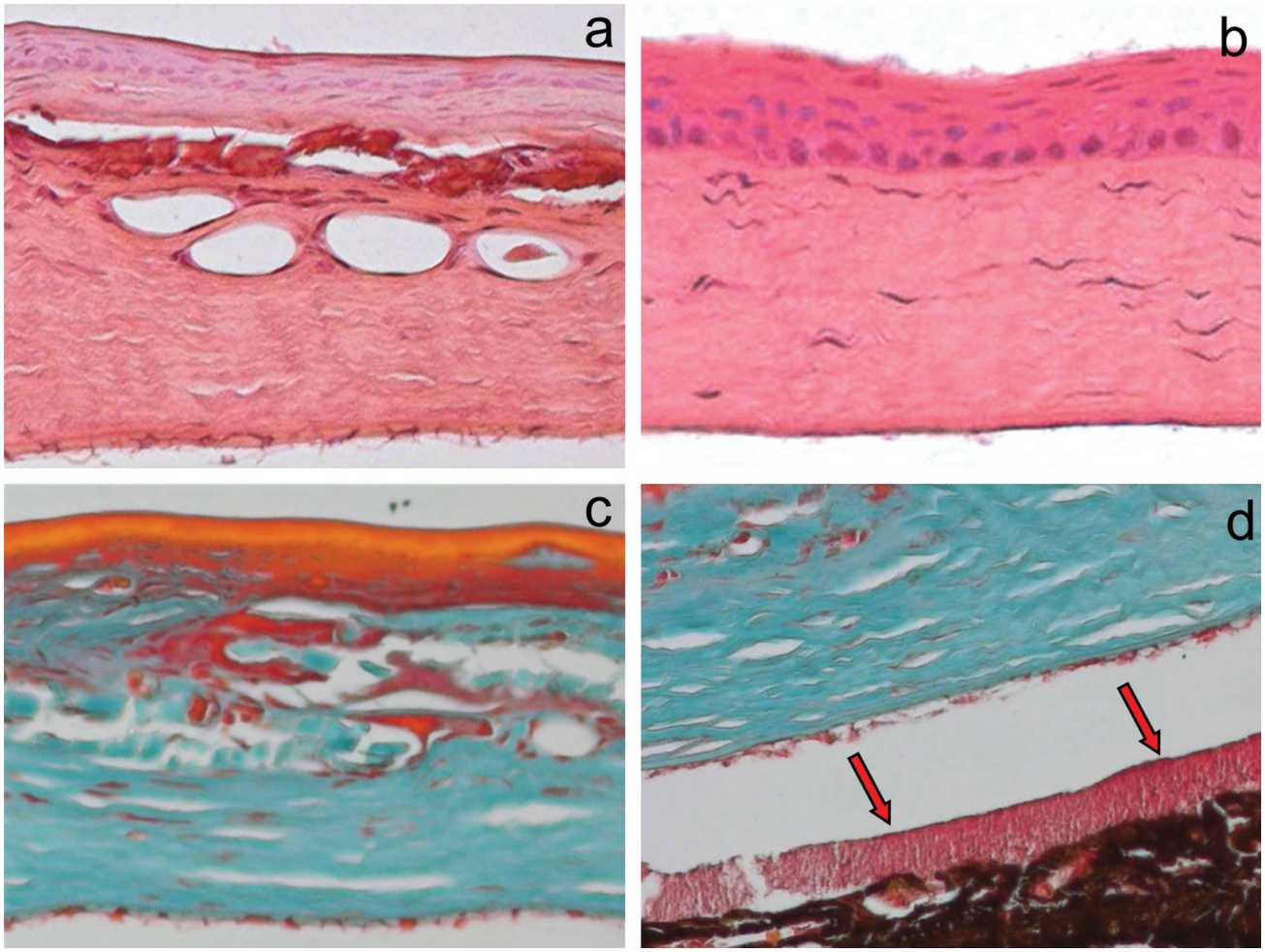

FIGURE 3. Light microscopy of CAP-induced keratouveitis. (**a**) With H&E staining, the cornea of the CAP-treated eye showed a thin and irregular epithelium, superficial scar tissue, and neovascularization. (**b**) The cornea of the CAP+PEDF 6.4 group had a normal appearance with H&E staining (day 42). (**c**) Both scar tissue of the cornea and (**d**) exudates on the surface of iris were clearly visible with Masson-trichrome staining (day 28). Magnification, ×200.

(3.50 ± 0.55), 28 (3.50 ± 0.55), and 42 (3.67 ± 0.52) compared with the control values (6.83 ± 1.33, 6.50 ± 1.38, 6.67 ± 0.52, 6.67 ± 0.52, and 7.17 ± 0.98, respectively). Treatment with 3.2 or 6.4 μg/kg PEDF from day 0 attenuated the effect of CAP at days 28 and 42 ($P < 0.001$) only, and significantly decreased tear secretion was measured in group CAP+PEDF 3.2 at days 1 (2.67 ± 0.52), 7 (3.33 ± 0.82), 14 (4.17 ± 0.75), 28 (4.83 ± 0.41), and 42 (5.33 ± 0.52), and in group CAP+PEDF 6.4 at days 1 (2.50 ± 0.55), 7 (3.83 ± 0.41), 14 (4.33 ± 0.52), and 28 (5.33 ± 0.52) compared with the controls. PEDF treatment initiated at day 14 also attenuated the effect of CAP pretreatment on tear secretion, but these effects are not significant at any time point (Fig. 1).

Clinical Picture

A single retrobulbar injection of CAP into young rats caused a clinically marked inflammation of the anterior segment progressing from slight punctate vacuolization in the epithelium at the second or third days to diffuse edematous opacities and neovascularization in the stroma, at the third or fourth week, persisting at least for 6 weeks. These alterations of the cornea showed continuous progression by the end of follow-up. In contrast, with treatment with PEDF, both corneal opacities and scar formation were prevented, and the cornea became completely transparent at the end of the experimental period (Fig. 2).

Histopathology

The most prominent histopathologic feature of the affected corneas was a marked disorganization of the epithelium, followed by marked polymorphonuclear leukocyte influx as well as by edema and disorganization of the corneal stroma with degeneration and loss of the central epithelium These alterations were accompanied with fibrinous and cellular exudation into the anterior chamber (Fig. 3). Electron microscopy showed more details of both epithelial edema and disorganization as well as stromal alterations. Intercellular edema in the corneal epithelium was particularly evident, even in the basal and intermediate layers of the epithelium of CAP-treated animals (Fig 4). There was extensive corneal neovascularization at approximately 14 days. Myeloid cell infiltration was also evident within the angle, the anterior chamber, and the iris. Treatment with PEDF prevented corneal epithelial and stromal damage and significantly attenuated leukocyte infiltration of the uvea and anterior chamber. CAP-induced keratouveitis was not confined to the anterior segment of the eye. A moderate number of leukocytes was also found in the posterior chamber, peripheral retina (pre-equatorial area), and corresponding vitreous body (Fig. 5). PEDF treatment decreased leukocyte infiltration in a dose-dependent manner.

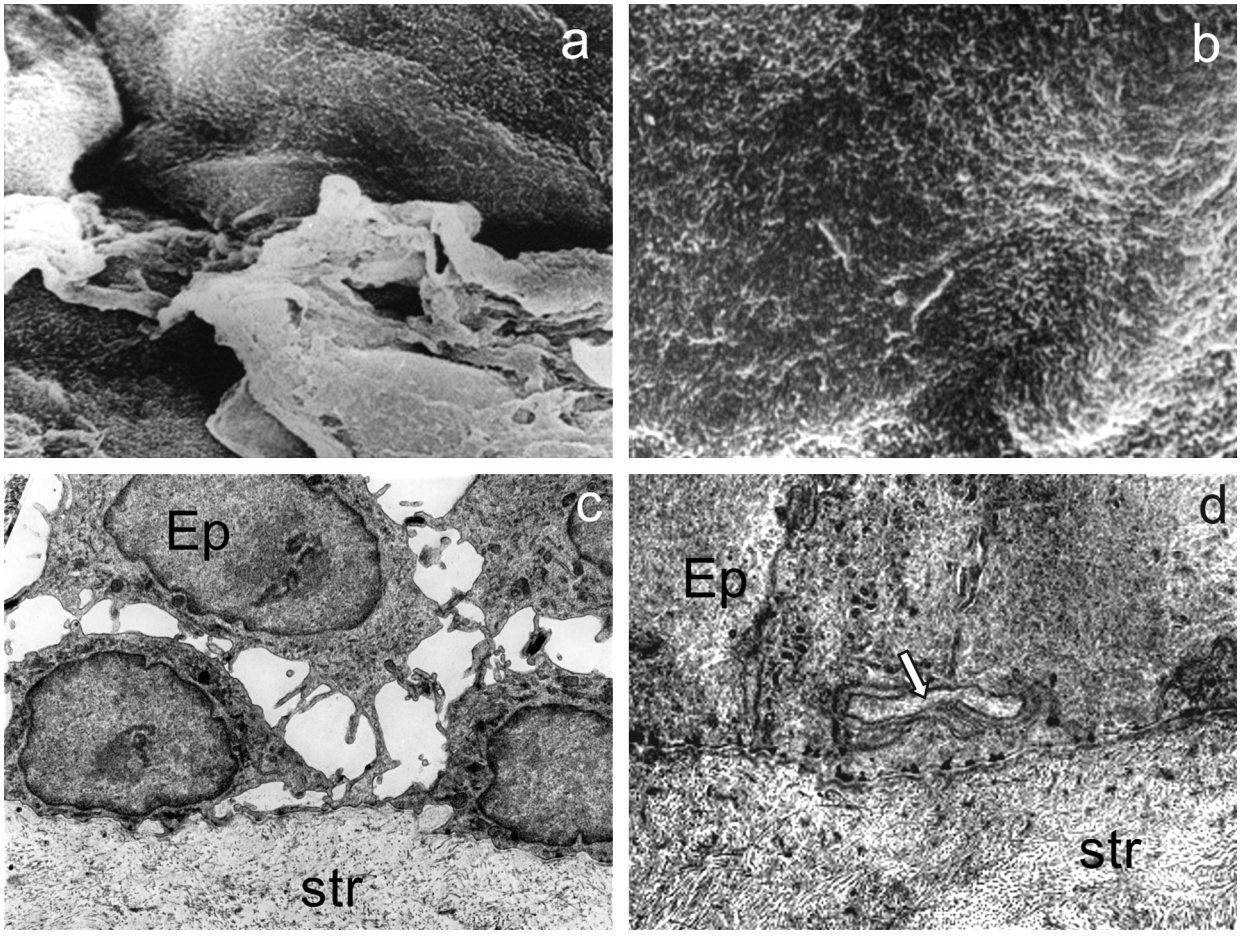

FIGURE 4. Electron microscopy of the CAP-induced epithelial alterations as seen with a scanning electron microscope. (**a**) Highly irregular corneal surface of the CAP-treated eye, evident reduction of microvilli, and loss of some superficial cells. (**b**) Normal appearance of the epithelial surface of a CAP+PEDF 6.4–treated eye. (**c**) Intercellular epithelial edema and disorganized epithelial–stromal junction of the CAP-treated eye, as shown by transmission electron microscopy. *Arrow*: a nerve fiber in the corneal epithelium of the CAP+PEDF 6.4–treated eye (day 42). Ep, epithelium; str, stroma. Magnification: (**a**, **b**) ×3,000; (**c**) ×15,000.

Quantitative Evaluation of Leukocyte Infiltration

CAP treatment resulted in a statistically significant increase in the number of myeloid cells in the anterior chamber, posterior chamber, peripheral vitreous, and retina compared with control eyes (Fig. 6). Treatment with 3.2 or 6.4 μg PEDF either from day 0 or day 14 significantly attenuated this myeloid cell infiltration at different time points. Total abolishment of CAP-induced myeloid cell infiltration was observed at day 28 in the posterior segment and at day 42 in the anterior segment after PEDF treatment was initiated at day 0. These data are provided in detail in Table 1.

DISCUSSION

Our data demonstrated that a retrobulbar injection of CAP into young rats resulted in decreased tear secretion and neurotrophic keratouveitis characterized by epithelial alteration, stromal edema, and scar formation accompanied with neovascularization of the cornea. These alterations are essentially similar to those described in previous studies.[28,36] In contrast to those, we observed leukocyte infiltration in the posterior chamber, peripheral retina, and corresponding vitreous. A further novel finding in our study was that all these CAP-induced alterations were attenuated in a dose-dependent manner by retrobulbar injection of PEDF. The difference was statistically significant at the end of the study compared with the CAP-treated group, and neither of the parameters returned to normal. Delayed application of PEDF resulted in significantly decreased infiltration of the eye tissue with myeloid cells; however, in contrast to application from day 0 there was no effect on post-CAP tear secretion.

CAP exerts its effects through binding to transient receptor potential vanilloid type 1 (TRPV1), which is a Ca^{2+}-permeable ion channel.[37] Current knowledge suggests two effects of CAP on TRPV1: (1) CAP may act on TRPV1 receptors of *sensory nerves* of the cornea, conjunctiva, lacrimal glands, ciliary body, and choroids, all of which have rich CAP-sensitive sensory innervation. On activation of TRPV1, pain and heat sensation are transmitted to the specific brain center. At the same time, from the sensory nerve endings, the proinflammatory neuropeptides SP and CGRP are released, resulting in *neurogenic inflammation*. Although the retina has no direct sensory innervation, proinflammatory neuropeptides may reach the peripheral retina through the aqueous and vitreous humors. Thus, generation of neurogenic inflammation may be a leading contributing factor to the CAP-induced keratouveitis in our model. (2) Recent studies have revealed TRPV1 receptors of several *non-neuronal cells*. These include epithelial cells (e.g., keratinocytes, urothelium, gastric epithelial cells, enterocytes, and

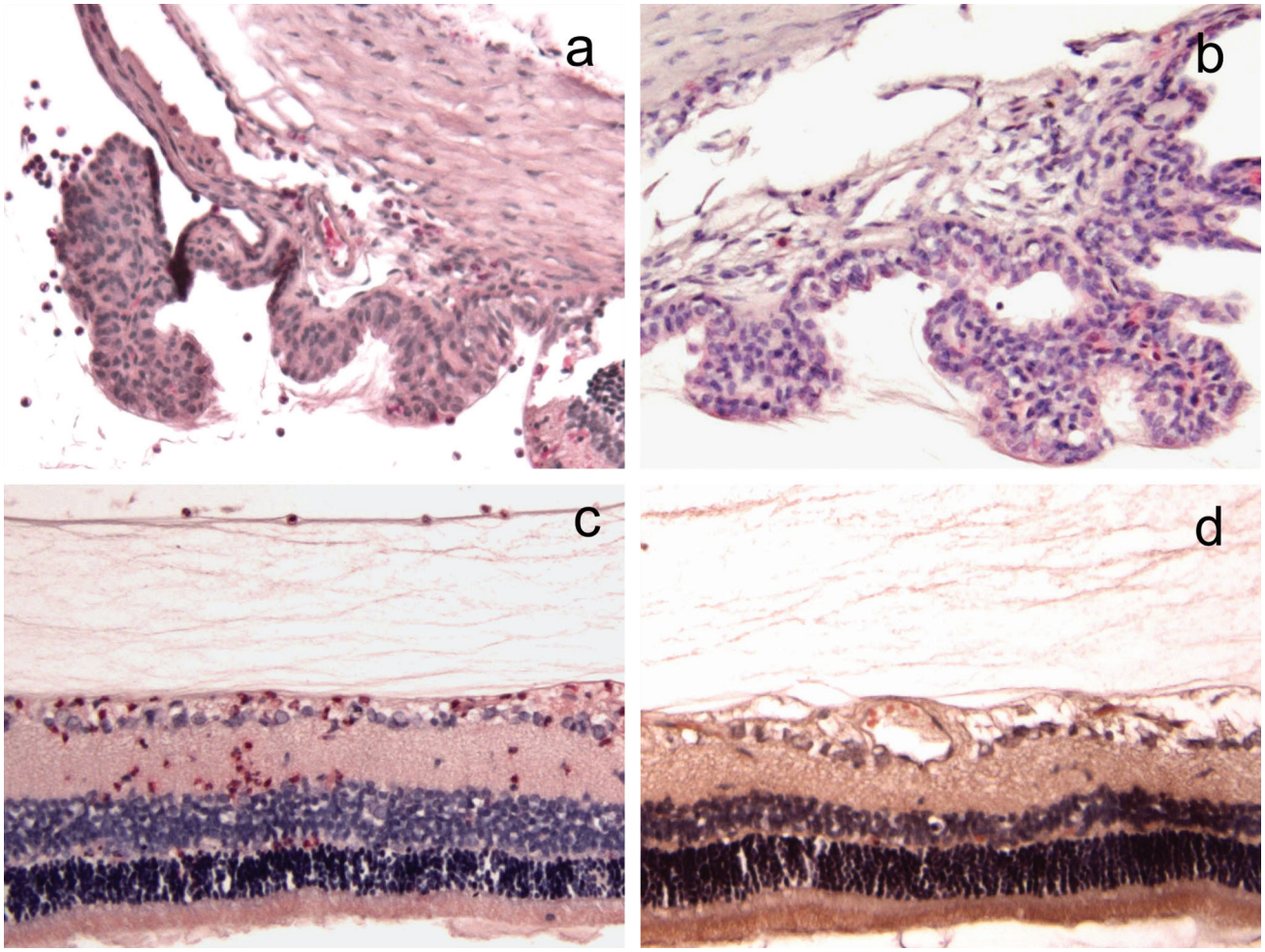

FIGURE 5. Light-microscopy of uveoretinal involvement (day 42) in CAP-induced keratouveitis. (**a**) Numerous leukocytes were present in the chamber angle, iris, and ciliary body. (**b**) In CAP+PEDF 6.4–treated eyes, very few leukocytes were visible in the chamber angle and ciliary body. (**c**) Numerous leukocytes were present in the retina and in the vitreous body of CAP-treated eyes. (**d**) The retina of the CAP+PEDF 6.4–treated eyes had a normal appearance. Note the numerous bright *red* chloroacetate-esterase-positive myeloid cells. H&E staining. Magnification, ×300.

pneumocytes), vascular endothelium and cells of the immune system (e.g., T cells and mast cells) as well as smooth muscle cells, fibroblasts, and hepatocytes.[38] TRPV1 receptors have been found in corneal epithelial cells of humans and rabbits,[39] in amacrine cells of the retina,[40] and, most recently, in retinal microglial cells.[41] Activation of TRPV1 receptors of these cells upregulates expression of immunoreactivity for proinflammatory neuropeptides by autocrine regulation. All these data justify an assumption that activation of TRPV1 receptors of non-neuronal cells and the subsequent release of proinflammatory neuropeptides like SP and CGRP may also contribute to CAP-induced keratouveitis. Our ongoing studies are dedicated to the investigation of this hypothesis.

PEDF accelerated the recovery of tear secretion, and prevented neurotrophic keratouveitis (corneal ulcerations, scar formation, and neovascularization) and peripheral vitreoretinal inflammation. Indirect evidence suggests that both the neurotrophic and antiangiogenic effects of PEDF are involved in these mechanisms. In vitro studies have shown that neuronal growth factors exert their effects through modulating TRPV1 expression and activity of sensory nerve cells.[42–46] Recently, topical treatment with nerve growth factor was also shown to restore corneal integrity in humans with corneal neurotrophic ulcers or keratitis.[47,48] The antiangiogenic effects of PEDF are less clear. There is accumulating evidence that in normal conditions there may be a balance between the release of PEDF and proangiogenic cytokines—first of all, vascular endothelial growth factor (VEGF). A decrease in the levels of PEDF or an increase in VEGF may be responsible for neovascularization in the exudative form of age-related macular degeneration, diabetic retinopathy,[46] and ischemia-induced retinal neovascularization.[49] These findings suggest a certain *antagonism* between PEDF and VEGF in angiogenesis. However, this scenario is controversial, as several studies have shown direct neurotrophic effects of VEGF similar to nerve growth factors suggesting a *synergy* between them in providing neuroprotection.[50–52] Further studies are certainly needed to reveal the molecular mechanism of the association between PEDF and VEGF in both neuroprotection and angiogenesis.

In conclusion, our studies of CAP-induced neurotrophic keratouveitis described involvement of the peripheral retina and adjacent vitreous. At the same time, this is the first experimental study to suggest a neuroprotective and antiangiogenic effect of PEDF in CAP-induced keratouveitis. Although the involvement of TRPV1 is presumed, the molecular mechanisms of PEDF remain to be elucidated.

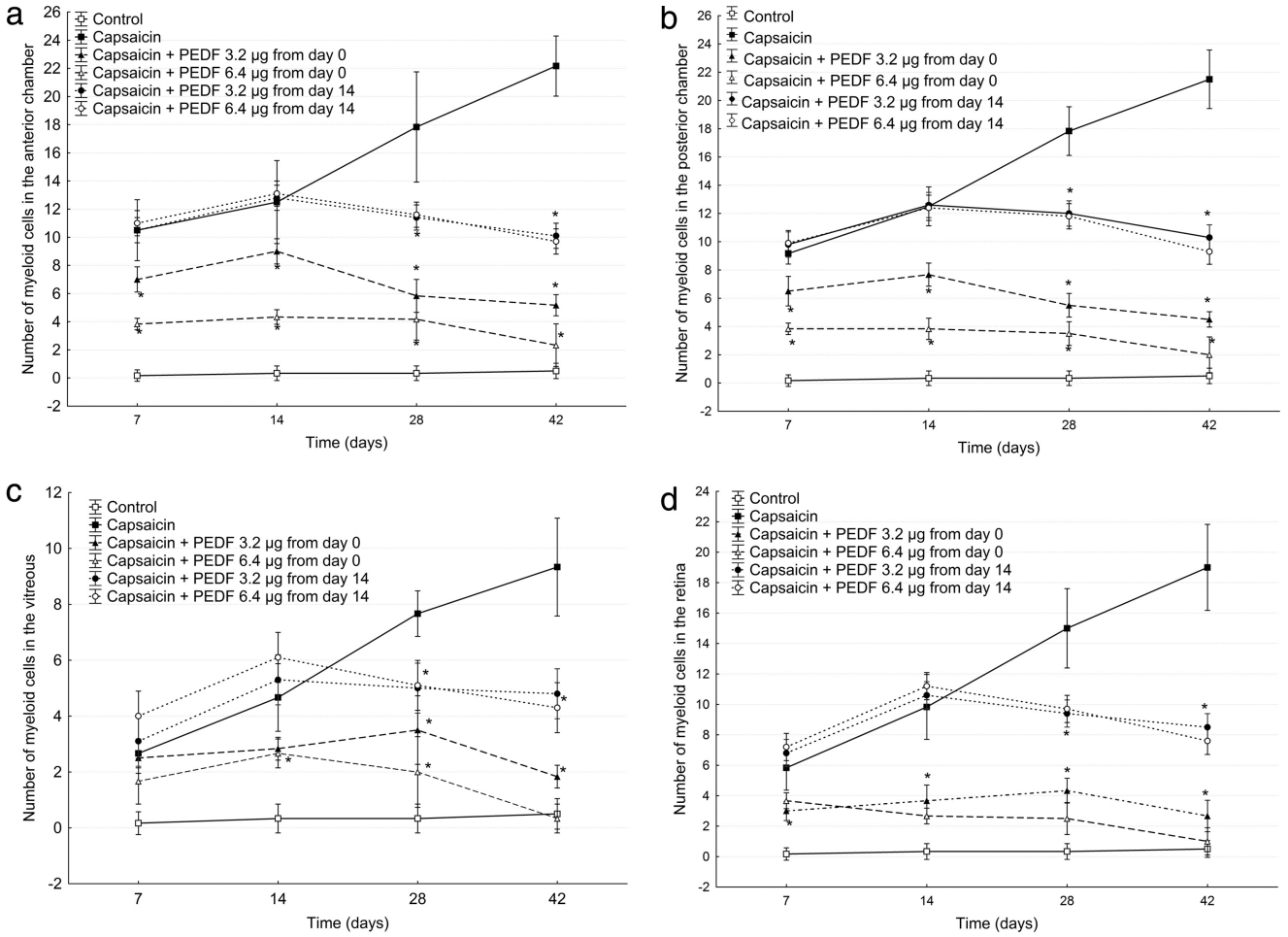

Figure 6. Quantitative analysis of leukocytes in the (**a**) anterior chamber, (**b**) posterior chamber, (**c**) peripheral vitreous body, and (**d**) peripheral retina. CAP treatment increased leukocyte infiltration in all four localizations up to the end of the study period, whereas PEDF attenuated this effect in a dose-dependent manner. *$P < 0.001$ compared with the CAP group at the given time points ($n = 6$).

Table 1. Number of Myeloid Cells in the Eyes at Different Time Points of the Study

Group	Localization	Day 7	Day 14	Day 28	Day 42
Control	AC	0.17 ± 0.40	0.33 ± 0.51	0.33 ± 0.51	0.5 ± 0.55
	PC	0.16 ± 0.41	0.33 ± 0.51	0.33 ± 0.51	0.5 ± 0.55
	Vitreous	0.16 ± 0.41	0.33 ± 0.51	0.33 ± 0.51	0.5 ± 0.55
	Retina	0.16 ± 0.41	0.33 ± 0.51	0.33 ± 0.51	0.5 ± 0.55
CAP	AC	10.5 ± 2.16*	12.5 ± 2.95*	17.83 ± 3.92*	22.16 ± 2.14*
	PC	9.17 ± 0.75*	12.5 ± 1.38*	17.83 ± 1.72*	21.5 ± 2.07*
	Vitreous	2.66 ± 0.52*	4.67 ± 1.21*	7.67 ± 0.82*	9.33 ± 1.75*
	Retina	5.83 ± 1.47*	9.83 ± 2.14*	15 ± 2.61*	19 ± 2.83*
CAP+3.2 µg PEDF from day 0	AC	7 ± 0.89*†	9 ± 0.89*†	5.83 ± 1.16*†	5.16 ± 0.75*†
	PC	6.5 ± 1.05*†	7.67 ± 0.82*†	5.5 ± 0.83*†	4.5 ± 0.55*†
	Vitreous	2.5 ± 0.55*	2.83 ± 0.41*†	3.5 ± 1.22*†	**1.83 ± 0.41†**
	Retina	3 ± 0.63*†	3.67 ± 1.03*†	4.33 ± 0.82*†	**2.67 ± 1.03†**
CAP+6.4 µg PEDF from day 0	AC	3.83 ± 0.41*†	4.33 ± 0.52*†	4.16 ± 1.47*†	**2.33 ± 1.51†**
	PC	3.83 ± 0.41*†	3.83 ± 0.75*†	3.5 ± 0.83*†	**2 ± 1.26†**
	Vitreous	1.66 ± 0.82*	2.66 ± 0.52*†	**2 ± 1.26†**	**0.33 ± 0.52†**
	Retina	3.67 ± 0.52*†	2.67 ± 0.51*†	**2.5 ± 1.04†**	**1 ± 0.89†**
CAP+3.2 µg PEDF from day 14	AC	10.5 ± 0.89*	12.8 ± 0.89*	11.4 ± 0.89*†	10.0 ± 0.89*†
	PC	9.8 ± 1,03*	12.6 ± 0.98*	12 ± 0.89*†	10.3 ± 0.83*†
	Vitreous	3.1 ± 0.75*	5.3 ± 0.83*	5 ± 0.83*†	4.8 ± 0.89*†
	Retina	6.8 ± 0.89*	10.6 ± 0.89*	9.4 ± 0.98*†	8.5 ± 0.98*†
CAP+6.4 µg PEDF from day 14	AC	11 ± 0.98*	13.1 ± 0.89*	11.6 ± 0.89*†	9.7 ± 0.89*†
	PC	9.9 ± 0.89*	12.4 ± 1.14*	11.8 ± 0.89*†	9.3 ± 0.82*†
	Vitreous	4 ± 0.83*	6.0 ± 0.82*	5.1 ± 0.75*†	4.3 ± 0.83*†
	Retina	7.2 ± 0.89*	11.2 ± 0.89*	9.7 ± 0.98*†	7.6 ± 0.89*†

Bold data: PEDF treatment totally abolished the effect of CAP pretreatment. AC, anterior chamber; PC, posterior chamber.
* $P < 0.001$ compared with the control group.
† $P < 0.001$ compared with the CAP group at the given time point.

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