Effects of Periocular Administration of Triamcinolone Acetonide on Leukocyte–Endothelium Interactions in the Ischemic Retina

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**PURPOSE.** Recent studies have reported that intravitreal or posterior sub-Tenon’s injection of triamcinolone acetonide (TA) is effective in the treatment of macular edema resulting from retinal microcirculatory disturbances such as diabetic retinopathy and retinal vein occlusion. The effects of periocular administration of TA on leukocyte–endothelium interactions were studied after transient retinal ischemia.

**METHODS.**Transient retinal ischemia was induced by temporary ligation of the optic nerve sheath for 60 minutes in male Long-Evans rats. After the induction of ischemia, experimental eyes received a periocular injection of TA (2 mg). In control animals, the same volume of saline was administered. Leukocyte dynamics were evaluated in the retinal microcirculation using acridine orange digital fluorography. Also, retinal thickness was studied by using optical coherence tomography and a histologic method. The retinal mRNA expression of P-selectin and intercellular adhesion molecule (ICAM)-1 was semiquantitatively studied with RT-PCR.

**RESULTS.** The leukocytes rolling along retinal vein linings increased after ischemia in the vehicle-treated rats (32.5 ± 2.1 cells/min). No rolling leukocytes, however, were seen in the TA-treated rats. The number of accumulated leukocytes was significantly lower in the TA-treated rats (831 ± 99 cells/mm²) than in the control (971 ± 81 cells/mm², *P* < 0.05). The treatment decreased the retinal thickness and the mRNA expression of P-selectin and ICAM-1.

**CONCLUSIONS.** The present study demonstrated that the periocular injection of TA effectively decreased retinal thickness and inhibited leukocyte–endothelium interactions in the retina after ischemia. Downregulation of adhesion molecules of retinal vascular endothelium induced by TA may play a role in the course. 

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Macular edema is a major condition that leads to reduced visual acuity and is caused by retinal vascular disease, intraocular inflammatory disease, surgical procedures, drugs, retinal dystrophies, vitreomacular traction syndrome, and so on. Though many treatments have been proposed, none has been shown to improve visual outcome. Corticosteroids are known to reduce inflammation and tissue edema. The safety and efficacy of intravitreal triamcinolone acetonide (TA), a corticosteroid suspension, have been studied experimentally.1-3 Recently, intravitreal or posterior sub-Tenon’s administration of TA has been used in clinical studies for the treatment of intraocular edema or neovascular diseases due to retinal vein occlusion,4,5 diabetic retinopathy,6-8 uveitis,9-11 age-related macular degeneration,12,13 and other diseases.14,15 These reports suggest that the therapeutic potential of TA. However, little is known about the mechanism of the effect of TA.

Secondary to retinal ischemic disease, retinal edema is usually caused by obliteration of parts of the microvascular circulation and the breakdown of the blood-retinal barrier. Leukocytes are thought to play critical roles in ischemia-reperfusion injury.16 We have established a method using acridine orange digital fluorography that permits the clear visualization of leukocytes and quantitative evaluation of their dynamics in the retinal microcirculation in vivo.16-18 Leukocyte dynamics are known to be mediated by adhesion molecules.17-21 Prevention of the adhesion of leukocytes to retinal tissue by blocking the leukocyte adhesion molecule P-selectin and intercellular adhesion molecule (ICAM)-1 reduces ischemia-reperfusion injury.

We investigated the role of TA in the reduction of retinal edema induced by ischemia, evaluating the effects of posterior periocular administration of TA (as a model of posterior sub-Tenon’s administration), by measuring retinal leukocyte dynamics, retinal thickness, and the expression of adhesion molecules after ischemia-reperfusion injury.

**MATERIALS AND METHODS**

**Animal Model**

Transient retinal ischemia was induced by a method previously described,16,22 with minor modifications. Male pigmented Long-Evans rats (200–250 g) were used. Only the right eye of each rat was subjected to ischemia. The rats were anesthetized with a mixture (1:1) of xylazine hydrochloride (4 mg/kg) and ketamine hydrochloride (10 mg/kg). The pupils were dilated with 0.5% tropicamide and 2.5% phenylephrine hydrochloride. After lateral conjunctival peritomy and disinsertion of the lateral rectus muscle, the optic nerve head of the right eye was exposed by blunt dissection. A 6-0 nylon suture was passed around the optic nerve and tightened until the blood flow ceased in all the retinal vessels. After 60 minutes of ischemia, complete nonperfusion was confirmed through an operating microscope, and the suture was removed. Reperfusion of the vessels was also observed microscopically. Eyes that failed to reperfuse within 5 minutes were excluded from the experiment. Sham-operation rats (*n* = 26) underwent the same surgery, but without tightening of the suture. A TA suspension (40 μg/mL; Bristol-Myers Co., Tokyo, Japan) was administered into the posterior periocular space to TA-treated rats (*n* = 26) after reperfusion (2 mg, 50 μL). Vehicle-treated rats (*n* = 26) were given the same volume (50 μL) of saline. Sham-operation rats were used as the control. All experiments were performed in accor-

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dance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Acridine Orange Digital Fluorography

Acridine orange (AO) digital fluorography was performed as previously described.16,23–24 In this technique, a scanning laser ophthalmoscope (SLO; Rodenstock Instruments, Munich, Germany), coupled with a computer-assisted image-analysis system, makes continuous high-resolu-
tion images of the fundus, which has been stained with the meta-
chronic fluorochrome (Wako Pure Chemical, Osaka, Japan), which
emits green fluorescence when it interacts with DNA. The spectral
properties of AO-DNA complexes are very similar to those of sodium
fluorescein, with an excitation maximum at 502 nm and an emission
maximum at 522 nm. An argon blue laser was used as the illumination
source, with a regular emission filter for fluorescein angiography.
Immediately after the AO solution was infused intravenously, the
leukocytes were stained selectively among circulating blood cells.
Nuclei of vascular endothelial cells were also stained. The obtained
images were recorded digitally (DVD-RAM) at a rate of 30 frames/s for
further analysis.

Experimental Design

AO digital fluorography was performed at 12 and 24 hours after
reperfusion in all groups. Sham-operation rats were evaluated as the
test group. Five different rats were used at each time point in each
group. Immediately before AO digital fluorography, the rats were anesthe-
tized, and the pupils were dilated. A contact lens was used to retain
corneal clarity throughout the experiment. Each rat had a catheter
inserted into the tail vein and was placed on a stereotaxic platform.
Body temperature was maintained at 38 ± 0.5°C. AO (0.1% solution in
glycerol) was injected continuously through the catheter for 1 minute at
a rate of 1 mL/min. The fundus was observed with the scanning laser
ophthalmoscope in a 40° field for 5 minutes. At 30 minutes after the
injection of AO, the fundus was observed again, to determine leuko-
cyte accumulation in the retinal microcirculation. After the experi-
ment, the rat was killed with an anesthetic overdose, and the surgical eyes were imme-
diately enucleated. The bisected eyes were immediately frozen in
cutting compound at an optimal temperature (Tissue-Tek; Sakura Fi-
netek, Tokyo, Japan) on dry ice. The cryostat sections were cut sagit-
tally through the optic nerve at 10-μm thickness and fixed in 4% paf-ormaldehyde in PBS for staining by hematoxylin and eosin. The
sections were cut perpendicular to the retinal surface. To quantify the
retinal damage induced by ischemia–reperfusion injury, we measured
changes in thickness of various retinal layers, according to the method
described by Hughes.25 The thickness of the inner plexiform layer
(IPL), inner nuclear layer (INL), outer nuclear layer (ONL), and the
overall retina from outer to inner limiting membrane (OLM–ILM) were
measured. The thickness of these layers in each section was measured
in the retina at a distance of 1.5 mm from the center of the optic nerve
head. The value of each retinal thickness was averaged from eight
measurements of four sections from each eye.

Image Analysis

The DVD-RAM recordings were analyzed with an image-analysis sys-
tem, described in detail elsewhere.16,23–24 In brief, we used a computer
equipped with software (DVD-MOVIE Album; Matsushita Electric In-
dustrial, Osaka, Japan) that enters the digital images into a personal
computer in real time (30 frames/s) in 640 horizontal and 480 vertical
pixels, with an intensity resolution of 256 steps. We evaluated the
diameters of major retinal vessels, the number of rolling leukocytes
among the major retinal veins, and the number of leukocytes accumu-
lated in the retinal microcirculation through use of this system.

The diameters of major retinal vessels were measured at 1 disc
diameter from the center of the optic disc in monochromatic images
recorded before AO injection. Each vessel diameter was calculated in
pixels as the distance between the half-height points determined sepa-
ately on each side of the density profile of the vessel image and was
converted into a real value by using the calibration factor. The averages
of the individual arterial and venous diameters were used as the arterial
and venous diameters for each rat.

Rolling leukocytes were defined as leukocytes that moved at a
velocity much slower than that of free-flowing leukocytes. The process
of differentiating such leukocytes from free-flowing leukocytes has
been described in a previous article.16 In brief, leukocytes that were
rolling along the major retinal veins were easily recognized on the
video monitor, because even the fastest rolling leukocyte moved al-
most 300 times slower than the average free-flowing leukocytes. Be-
cause no leukocytes with an intermediate velocity were observed, it
was not difficult to distinguish semiattached rolling leukocytes from
free-flowing leukocytes. The number was calculated by counting the
number of rolling cells passing a fixed line in all major veins (four to
seven veins) at a distance of 1 disc diameter from the center of the optic
disc per minute. The average number of rolling leukocytes in individual
major veins was used as the value for each rat.

The number of leukocytes accumulated in the retinal microcircu-
lation was evaluated at 30 minutes after AO injection. The number of
fluorescent dots in the retina within eight areas of 100 pixels square at
a distance of 1 disc diameter from the edge of the optic disc was
counted. Averages for individual areas were used values for each rat.

Retinal Thickness

Four eyes from four rats each in the TA-treated, vehicle-treated, and
sham-operation control groups were obtained 12 hours after reperfu-
sion to evaluate retinal edema. At 12 hours after reperfusion, we evalua-
ted retinal thickness using optical coherence tomography (OCT; Carl Zeiss Meditec, Tokyo, Japan) under anesthesia. The rats were
ekilled with an anesthetic overdose, and the surgical eyes were imme-
diately enucleated. The bisected eyes were immediately frozen in
cutting compound at an optimal temperature (Tissue-Tek; Sakura Fi-
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Semi-quantification of P-selectin and ICAM-1 Gene Expression by Reverse Transcription-PCR

RT-PCR methods for examine the expression of mRNA of adhesion
molecules were performed as described previously.26

At 24 hours after reperfusion, the TA-treated, vehicle-treated, and
sham-operation control groups rats were killed. The sensory retinas
were dissected after enucleation and frozen immediately in liquid
nitrogen. Total RNA was prepared from frozen tissue samples (Isogen;
Nippon Gene Inc., Tokyo, Japan) in accordance with the manufactur-
er’s manual. Residual DNA was removed with RNase-free DNase (RQI;
Promega Madison, WI). Total RNA was reverse transcribed into cDNA
(Superscript II; Invitrogen-Gibco, Grand Island, NY) and oligo (dT)
primers. The cDNA was amplified by using specific primers with a PCR
system (Gene Amp PCR System 2400; Applied Biosystems, Inc., Foster
City, CA). The sequences for the P-selectin and ICAM-1 were as follows:
P-selectin upstream primer, 5'-CGAGAGGAACACGAGACT-3';
downstream primer, 5'-AATGGCTTCACAGGTTGGCA-3'; and ICAM-1 up-
stream primer, 5'-AGACAGAAACGAAAGAAGA-3'; downstream primer,
5'-GAGAACGCCAAAACCGTATG-3'. Oligonucleotide primer pairs from
separate exons were prepared for P-selectin and ICAM-1. The following
conditions were used: denaturing at 94°C for 30 seconds, annealing at
54°C for 30 seconds, and polymerization for 30 seconds. The reaction
was performed for 30 cycles. In addition, 28S mRNA was amplified as
a reference marker by using the same RT-PCR technique (25 cycles: 30
seconds at 94°C, 30 seconds at 54°C, and 30 seconds at 72°C). For 28S, a pair
of oligonucleotide primers 5'-TGGTAGCCCGAGTTGATTCTCTGC-3' (forward) and
5'-TCTACACCTCTCATGTCTCTCCA-3' (reverse) were prepared. We quantified PCR
products during the exponential phase of amplification. As the negative
control of 28S amplification, isolated total RNAs were treated in the same
way, except that no reverse transcriptase was added. PCR products were run on 2% agarose gels
and stained with ethidium bromide, and bands were visualized by
scanning laser densitometry (FMBIO II; Hitachi, Tokyo, Japan). Molec-
ular identity and homogeneity of the resultant PCR fragments were checked by DNA sequencing. Analysis of the DNA-stained agarose gels was evaluated by band intensity comparison of 28S expression versus each molecule with NIH Image. Each PCR reaction was repeated three times in all four eyes in each time group.

Statistical Analysis

All values are presented as the mean ± SEM. Statistical comparisons between two groups were performed by unpaired t-test. ANOVA was used to compare three or more conditions, with post hoc comparisons tested using the Bonferroni procedure. *P < 0.01; **P < 0.05.

RESULTS

Diameters of Major Retinal Vessels

Figure 1 shows the changes in major retinal vessel diameters in sham-operation, vehicle-treated, and TA-treated ischemic rats at 12 and 24 hours after reperfusion. In vehicle- and TA-treated rats, significant arterial vasoconstriction occurred 12 and 24 hours after reperfusion compared with sham-operation rats; in addition, posts ischemic arterial vasoconstriction was significantly less in TA-treated rats than in vehicle-treated rats at 24 hours after reperfusion (Fig. 1A). In veins, posts ischemic vasodilation was somewhat marked in vehicle-treated rats compared with sham-operation rats, but there was no significant difference. In TA-treated rats, venous vasodilation was significantly suppressed only at 12 hours after reperfusion, compared with that in vehicle-treated rats (Fig. 1B).

Leukocyte Rolling

Immediately after AO was infused, many free-flowing leukocytes were visualized. In ischemic eyes, some leukocytes were observed rolling slowly along major retinal veins, but not along any major retinal arteries throughout the experiments. In nonischemic sham-operation rats no rolling leukocytes were seen (data not shown). In saline-treated rats, a significant number of rolling leukocytes were observed 12 and 24 hours after reperfusion (Fig. 2). In contrast, none were observed in TA-treated rats at 12 or 24 hours after reperfusion.

Leukocyte Accumulation in the Retinal Microcirculation

Figure 3 indicates leukocyte accumulation in the retinal microcirculation. In sham-operation rats, few leukocytes were recognized at any time point. In vehicle-treated rats, they began to increase with time after reperfusion and peaked at 971 ± 81 cells/mm² at 24 hours after reperfusion. TA treatment significantly inhibited leukocyte accumulation during the reperfusion period (P < 0.01). After treatment with TA, the number of accumulated leukocytes was reduced by 20.3% (P < 0.01) and 14.4% (P < 0.05) at 12 and 24 hours after reperfusion, respectively.

Gene Expression of P-Selectin and ICAM-1 in the Retina

To investigate the mechanism of TA-mediated inhibition of the leukocyte-vascular endothelium interaction, we determined mRNA expression of adhesion molecules in TA-treated and saline-treated rat retina (Fig. 4). P-selectin and ICAM-1 gene expression was significantly decreased with TA treatment, sug-

![Figure 1](image1.png)

**Figure 1.** The major (A) arterial and (B) venous diameters at 12 and 24 hours after reperfusion. Data are the mean ± SEM. In vehicle- and TA-treated rats, significant arterial vasoconstriction occurred 12 and 24 hours after reperfusion compared with that in sham-operation rats. Posts ischemic arterial vasoconstriction was significantly suppressed in TA-treated rats compared with that in vehicle-treated rats at 24 hours after reperfusion. Venous vasodilation in TA-treated rats was significantly suppressed at 12 hours after reperfusion, compared with that in vehicle-treated rats. Five rats were used at each time point in each group.

*P < 0.01; **P < 0.05.

![Figure 2](image2.png)

**Figure 2.** The flux of rolling leukocytes along major retinal veins at 12 and 24 hours after reperfusion. Leukocyte rolling was inhibited significantly in TA-treated rats. Data are the mean ± SEM. Sham- and TA-treated rats showed significant suppression compared with vehicle-treated rats (P< 0.01).
gesting that TA suppressed the activation of endothelial cells after ischemia–reperfusion induction.

Retinal Thickness

To further analyze the effect of TA against retinal ischemia–reperfusion injury, changes in the retinal thickness were measured with OCT (Fig. 5) and were also evaluated histologically (Fig. 6).

After ischemia, the retinal thickness with OCT was increased in the vehicle-treated rats (336 ± 31 μm, \( P < 0.01 \)) compared with normal rats (174 ± 14 μm). Treatment with TA significantly reduced the postischemic retinal thickness (215 ± 12 μm, \( P < 0.01 \)).

Histologic study of the same retinas as examined with OCT demonstrated increase of the thickness of IPL without any significant changes of INL and ONL. This was the cause of the increase in retinal thickness in ischemic eyes.

Although thicknesses in IPL and from OLM to ILM increased in vehicle-treated and TA-treated rats compared with sham-operation rats, the TA treatment reduced the increase of IPL and OLM-ILM thicknesses significantly compared with those in vehicle-treated rats. The thickness of the ILM-OLM in vehicle- and TA-treated rats was 148.1% and 114.4%, respectively, of that in sham-operation rats at 12 hours after reperfusion.

The effect of TA was characteristic in the inner retina. The thickness of the IPL in vehicle- and TA-treated rats was 177.7% and 132.6%, respectively, of that in sham-operation rats. The increase of IPL-induced ischemia decreased significantly with eyes treated with TA (\( P < 0.01 \)). We also observed pyknotic nuclei (Fig 6A), which were probably migrating leukocytes in the IPL. There were no significant differences in the count of pyknotic nuclei between vehicle- and TA-treated retinas (data not shown).

DISCUSSION

The exact pathogenesis of macular edema induced by retinal vascular disease has not been identified. Several mechanisms may be responsible, including an altered blood-retinal barrier (BRB) due to hemodynamic change, alterations in capillary basement membranes, and pericyte loss. Leukocytes adhere to vascular walls and are trapped in retinal capillaries by ischemic injury,16 this may cause BRB breakdown by blocking blood flow, releasing various kinds of inflammatory cytokines. In the present study, periocular injection of TA effectively suppressed the ischemia-induced leukocyte–endothelium interactions in vivo.

![Figure 3](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933239/)

**FIGURE 3.** (A) Fundus image with AO digital fluorography of vehicle- and TA-treated rat retinas at 24 hours after reperfusion. Leukocytes accumulating in retinal microcirculation appeared as fluorescent dots at 30 minutes after AO injection. (B) The number of leukocytes that accumulated in the retina at 12 and 24 hours after reperfusion. Data are the mean ± SEM. *\( P < 0.01 \); **\( P < 0.05 \).

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933239/)

**FIGURE 4.** (A) Expression of adhesion molecules at 24 hours after reperfusion. ICAM-1 and P-selectin levels were significantly suppressed in TA-treated rats. (B) Expression of adhesion molecules normalized to 28s RNA. Data are the mean ± SEM. *\( P < 0.01 \); **\( P < 0.05 \).
Leukocyte transmigration from blood vessels requires sequential interactions of adhesion molecules between leukocytes and endothelial cells. Leukocyte–endothelium interactions are regulated by a multistep process. Leukocyte rolling is the first step in a cascade of events that lead to firm adhesion and transmigration through the endothelium. It has been established that selectins can rapidly arrest free-flowing leukocytes and mediate their rolling along the endothelium of blood vessels. Subsequently, firm adhesion and emigration are mediated by \( \beta_2 \)-integrin and its counter ligand ICAM-1.

Penfold et al. reported that TA modulated permeability and ICAM-1 expression in human choroidal endothelial cells and in the human epithelial cell line that was used as a BRB model. Inhibition of P-selectin or ICAM-1 has been suggested as a possible treatment for ischemic injury. Experimental studies have brought improvement in retinal ischemia–reperfusion injury with inhibition of adhesion molecules. The current data also show a significant reduction of P-selectin and ICAM-1 expression and inhibition of retinal edema via periorcular TA injection. These findings suggest that TA administration reduces macular edema by the suppression of leukocyte–endothelium interaction via the reduction of P-selectin and ICAM-1 expression.

A previous study has shown that ICAM-1 mediates vascular endothelial growth factor (VEGF)–induced retinal vascular permeability, and VEGF increases retinal vascular ICAM-1 expression. It has been established that hypoxia is the major stimulator of VEGF induction in various cell types. VEGF is a potent factor involved in retinal neovascularization and vascular leakage. Glucocorticoids such as TA are known to display differential capacities to mediate anti-angiogenic, anti-inflammatory, and permeability reductions. TA-modulated permeability and ICAM-1 expression, suppressed VEGF in vitro and blocked VEGF-induced BRB breakdown in vivo. It has been reported that vitreous levels of VEGF and ICAM-1 are related to diabetic macular edema. In our experiment, TA may affect retinal edema through the suppression of VEGF in connection with ICAM-1 suppression.

A recent study demonstrated that pigment epithelium-derived factor (PEDF), a potent angiogenic inhibitor, reduced the expression of hypoxia-induced VEGF and ICAM-1. It was further reported that dexamethasone is effective at increasing PEDF RNA levels. Periorcular administration of TA may decrease the expression of VEGF and ICAM-1 through the up-regulation of PEDF in the retina. To elucidate the molecular mechanism involved in the pathogenesis of retinal edema and the effect of TA, future analysis of VEGF, PEDF, and other factors by using our experimental model is needed.

In the present study, we analyzed the retinal thickness with OCT and histologic techniques. Both of these analyses showed that the retina thickened with ischemic injury and that TA reduced the thickness of the ischemic retina. OCT was useful in evaluating retinal thickness noninvasively in the rat, as previously reported.

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In conclusion, in the present study, pericentral injection of TA effectively decreased retinal thickness and inhibited leukocyte–endothelium interactions in the retina after ischemia. Downregulation of adhesion molecules of the retinal vascular endothelium induced by TA may play a role in the course.

References