Eye (2009) 23, 215–221

www.nature.com/eye

LABORATORY STUDY

The effects of glaucoma filtering surgery on anterior chamber-associated immune deviation and contribution of lymphatic drainage in rats

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Abstract

Purpose **To investigate whether glaucoma filtering surgery (GFS) in rats would impair the eye's capacity to induce anterior chamber-associated immune deviation (ACAID) and assess the possible mechanism involved.**
Methods **Rats subjected to GFS were injected with bovine serum antigen (BSA) into the anterior chamber to induce ACAID. Animals that had their cervical lymph nodes (CLNs) excised before filtering surgery and those that had sham filtering surgery served as control comparison groups. Antigen-specific delayed-type hypersensitivity (DTH) was used to identify the induction of ACAID. Antigen level in the CLNs was indicated by the percentage of FITC-positive cells in CLNs after FITC dextran was injected into the anterior chamber. Statistical analyses were performed using the student's *t*-test for comparison of data between control and experimental groups. A $P<0.05$ was required for results to be considered statistically significant.**
Results **Rats undergoing GFS demonstrated antigen-specific DTH, while those in the sham filtering surgery or CLNs excised groups failed to acquire an antigen-specific DTH response. The percentage of FITC-positive cells in CLNs was significantly increased ($P=0.001$) in GFS (mean ± SD: 2.96 ± 0.67%) *vs* controls (1.57 ± 0.48%) at 1 day, but not at 3, 5, 7, or 12 days post-antigen injection**
Conclusions **GFS prevents the induction of ACAID in rats, and the antigen drainage to CLNs plays a critical role in this process. The results suggest that the ocular immune status might be altered by GFS.**

Eye (2009) **23,** 215–221; doi:10.1038/eye.2008.151; published online 23 May 2008

Keywords: ACAID; aqueous humour drainage; cervical lymph nodes; glaucoma filtering surgery

Introduction

Anterior chamber-associated immune deviation (ACAID) is defined as an antigen-specific systemic response evoked by intracamerally injected soluble antigens, characterized by a deficiency of delayed-type hypersensitivity (DTH)-mediating T cells and of complement-fixing antibodies. The stereotypical ACAID response is distinct from the convention immune response, such as that seen with subcutaneous inoculation of antigen, in that it leads to a long-lasting systemic suppression of antigen-specific DTH. ACAID effectively eliminates ocular pathogens, while minimizing tissue damage that can cause blindness.[1] The extraordinary success of corneal transplants has been related to the privileged immune status in the ocular microenvironment,[2] where orthotopic corneal allograft can experience prolonged survival even without immunosuppressive treatment.[3,4] It has been suggested that induction of donor-specific ACAID plays a critical role in long-term corneal transplant survival.[5]

Interestingly, many patients with significant corneal disease requiring penetrating keratoplasty have concurrent glaucoma. Previous investigators have reported a high

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Received: 19 November 2007
Accepted in revised form: 21 April 2008
Published online: 23 May 2008

incidence of corneal graft failure, varying between 20 and 50% after drainage tube insertion for the treatment of glaucoma.[6–8] The presence of a glaucoma drainage device was considered by many to be associated with a worse prognosis for graft survival,[6,7,9–11] and a significant and independent risk factor for corneal graft failure.[12] The mechanism for graft failure in these patients is not fully understood. One possible explanation for the graft failure associated with penetrating keratoplasty is a change in ocular immune status after the creation of a communication between the anterior chamber and the subconjunctival space. We hypothesized that glaucoma filtering surgery (GFS) allows for aqueous matter to contact local lymph nodes through the conjunctiva lymphatic vessels, which destroys the induction of ACAID, and increases the risk of graft rejection.

The purpose of this study was to determine whether the induction of ACAID could be interrupted by GFS. To establish the involvement of cervical lymph nodes (CLNs), rats that had their CLNs excised before filtering surgery served as a comparison control group. In addition, FITC dextran was injected into the anterior chamber, and we compared the percentage of FITC-positive cells in CLNs between rats with and without GFS to demonstrate the role of CLNs in this response.

Materials and methods

Animals

Female Sprague–Dawley rats at 8–11 weeks of age were obtained from the Animal Resources Center of Capital University of Medical Science. The rats were maintained under pathogen-free conditions in chaff-lined cages and housed in a 12-h day/night cycle. Each animal was anaesthetized with an intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg). All animals were treated in compliance to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. We certify that all applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research.

Glaucoma and sham filtering surgery

The method for rat GFS has been described in detail elsewhere.[13] Briefly, a limbus-based conjunctival flap was created 3–4 mm behind the limbus by making a conjunctival incision and elevating the underlying Tenon's capsule by blunt dissection. No antimetabolites were applied. A full-thickness scleral tunnel was then created with a 29-gauge needle, which was inserted into the anterior chamber, taking extreme care to avoid the iridal blood vessels. Rats exhibiting any hyphema were not included in the study. Viscoelastic (Healon, 10 mg/ml, Alcon, USA) was injected through the needle to maintain the anterior chamber and the needle was then withdrawn. A beveled 29-gauge over-the-needle microcannula was then inserted through the sclera tunnel. After insertion into the anterior chamber, the needle was withdrawn. The tight fit of the cannula between the sclera tunnel and iris tissue made further anchoring suture unnecessary, and none of the eyes in which surgery was performed exhibited subsequent slippage or dislocation of the cannula. The proximal end of the cannula was trimmed flat approximately 1 mm behind the limbus and a bead of visualized fluid confirmed patency. The bead of fluid was not present in rats with sham filtering surgery, in which the proximal end of the cannula was ligated using a nonabsorbable 5–0 suture (Ningbo Medical Needle Co. Ltd, China). The conjunctiva and Tenon's capsule were closed in a single layer, using a nonabsorbable 10–0 suture attached to a BV needle (Ningbo Medical Needle Co. Ltd, China) in a continuous locking pattern. A filtering bleb formed immediately after surgery, but was not seen in sham filtering surgery rats. A single drop of erythromycin eye ointment (Shenyang Xingqi Pharmaceutical Co. Ltd, China) was instilled after surgery. No additional medications were administered.

Surgical removal of CLNs

After induction of deep anaesthesia, an incision was made in the neck, and the following CLNs (named accordingly to the nomenclature of Tilney) were removed for generation of CLNs in the rat: right and left submandibular lymph nodes, superficial CLNs, and facial lymph nodes. The incision was then closed with several 1–0 nylon sutures (Ningbo Medical Needle Co. Ltd, China).

ACAID induction and DTH assessment

Anaesthetized rats of the unoperated control groups were injected with 50 μg of soluble antigen BSA (Sigma, USA) per 10 μl volume of sterile phosphate-buffered saline (PBS 0.01 M, pH 7.2, positive control, group I) or 10 μl PBS (negative control, group II) into the anterior chamber of the right eye. Animals with GFS (group III), sham filtering surgery (group IV) and excised CLNs before GFS (group V) received the anterior chamber injection of BSA (50 μg/10 μl PBS) at 2 days after the operation. There were six animals in each group. Seven days after the anterior chamber injection, rats received an immunizing dose of 100 μg BSA (emulsified in complete Freund's adjuvant) for a total volume of 200 μl injected

subcutaneously into the forearm. Seven days after subcutaneous immunization, the rats received an intradermal inoculation of the antigen (200 μg/20 μl PBS) into the pinna of their right ear and PBS alone into the left. Before injection, as well as 24 h after injection, the ear-swelling response, as a measure of DTH, was assessed using an engineer's micrometre (Tianjin container factory, China). Ear swelling was expressed as follows: specific ear swelling = (24-h measurement of right ear − 0-h measurement of right ear) − (24-h measurement of left ear − 0-h measurement of left ear). Ear swelling responses at 24 h after injection were presented as group means ± SD. The investigator performing ear measurements was blind as to the treatment of the animals.

FITC-dextran injection into anterior chamber

To perform intracameral injections, a drop of 0.5% proparacain (Alcon, USA) was applied to the right eye of GFS and unoperated control rats. There were 15 animals in each group. Aqueous humour (6–8 μl) was removed from the anterior chamber with a fine glass microcannula (BD, USA) inserted obliquely through the paracentral cornea. A second glass micropipe was then inserted through the same wound and 3 μl (30 μg) of lysine-fixable FITC dextran (molecular probes, USA) was injected into the anterior chamber. Negative control animals (two) were injected PBS alone into the anterior chamber. Leakage of antigen was minimized by introducing a small air bubble into the anterior chamber.

Tissue collection and processing

Animals were euthanized and the following CLNs were collected: right and left submandibular lymph nodes, superficial CLNs, and facial lymph nodes at different times after dextran injection: 1, 3, 5, 7, 12 days, with three animals at each time point in each group. The tissues were assayed for both conventional morphologic examination and flow cytometric assay of FITC-positive cells. One half of the tissues were post-fixed in 4% paraformaldehyde overnight before they were embedded in optimal cutting temperature compound and stored at −80°C. Frozen sections (8 μm thick) were cut with a cryostat (CM1850; Leica, Heidelberg, Germany). Sampling of lymphoid organs in the search for fluorescent antigen was performed on unstained sections by epifluorescence microscopy (DM4000; Leica, Heidelberg, Germany). The other half of the tissues to be used for flow cytometric assay was placed in cold RPMI 1640 medium. The lymph nodes obtained were pressed through nylon mesh to produce a single-cell suspension. After red blood cells lysed, cells were washed twice in 1% BSA/PBS and analysed by Flow cytometry (BD FACSAria, BD Biosciences, USA) using BD FACSDiva. The threshold for the detection of FITC-positive cells was set at 0.2% above the negative control. The percentage of FITC-positive cells was presented as group means ± SD.

Statistical methods

The data of DTH responses were analysed by ANOVA using SPSS. A value of $P<0.05$ was considered significantly different. Statistical analyses were performed using the student's t-test for comparison of the percentage of FITC-positive cells in CLNs between the GFS and the control groups. A $P<0.05$ was required for results to be considered statistically significant.

Results

Induction of ACAID after GFS

The rats with GFS in the right eye exhibited a heightened antigen-specific DTH response (mean ear swelling at 24 h ± SD, 0.28 ± 0.04 mm), which was not statistically distinguishable from the negative control (0.25 ± 0.07 mm; $P>0.05$). The antigen-specific DTH response in rats with sham filtering surgery (0.08 ± 0.02 mm) and rats with CLNs excised before GFS (0.09 ± 0.02 mm), as well as in positive control animals (0.07 ± 0.03 mm), were significantly suppressed compared with the negative control ($P<0.001$—Figure 1). These results demonstrate that the ACAID response is absent in rats with GFS, but is induced successfully in rats with sham filtering surgery and those where CLNs were excised before GFS.

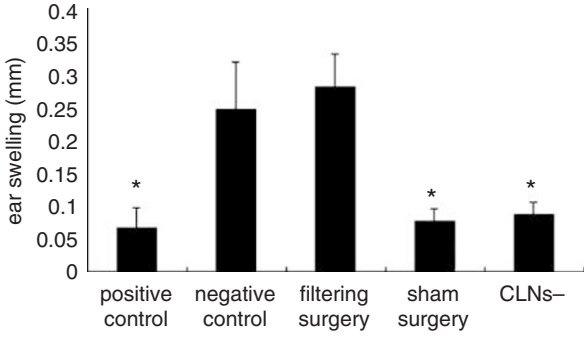

Figure 1 Capacity for ACAID induction, measured as the ability to show DTH downregulation after antigenic challenge. The control animals received antigen (positive) or PBS (negative) inoculations in untreated normal eyes. The ACAID induction capacity is no longer present in rats with GFS, and it is normal in rats with sham filtering surgery and under conditions where CLNs excised before GFS. (*$P<0.001$, compared with negative control).

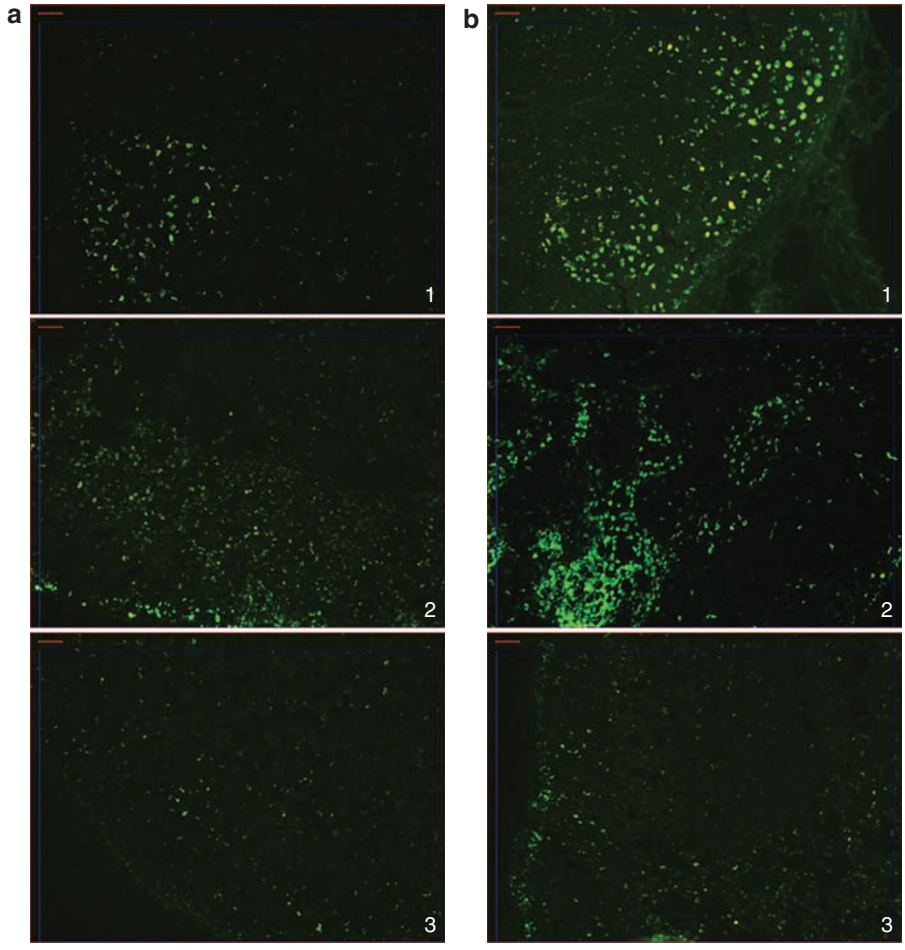

Figure 2 Distribution of fluorescent dextran (green) in frozen sections of a variety of ipsilateral CLNs 24 h after injection into the anterior chamber of normal (a) and GFS (b) rats. Note FITC-positive cells in the subcapsular sinus of the submandibular lymph nodes (1), superficial CLNs (2), and facial lymph nodes(3). Bars represent 50 μm in each image.

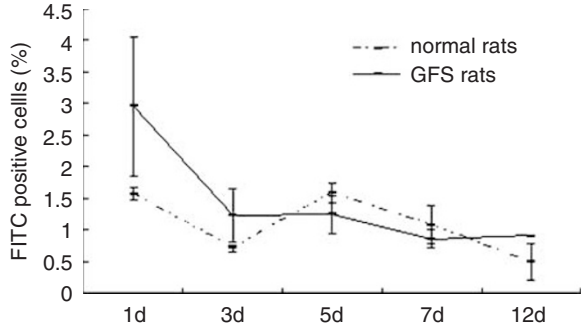

Figure 3 Flow cytometric assay of FITC-positive cell percentage in CLNs at different time points after anterior chamber injection in normal and GFS rats. The percentage of FITC-positive cells in CLNs was significantly increased ($P = 0.001$) in rats of the GFS group (mean ± SD: 2.96 ± 0.67%) *vs* control group (1.57 ± 0.48%) at 1 day after injection. There was no statistically significant difference between the two groups at 3, 5, 7, or 12 days.

Distribution of antigen in the secondary lymphoid organs after intracameral injection

Examination of CLNs from animals that received an injection of fluorescent dextran into the anterior chamber of GFS and normal rats revealed FITC-positive cells in the bilateral CLNs. In the CLNs, FITC-positive cells were located in the subcapsular sinus (Figure 2). The pattern of antigen distribution after intracameral injection in the CLNs was similar at all time periods tested. No fluorescence could be detected within CLNs of animals injected with PBS.

Percentage of FITC-positive cells in CLNs

Flow cytometry analysis of the percentage of FITC-positive cells in CLNs of rats with GFS and control animals was performed. Statistical analysis revealed a significantly higher frequency of FITC-positive cells in

CLNs from GFS (mean ± SD: 2.96 ± 0.67%) *vs* control animals (1.57 ± 0.48%) at 1 day after injection ($P = 0.001$). No statistically significant differences were obtained at 3, 5, 7, or 12 days (Figure 3).

Discussion

Although ACAID induction is the expected outcome when antigen is placed within a normal eye of a rodent, ACAID may be prevented if the antigen is placed in an abnormal eye, or an eye that has been experimentally altered. It has been reported that ACAID is not induced, if antigen is injected into murine eyes where increased numbers of Langerhans cells are present within the central corneal epithelium.[14] Moreover, intrastromal and intracameral injection of interleukin-1 prevents ACAID induction,[15] and both local and systemic administration of interleukin-2 at the time of intracameral antigen injection aborts ACAID induction.[16] Dana *et al*[17] demonstrated that corneal neovascularization leads to an inability to sustain ACAID in anterior segment of the eye. Within this context, GFS also prevents ACAID induction in rats. Since the operation and the microcannula as a foreign body might affect the induction of ACAID, a sham control for this experiment was included that consisted of rats undergoing the microcannula implant in the anterior chamber, but this microcannula is obstructed and failing drainage into subconjunctival space. The demonstration that ACAID can be induced in rats with sham filtering surgery indicates that it is aqueous humour drainage to the subconjunctiva space that affects the induction of ACAID.

Under normal circumstances, the bulk of aqueous humour escapes the anterior chamber of the eye by passing through the trabecular meshwork into the Canal of Schlemm, and thereby directly into the blood. Considerable experimental evidence supports the hypothesis that induction of ACAID requires that antigen-bearing cells from the eye enter the blood stream directly through the trabecular meshwork and first address the immune system on entry into the spleen.[18] As a means to assess this hypothesis. it is important that no lymph node stands between eye-derived ACAID-inducing cells and the spleen. Therefore, we excised the CLNs before GFS to determine the CLN's role in the loss of ACAID. In rats with excised CLNs, GFS cannot prevent the induction of ACAID. In this way, when the CLNs were excised, no effect of GFS on ACAID was observed.

The absence of lymphatics within the eye[19,20] appeared to support the hypothesis that no communication exists between the anterior chamber and regional lymph nodes. Camelo *et al*[21] provided evidence for the existence of an antigen in CLNs, as revealed following injection into the anterior chamber of the eye, and antigen reaches CLNs through both the lymphatic system and the intravenous route. Bill[22] have demonstrated that in an eye with a relaxed ciliary muscle, a significant fraction of aqueous humour (up to 25%) escapes through the uveoscleral pathway. Moreover, while corneal grafts in normal mice show a 50% rejection rate, none of the transplants showed rejection in mice without CLNs.[23] Hoffman *et al*[24] demonstrated that 16% of nanocoll injected intraocularly accumulated in the lymph nodes in mice, which might explain why draining CLNs played a critical role in corneal graft rejection. Such findings are of significant immunologic interest, because ocular contents transported through the uveoscleral pathway escape from the eye through the perivascular spaces of the episcleral tissues and therefore may have direct access to draining CLNs. In this regard, an important issue to address is the means by which CLNs are involved with preventing the induction of ACAID following GFS treatment. As one approach to examine this relationship, in this current experiment, we determined whether the volume of antigen drained to the CLNs from anterior chamber is altered in rats with GFS.

To investigate the role of CLNs more directly, FITC dextran was injected into the anterior chamber of GFS and normal rats. Advantages of using dextran studies of antigen uptake or movement include the ability to use fixable forms, which are stabilized following fixation in paraformaldehyde, thereby preventing their further movement during tissue preparation. Moreover, dextran has recently been used successful to investigate antigen and lymph flow through lymph nodes where it was reported to remain in the subcapsular sinus.[25] Immunofluoresent tracer molecules are very sensitive and this has allowed us to detect minute amounts of antigen in CLNs. The result of our current experiment was similar with that reported previously.[21] The flow cytometric assay allowed us to analyse quantitatively dextran in CLNs derived from ocular tissues at different time points that previously had not been reported. FCM analysis revealed that the percentage of FITC-positive cells in CLNs of GFS rats was significantly increased as compared with normal rats at 1 day after injection ($P = 0.001$). These results demonstrated that the GFS enables the antigen injected into the anterior chamber to have an enhanced degree of contact with the CLNs due to the communication between the anterior chamber and the subconjunctival space.

The immunologic role of the head-and-neck lymph nodes in the induction of ocular immune tolerance is controversial. A tolerogenic role for ipsilateral submandibular lymph nodes has been suggested after peptide immunization in the eye caused activation and anergy of peptide-specific T cells in these nodes.[26–29] The

results of our study show that CLNs play a critical role in GFS prevention of ACAID induction. However, the exact mechanism involved remains to be determined. In addition to CLNs, other possible mechanisms exist and should be explored. For example, the proportion of aqueous humour drainage between the spleen and regional lymph nodes may affect ACAID induction. An important question that follows from our results is whether GFS effects upon ACAID occur under conditions of corneal transplant.

In summary, the results indicated that GFS can prevent the induction of ACAID due to the aqueous drainage to subconjunctival space, and the antigen's draining to CLNs plays a critical role in this effect. The results of our current experiments are important, because they indicate that ACAID may be prevented, if the antigen is placed in an eye where the aqueous humour drainage pathway is altered. Since the aqueous drainage pathway can be altered by disease and medications to reduce intraocular pressure and choroidal detachment, our findings have important implications regarding the resultant immune effects that can occur in these conditions and indicate that the immune status of the eye might be changed after GFS. Such implications may aid in understanding the low success rates of corneal grafts in patients with glaucoma drainage tube implant.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (30600688).

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