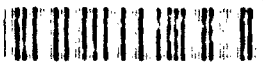

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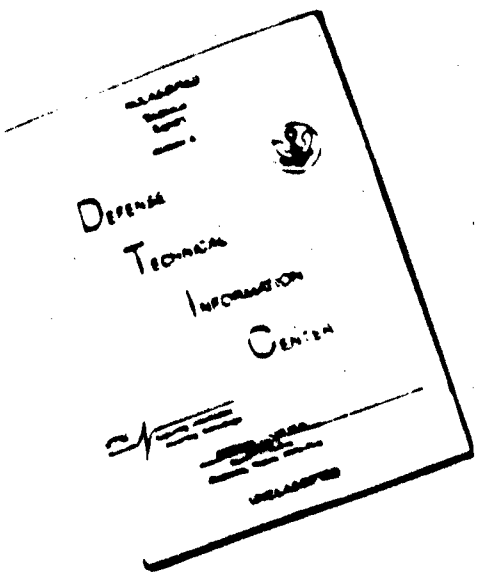

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The project was carried out in response to the increasing incidence of eye injuries, particularly of the retina and choroid, as a result of accidental and other exposures to medium to high energy lasers in the military environment. This research was directed at developing techniques of surgical intervention in an attempt to prevent visual loss which might occur secondary to subretinal and vitreous hemorrhage following exposure to laser energy.

The technical objectives of the research were:

1. Creation of subretinal hemorrhages in the cat model of human laser eye injuries.
2. Comparison of the histopathology of laser injury in the cat and determination of the time interval post-laser injury for optimal surgical intervention.

14. SUBJECT TERMS
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3. The development of improved subretinal microsurgical techniques to evacuate subretinal hemorrhages.

4. The study of the toxicity and efficacy of tissue plasminogen activator (TPA) as a fibrinolytic agent to assist in removal of laser-induced subretinal blood clots.

These objectives were all achieved. We were able to show that it is possible to treat laser induced eye injuries using ultramicrosurgical techniques which we developed. Subretinal hemorrhages were removed with minimal subretinal trauma using TPA injected into the subretinal space in experimental animals. These ultramicrosurgical techniques are now being used in human patients. We found that a window of time exists following laser eye injury extending from day one to day six, during which ultramicrosurgical evacuation of laser induced subretinal hemorrhages has an excellent chance of reducing long term scarring and secondary loss of vision.

Maurice B. Landers, III, M.D. - 5

Final Report

USAMRDC Contract #DAMD 17-89-C-9026

Surgical Treatment of Laser Induced Eye Injuries

April 17, 1989 - 1 July 1992

INTRODUCTION

1. Background:

(a) Basis:

Damage in the human retina/choroid resulting from medium to high energy lasers can be divided into two phases. The first phase constitutes the acute effects of the laser itself, and the second phase is composed of the histological response of the eye to the initial damage. Clearly, little can be done about initial damage after the injurious event. However, it is quite probable that surgical intervention to remove blood and other substances which stimulate tissue proliferation can avoid the development of cellular membranes in the vitreous body and on the inner and outer surfaces of the retina. Such membranes invariably lead to mechanical forces on the retina and underlying structures which lead to loss of vision and blindness. The research on surgical intervention proposed here will include the development of new microsurgical techniques for the removal of subretinal blood, blood clots, and membranes, as well as the use of novel adjuvant enzymes such as tissue plasminogen activator to liquify clots so that they may be removed more easily without destruction of the retina.

(b) Previous work:

The literature now contains many reports of accidental laser exposure leading to ocular damage (Curtain, et al., 1968; Dannhein, et al., 1977; Gass, et al., 1984; Marshall, et al., 1978; Fowler, et al., 1983; Beatrice, et al., 1973; Zweng, et

al., 1967; Bleckmann, et al., 1981; Kearney, et al., 1987; Lang, et al., 1985). The case reported by Kearney, et al. (1987) is representative of many of the cases found in the literature. An accidental eye injury produced by a q-switched Nd:YAG laser target designator was reported in a 21-year old soldier. The Nd:YAG laser target designator had a nominal output of 50 Mj ta 1064 nm and a beam diameter of 4 cm, and was operating with a pulse duration of 20 ns at 10 Hz. The soldier noted a bright flash of light, turned his head, attempted to look away, and covered his eyes with his hands. He immediately noticed a red globular haze in the vision in his right eye and visual acuity rapidly decreased to 20/400. He was found to have a small vitreous hemorrhage, three visible retinal lesions, and each lesion was accompanied by varying degrees of subretinal hemorrhage. The subretinal hemorrhage was confirmed by fluorescein angiography. The vitreous hemorrhage gradually settled and cleared, whereas the laser burns and subretinal hemorrhage resulted in chorioretinal scars. Vision improved to 20/25 one month after the injury, as the chorioretinal scars were not in the central macula. However, four months following injury, the vision had decreased again and there was evidence of striate retinopathy with a fibrous epiretinal membrane. Similar reports by Bleckmann, et al., (1981), Boldrey, et al., (1981), and Manning, et al., (1986) indicate that subretinal and vitreous hemorrhage are common events in medium to high energy laser burns to the retina and choroid. These literature citations suggest a composite picture of the pathological sequelae of medium to

high energy laser exposure to the eye. First, tissue in the retina and choroid is destroyed by immediate thermal explosive injury. This is an acute event which is not reversible. Immediately after such an exposure, hemorrhage occurs at the level of the choriocapillaris, leading to subretinal deposition of blood and often hemorrhage into the vitreous body as well. Such mobile pools of blood then go on to clot, and subsequently become organized as cellular elements replace the thrombus. Eventually, cellular membranes appear in the vitreous body and on the inner and outer surfaces of the retina. When such membranes mature and deposit extracellular matrix, tractional forces are exerted on the retina and underlying structures which lead to detachment of the retina and blindness.

The clinical course and management of subretinal hemorrhage has been addressed by de Juan and Machemer (1988). These authors note similar progression of hemorrhage to fibrotic tissue, although the etiologies of the subretinal hemorrhages considered were different. In general, it appears that once liquid blood becomes clotted, surgical removal of such material, or of cellular scars representing later stages of the histological response, most often leads to severe mechanical damage of the retina and retinal pigmented epithelium. Endosurgical techniques to remove subretinal strands occurring in the natural progression of proliferative vitreoretinopathy are now appearing in the literature (Eckardt, et al., 1985; Trese, et al., 1985). These approaches involve the use of

intentional retinotomy in order to gain access to subretinal strands, which are then grasped with an instrument such as the de Juan forceps, and removed. Intentional retinotomy is also frequently used in order to gain access to the subretinal space in order to remove subretinal fluid (Doft, 1986). In general, de Juan's comments on surgical intervention for subretinal hemorrhage encapsulate the problem. Once a thrombus is formed, and the clot becomes organized by cellular invasion, surgical intervention often involved both destruction to the retina and underlying structures as well as the initiation of proliferative vitreoretinopathy.

Nonsurgical approaches to the problem of intraocular hemorrhage have also appeared in the literature. Several studies have proposed the use of a plasminogen activator, either streptokinase, urokinase, or tissue plasminogen activator (TPA), for the lysis of intravitreal clots or clots in the anterior chamber (Gundorova, et al., 1985; Textorius, et al., 1983; Belkin, et al., 1983; Chen, 1983; Kokacek, 1983; Chattopadhyay, et al., 1982; Forrester, et al., 1982; Bransen, 1978; Peyman, et al., 1978; Chapman-Smith, et al., 1977; Romashchenko, et al., 1985). Although plasminogen activators clearly possess the ability to lyse fresh clots, and in some cases prevent attendant fibrosis, many authors felt that either streptokinase or urokinase were potentially too toxic for ocular structures. With the advent of recombinant tissue plasminogen activator, which is apparently clot-specific, hopes are on the rise that this modality of therapy may become a useful adjunct to surgical therapy for intraocular hemorrhage.

Several articles have dealt with animal models of medium to high energy laser damage and/or subretinal hemorrhage. Although one of these articles, (Glatt, et al., 1982) deals with injection of autologous blood, most studies utilize laser energy to produce subretinal hemorrhage (Chino, et al., 1986; Ishibashi, et al., 1987, Borges, et al., 1987; Mosier, et al., 1987; Docchio, et al., 1987, van der Zypen, et al., 1985; Blankenstein, et al., 1986; van der Zypen, et al., 1986; Brown, et al., 1986; Sameshima et al., 1986, Mosier, et al., 1985; Yumita, et al., 1986; Lorenz, et al., 1986; Yew, et al., 1983). In the vast majority of reports, either the q-switched Nd:YAG or the q-switched ruby laser were used to study ocular damage, although reports on the Nd:YAG in its free running mode, the Krypton laser, and various dye lasers also appeared. In general, with the longer visible wavelengths and near infrared wavelengths, the histological results were very similar. As summarized above, these wavelengths cause deep burns in the choroid, and if applied with enough energy, also lead to subretinal and vitreous hemorrhage. Most if not all of the reactive proliferation which causes further damage to ocular structures can then be directly related to the presence of these hemorrhages.

2. Hypothesis

The hypothesis which was tested in this work is that early surgical intervention for vitreous and subretinal hemorrhage resulting from medium to high

energy laser burns to the retina/choroid would lead to improved visual recovery when compared to untreated controls. This study developed surgical and adjuvant techniques which are directly applicable to the management of vitreous and subretinal hemorrhage in the human eye, resulting from both laser injury and other etiologies such as age-related macular degeneration.

3. Technical Objectives:

The technical objective of the first research milestone was to create a subretinal hemorrhage by injection of autologous blood in the cat which clinically resembled as nearly as possible subretinal hemorrhage seen in human patients. Although the cat retina does not have a macula, per se, it does have an area centralis and is considered to be euangiotic, and is thus an appropriate model. In further experiments with the cat, in which subretinal and choroidal hemorrhages were produced with a q-switched Nd:YAG laser, a comparison was made between the ultrastructure and the burn and resulting histological changes in the cat retina/choroid with such features already demonstrated in other species. Nd:YAG laser-produced injuries in the cat should provided a direct working model for the evaluation of experimental surgical techniques. The objective of the development of new surgical techniques was to overcome damage that may result from the surgical removal of clotted blood or subretinal fibrotic membranes resulting from previous hemorrhage. Among the techniques developed were better microsurgery

under the retina and the use of adjuvant substances, such as tissue plasminogen activator, to dissolve clots. Techniques developed in the cat are directly applicable to human surgery.

4. Military Significance:

The goal of this project was to develop and test surgical techniques for the treatment of hemorrhagic complications of laser induced eye injuries. With the steadily increasing use of lasers for military applications, both as aiming devices and possible weapons, as well as the possibility of exposure of military personnel to other bright light sources, the risk of injury is very clear. This new treatment will speed visual rehabilitation, decrease the extent of injury, and prevent late complications. It can also be used as a therapy for age-related/senile macular degeneration which has similar subretinal hemorrhage of spontaneous origin.

5. Methods:

(a) Preparation of Animals:

Domestic cats, 2-4 kg, either sex, was obtained from the University of California, Davis Animal Resource Services. The cats were given standard veterinary care and vaccinations. For brief procedures of up to 0.5 hours, the cats were given 30 mg/kg Ketamine and 0.3 mg/kg Acepromazine as an intramuscular injection. For longer procedures, the animals was anesthetized with 25 mg/kg

Ketamine and 0.04 mg/kg Acepromazine, and then intubated for Halothane anesthesia with an Ohmeda small animal anesthesia machine. Laryngospasm will was avoided by the topical application of 4% Lidocaine to the vocal cords. The pupils were dilated with a mixture of 5% phenylephrine and 0.25% tropicamide applied to the cornea. Color fundus photographs were made using a fundus camera.

(b) The Injection of Subretinal Blood:

Cats were anesthetized as described above and the pupils dilated. One eye was prepared for eye surgery in sterile operating room conditions. A conjunctival peritomy was performed and the recti muscles secured with 4-0 silk sutures. A 27 gauge needle was placed through the sclera posterior to the equator and the needle placed through the choroid into the subretinal space. The needle was observed through the operating microscope (Zeiss OPMI 6) and a flat-faced contact lens on the cornea. When the needle was visualized in the subretinal space, 0.1 ml of autologous blood was injected into the subretinal space in the area centralis. Pre- and post-operative fundus photographs were obtained with a Topcon fundus camera. Following the creation of the subretinal hemorrhage, the cat was randomly assigned to observation or surgical evacuation of the subretinal hemorrhage.

(c) Creation of Subretinal Hemorrhages:

Subretinal blebs were created in the tapetal retina with a glass micropipette stabilized with a stereotactic micro-manipulator. Glass micropipettes of 550 μm in outside diameter (In Vitro Dynamics, Rahway, NJ) were fabricated to a 40-60 μm outside diameter tip and protected within a retractable 20-gauge sheath (Mellow Manufacturing, San Rafael, CA). Balanced salt solution (BSS) was infused in the subretinal space at a rate of 5 to 10 μl/min for one to two minutes to create a subretinal bleb. A neodymium YAG laser (VisuLas YAG, Zeiss, Germany) was focused through a flat fundus contact lens onto the tapetum just inside the edge of the bleb. The aiming beam was slightly defocused and the laser was discharged resulting in formation of a subretinal hemorrhage. Approximately two to ten laser shots from 10 to 25 mJ were required to create a subretinal hemorrhage. As blood rushed into the subretinal space, the size of the neurosensory retinal detachment variably expanded. Slit lamp biomicroscopy, indirect ophthalmoloscopy, and fundus photography were performed at selected time points. The sizes of the subretinal hemorrhages were measured from the fundus photographs in terms of optic disc diameters (one disc area of cat = 0.9 mm).

(d) Removal of the Subretinal Hemorrhage:

Removal of subretinal hemorrhage was performed using the standard vitrectomy approach. The techniques for removal of subretinal fluid by creating

small holes in the retina and aspirating fluid from underneath the retina using transvitreal aspiration needles have been used clinically. However, the viscosity and possible clotting of subretinal blood has created problems. In this case, the retinotomy needs to be enlarged or possibly a suction-cutting device used to evacuate the subretinal blood. Alternatively, solutions of tissue plasminogen activator were injected to lyse the clot and thus improve the ability of the clotted blood to be removed. Once this technique was perfected, the eyes that were selected for removal of subretinal hemorrhage underwent vitrectomy with removal of vitreous hemorrhage, if any, and also removal of subretinal heme with the retinal reattachment. The retinal reattachment was secured by filling the vitreous cavity with a temporary air bubble at the end of the operation.

(e) Surgical Removal of Vitreous Hemorrhage:

For removal of vitreous hemorrhage, we used the established vitrectomy techniques, at an earlier stage than is usual. Briefly, the cats were be placed under general anesthesia as described above in (a) Preparation of Animals, and prepared for eye surgery under sterile conditions. A conjunctival peritomy was performed and sclerotomies made 3.5 mm behind the limbus superonasally and superotemporally. An irrigation cannula was placed through one sclerotomy for continuous infusion of Lactated Ringer's solution. The Micro-Vit vitrectomy unit wwas placed through the other sclerotomy to remove vitreous blood and vitreous gel. This was done with the aid of the operating microscope and a flat corneal

contact lens. Following the vitrectomy, the sclerotomies were sutured with 7-0 Vicryl sutures and 20 mg of Gentamicin was administered subconjunctivally.

(f) Clinical Examination and Fundus Photographs:

A complete eye examination, including indirect ophthalmoscopy, wwas performed prior to the onset of experiments, immediately following each experimental procedure, and at one week, two weeks, four weeks, and monthly until a maximum of six months. Color fundus photography was performed at the same time points using a Topcon fundus camera.

(g) Histological Examination:

At the end of the experimental protocol, each cat was euthanized with 5 mg Acepromazine, followed by an IV overdose of Pentobarbital (100 mg/kg). Both eyes were enucleated and fixed in 4% glutaraldehyde for 15 minutes, followed by 10% formaldehyde fixation. Each eye was prepared for light electron-microscopy with hematoxylin eosin stain. Selected eyes were prepared for electron microscopy. In those cases, the eyes were placed in 4% glutaraldehyde fixative for at least 24 hours at 4^0C. After primary fixation, the part of the eye of interest was removed with a single edged razor blade. The tissue was then cut for embedding and placed back in the fixative. After washing the tissue with 0.1M cacodylate buffer in 5% sucrose, it was post fixed in 2% $Os0_4$ in 0.1% cacodylate buffer for at least 1.5 hours. The tissue was washed again and dehydrated in increasing concentrations of ethanol (50%, 70%, 80%, 85%, 90%, 95%, and 100%). The

tissues for transmission electronmicroscopy were embedded in Spurr's low viscosity medium.

I. Technical Objectives:

The USAMRDC Contract #DAMD 17-89-C-9026, Surgical Treatment of Laser Induced Eye Injuries was contracted for three years to meet the following technical objectives:

1. Creation of subretinal hemorrhages in the cat model of human laser eye injuries.
2. Comparison of the histopathology of laser injury in the cat, and determination of the time interval post-laser injury for optimal surgical intervention.
3. The development of improved subretinal microsurgical techniques to evacuate subretinal hemorrhages.
4. The study of the toxicity and efficacy of tissue plasminogen activator (tPA) as fibrinolytic agent to assist in removal of laser induced subretinal blood clots.

These objectives have all been achieved. Studies in our laboratory have shown that it is possible to treat laser induced eye injuries using ultramicrosurgical techniques which we have developed. Subretinal hemorrhages can be removed with minimal surgical trauma, using tissue plasminogen activator injected into the subretinal space in experimental animals. Reports of the use of these ultra-microsurgical techniques developed in our laboratory being used in human patients

are now appearing. We have demonstrated that a window of time exists following laser eye injury, extending from day one to day six, during which ultramicrosurgical evacuation of laser induced subretinal hemorrhages has an excellent chance of preventing long-term scarring and secondary loss of vision.

II. Summary of Scientific Progress During the Three Years

Year One

During the initial part of the first year, a fully functional experimental surgical suite and experimental pathology laboratory was established. Various procedures including halothane anesthesia, lateral canthotomy, conjunctival peritomy, globe immobilization, sclerotomy, pars plana phacoemulsificatior, and pars plana vitrectomy performed on cats and rabbits were perfected. Procedures for gross pathology examination and photography, tissue processing into plastic and paraffin for light and electron microscopy, histological staining and preparation of specimens for transmission and scanning electron microscopy were established.

During the middle of the first year, creation of subretinal hemorrhages in the cat model was developed using micropipette technology and micro-pumping system to pump fluid under the retina to create a bleb into which blood was pumped. It was found that a 30 - 60 micron tip of the pipette allowed placement of material under the retina without loss through a large retinal hole.

In the latter part of the first year, evaluation of the natural history of subretinal hemorrhage in the cat followed up to four weeks was completed. Analysis of the control and experimental cat retina with light and electron microscopy revealed that complete degeneration of the outer retina occurred beyond 21 days.

Year Two

The initial part of year two was used to evaluate the toxicity of tissue plasminogen activator (tPA) beneath the retina of the cat. No toxic changes of the retina was found at $\leq$ 200 ug/ml tPA. Irreversible toxicity was found at $\geq$ 1000 ug/ml tPA. The 1000 ug/ml dosage is 50 to 100 times that required for clot dissolution. Thus, tPA can potentially be used at a non-toxic level for dissolution of subretinal hemorrhages.

Studies from the latter part of the second year showed that thick subretinal hemorrhages $\geq$ 3 disc diameters in diameter will produce severe degeneration of the outer retina within 14 days. When followed to 28 days, the degenerative changes actually worsened without evidence of spontaneous recovery.

Concurrent with the discovery of the potential use of tPA, a more accurate, reproducible technique of creating subretinal hemorrhages with the use of the Nd:YAG laser was developed. Furthermore, the laboratory perfected techniques of making micropipettes to outer diameter of 0.35 - 0.55 mm.

Having completed many of the preliminary experimental milestones for the project, the focus of work was then turned to application of the experimental techniques into techniques for human subretinal surgery.

Year Three

Studies from the initial part of the third year evaluated the possible uses of tPA fibrolytic therapy in the setting of subretinal hemorrhages. We wanted to see if tissue plasminogen activator could be injected into the subretinal hemorrhage and 1) left alone to allow a more rapid dissolution/absorption of the hemorrhage or 2) be used to assist removal of the subretinal hemorrhage. When tPA fibrolytic therapy was injected into the subretinal hemorrhage without removal, the subretinal hemorrhage migrated inferiorly. More importantly, irreversible retinal

degeneration occurred at the initial and new subretinal hemorrhage sites. When tPA fibrolytic therapy was used to assist removal of subretinal hemorrhages, minimum to no retinal degeneration occurred. We have also demonstrated that a window of time exists following laser eye injury, extending from day one to day six, during which ultramicrosurgical evacuation of laser induced subretinal hemorrhages has an excellent chance of preventing long-term scarring and secondary loss of vision.

We continued to develop a variety of small gauge metallic needles ranging from 27 gauge to 34 gauge in size. Scanning electron microscopic studies showed that the 33 and 34 gauge needles made self-sealing retinotomies and cause minimal damage to the retinal pigment epithelium. Furthermore, the 33 and 34 gauge needles have been used to remove subretinal hemorrhage and subretinal fluid in the human eye resulting in less iatrogenic injury than when the usual large bore needles are used.

The laboratory also studied the toxicity of thrombin in the subretinal space. One hundred units of thrombin in the subretinal space showed less toxic retinal changes than when 1000 units of thrombin was injected in the subretinal space. Preliminary studies with basic fibroblast growth factor, a drug that might be used to reverse retinal degeneration, have shown no toxicity at a dose of 100 ug/ml.

III. Treatment of Laser Eye Injuries Future Directions

Although the findings from the three year contract demonstrated the feasibility of treating subretinal hemorrhages that are caused by laser injuries, currently there is no treatment available for the initial thermal damage after the laser injury. Direct thermal injury to the retina and retina pigment epithelium (RPE) is now irreversible.

Future studies will test the feasibility of actually treating injured retina and rescuing damaged retinal photoreceptor cells and RPE cells which have been impacted by laser energy. The local use of growth factors, such as basic fibroblastic growth factor, can potentially be used to stimulate the regrowth of injured photoreceptors. RPE transplantation can also potentially be used to replace injured RPE cells. The continued development of new instrumentations such as a micromanipulator to allow fine, microscopic, controlled movements within the eye to do ultramicroscopic surgery would also greatly assist in treating laser eye injuries. Laser lesions which have previously gone on to permanent retinal damage will be thus treated with the hope of returning the damaged retina to normal anatomical configuration and function.

IV. Publications

Publications and presentations directly resulting from USAMRDC Contract #DAMD 17-89-C-9026, Surgical Treatment of Laser Induced Eye Injuries:

1) Benner J, Magliocco MT, Toth CA, Hay A, Hjelmeland LM, Landers MB, and Morse LS. A retractable micropipette holder for subretinal and intravascular ophthalmic surgery. In preparation.

2) Benner JD, Morse LS, Hjelmeland LM, Landers MB. Fibrinolytic assisted removal of experimental subretinal hemorrhage in the cat. In preparation.

3) Morse LS, Benner JD, Dublin M, Hay A, Toth CA, Hjelmeland LM, Landers MB. A morphologic analysis of techniques for controlled detachment of the retina. In preparation.

4) Hay A, Benner J, Morse LS, Landers MB, and Hjelmeland LM. The natural history and effect of fibrinolysis on YAG laser induced subretinal hemorrhage in the cat. Ophthalmol. In press.

5) Benner JD, Huang M, Morse LS, Hjelmeland LM, Landers MB. A comparison of photocoagulation with the argon, krypton, and diode laser indirect ophthalmoscopes in the rabbit. Ophthalmol. In press.

6) Benner J, Morse LS, Hay A, Landers, MB. A comparison of argon and diode photocoagulation combined with supplemental oxygen for the treatment of retinopathy of prematurity. Retina. Submitted.

7) Benner J, Magliocco MT, Toth CA, Hay A, Hjelmeland LM, Landers MB, and Morse LS. Stainless steel micropipettes for subretinal surgery. Am. J. Ophthalmol. 113(6):716-718, June 1992.

8) Toth CA, Benner JD, Hjelmeland LM, Landers MB, Morse LS. Ultramicrosurgical removal of subretinal hemorrhage removal in cats. Am. J. Ophthalmol. 113:175, 1992.

9) Toth CA, Morse LS, Hjelmeland LM, and Landers MB. Fibrin directs early retinal damage after experimental subretinal hemorrhage. Arch Ophthalmol 109:723, 1991.

10) Benner JD, Morse LS, Toth CA, Landers MB, Hjelmeland LM. Evaluation of a commercial rt-PA preparation in the subretinal space of the cat. Arch Ophthalmol 109:1731, 1991.

11) Landers MB, Toth CA, Semple HC, and Morse LS. Treatment of retinopathy of prematurity with argon laser photocoagulation. Arch. Ophthalmol. 110:44, 1992.

12) Toth CA, Morse LS, Hjelmeland LM, and Landers MB. A natural history of subretinal hemorrhage in the cat. Invest. Ophthalmol. Vis. Sci. 31(4): 112, 1990.

13) Morse LS, Toth CA, Hjelmeland LM and Landers MB. Tissue plasminogen activator beneath the retina of the cat. Invest. Ophthalmol. Vis. Sci. 31(4): 307, 1990.

14) Morse LS, Benner JD, Toth CA, Hjelmeland LM, and Landers MB. Fibrinolytic therapy of experimental subretinal hemorrhage. Invest. Ophthalmol. Vis. Sci. 31(4): 4, 1990.

17) Toth CA, Morse LS, Hjelmeland LM, and Landers MB. A cat model for treatment of laser induced subretinal hemorrhage. Submitted to Society of Air Force Clinical Surgeons. Won second place Air Force Surgeon Generals Award, 1989.

18) Landers MB: Surgical treatment for laser induced eye injuries. Report of DARPA Program on Eye and Sensor Protection, Hyatt Regency, Crystal City Hotel, Arlington, Virginia 24 January 1989.

19) Landers MB: Surgical treatment for laser induced eye injuries. Report of DARPA Program on Eye and Sensor Protection, Hyatt Regency, Crystal City Hotel, Arlington, Virginia 26 November 1990.

20) Landers MB: Surgical treatment for laser induced eye injuries. Report of DARPA Program on Eye and Sensor Protection, Hyatt Regency, Crystal City Hotel, Arlington, Virginia November 2, 1991.

Several recent publications imminated from our laboratory and research group relating to USAMRDC Contract #DAMD17-89-C-9026, Surgical Treatment of Laser Induced Eye Injuries. These papers did not result directly from the USAMRDC Contract #DAMD17-89-C-9026, but were possible because of the ongoing research activity and critical mass of scientific personnel brought to our laboratory by USAMRDC Contract #DAMD17-89-C-9026:

1) Hanneken A, Lutty GA, McLeod DS, Robey F, Harvey AK, and Hjelmeland LM: Localization of basic fibroblast growth factor to the developing capillaries of the bovine retina. J Cell Physiol 138:115-120, 1989.

2) Aotaki-Keen A, Ishigooka H, and Hjelmeland LM: Basic fibroblast growth factor is localized to the nucleus of human retinal glia and pigmented epithelium. Invest Ophthalmol Vis Sci Suppl 31:199, 1990.

3) Ishigooka H, Aotaki-Keen A, and Hjelmeland LM: Subcellular localization of bFGF in human retinal glia and pigment epithelium. Exp Eye Res (In press), 1991.

4) Connolly SE, Hjelmeland LM, and LaVail MM: Localization of BFGF in developing retinas of normal and RCS rats. Invest Ophthalmol and Vis Sci Suppl 32:754, 1991.

5) Aotaki-Keen A, Harvey AK, de Juan E, and Hjelmeland LM: Primary culture of human retinal glia. Invest Ophthalmol Vis Sci 6:1733-1738,1991.

6) Ishigooka H, Nishikawa M, Ueno S, Honda Y, Landers MB, and Hjelmeland LM: High voltage cytochemical trials for three-dimensional observation of nuclei and calculation of total numbers of nuclear pores in mueller cells. Investigative Ophthalmology & Visual Science, ARVO Suppl. 31:37 (#183-6), 1990.

7) Hjelmeland LM, Li J, Toth CA, and Landers MB: Antifibrotic and uveitogenic properties of recombinant rabbit interferon gamma in the cell injection of tractional retinal detachment. Investigative Ophthalmology & Visual Science, ARVO Suppl. 31:27 (#137-109), 1990.

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Fi rin Directs Early Retinal Damage After E: perimental Subretinal Hemorrhage

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• bretinal blood within the macula may use visual loss in a number of mac r diseases. The clinical and histo- patt gic effects of experimental sub- retir emorrhage were evaluated in the cat. bretinal hemorrhages were pro- duc by creating a focal neurosensory retir detachment with micropipette tech ques, then inserting a needle tip tran lerally to allow choroidal blood to fill bleb. Experimental lesions were exar ed clinically and with light and elec n microscopy during a 14-day posi erative period. Initial observations incl ed clot organization with retraction of fi n strands. In six of nine clots more than hour old, fibrin was associated with earing of sheets of photoreceptor inne, and outer segments. Later degen- eration progressed to involve all retinal layers overlying the densest areas of fibrin in the clots. Hemorrhages into subretinal blebs containing tissue plas- minogen activator did not form fibrin strands or cause photoreceptor tearing. These findings highlight the potential for improved retinal survival if organized subretinal clot can be eliminated soon after formation.

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Aging macular degeneration is the leading cause of permanent blindness in people older than 60 years in industrialized world.[1] Subretinal hemorrhage from associated subretinal neovascular membranes may result in disciform macular scarring and permanent visual loss. Thus, there has been interest in the removal of subretinal hemorrhage to reduce the final disciform scar and the resultant area of visual loss.[2,3] The natural course and histopathologic features of retinal degeneration over subretinal hemorrhages remain poorly delineated to date. The mechanisms of injury may involve blood products, glial elements, or neovascular membranes.[4]

Studies of experimentally induced subretinal hemorrhages in animals have suggested that blood alone can induce degenerative changes in the overlying retina.[5-11] These studies did not, however, establish the time course of damaging events associated with clot formation beneath the holangiotic (fully vascular) retina.

This study demonstrates a model of subretinal hemorrhage that uses the holangiotic retina of the domestic cat and explores the natural course of early events after such a hemorrhage. The data suggest that fibrin directs the major mechanical injury and secondary degenerative processes in the early weeks after a subretinal hemorrhage.

MATERIALS AND METHODS

Study Design

In the study group, subretinal hemorrhages were created beneath the retina adjacent to the area centralis in one eye each of 10 domestic cats. In one eye, two subretinal hemorrhages were created, thus producing a total of 11 lesions for study. Lesions were observed through 25 minutes to 14 days, with enucleation for histopathologic evaluation at 25 minutes; 40 minutes; and 1, 2, 3, 7, and 14 days. For a control group, focal neurosensory retinal detachments (blebs) without hemorrhages were created in five additional cats by the subretinal microinjection of balanced salt solution (BSS) with transvitreal micropipette techniques. Finally, in two other eyes, a tissue-type plasminogen activator solution (tPA; Genentech, San Francisco, Calif) was used to create the initial bleb into which the hemorrhage was induced. This created two hemorrhages with fibrinolytic agents present before any clot formation.

Animals

All animals were purchased from the Animal Resource Service of the University of California, Davis, Sacramento. We adhered to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. Animals were fed and watered ad libitum and housed as a group with standard fluorescent lighting in 12-hour light-dark cycles. Laboratory studies included a complete blood cell count with differential count, platelet estimate, prothrombin time, and partial thromboplastin time. A dilated fundus examination was performed on all animals on entry into the study.

Creation of Subretinal Lesions

All animals were premedicated with 0.5 mg/kg of subcutaneous acepromazine maleate and 0.5 mL of atropine sulfate, then placed under halothane general endotracheal anesthesia for creation of lesions. A lateral canthotomy was performed. A peritomy was created, and the four rectus muscles were isolated. Bipolar cautery was applied to maintain hemostasis. A pars plana sclerotomy was created 5.5 mm posterior to the limbus and enlarged to 1.5 mm. A limited core vitrectomy was performed with an automated vitrectomy system (Storz, St Louis, Mo) without infusion, to soften the eye. Subretinal blebs were created with a micropipette microinfusion system. A syringe pump (Harvard Apparatus, South Natick, Mass) delivered fluid via intravenous extension tubing to an electrode coupler containing a glass micropipette that measured 40 to 50 μm (outer diameter) at the beveled tip. A stereotactic micromanipulator holding the electrode coupler allowed controlled manipulation of the micropipette within the eye. The micropipette was advanced through the pars plana inci-

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Fig 1.—Fundus photograph of a 1-hour-old subretinal hemorrhage in the area centralis.

Fig 2.—Fundus photograph of the same subretinal hemorrhage as in Fig 1, now 3 days old. A serum-erythrocyte interface is evident along with a central area of organized coagulum. Retinal wrinkling and opacification can be seen over the central clot.

sion and across the vitreous cavity until it penetrated the neurosensory retina. Continuous volumetric pumping of the fluid at 5 L/min for 1 minute was delivered beneath the retina to create a focal neurosensory retinal detachment. For both control and experimental eyes with subretinal hemorrhages, BSS was delivered under the retina. For the tPA group, tPA solution at 200 μg/mL was used to create the bleb. To create an autologous subretinal hemorrhage, the tip of a 20-gauge surgical knife (MVR, Rudolph Beaver Inc, Waltham, Mass) or a 25-gauge needle was passed transsclerally into the bleb and then withdrawn, allowing choroidal blood to fill the bleb and extend under the retina into the area centralis.

The sclerotomy was closed with polyglactin suture and the lateral canthotomy with nylon suture. The eye then received subconjunctival injections of dexamethasone sodium phosphate (2 mg) and gentamicin sulfate (20 mg) and topical applications of atropine sulfate and neomycin and polymyxin B sulfates and dexamethasone.

Postoperative Care

The animals were isolated for 8 to 16 hours after surgery. Topical atropine was applied twice daily and neomycin and polymyxin B sulfates and dexamethasone three times daily for 7 days to the operated-on eye. Animals were followed up with daily penlight examination. Slit-lamp examination, indirect ophthalmoscopy, and fundus photography were performed daily for 3 days and then every 2 to 4 days. For killing, animals were premedicated with an intramuscular injection of ketamine hydrochloride (33 mg/kg), then given an intravenous injection of pentobarbital (85 mg/kg), followed by immediate enucleation.

Tissue Processing

Eyes were incised at the pars plana and placed in 2.5% glutaraldehyde and 1.5% paraformaldehyde with 0.1-mol L sodium cacodylate buffer at 4°C. After 15 minutes, the anterior segment including the lens was excised, and both segments were replaced in fixative. After 24 hours, the fixed posterior eyecups were sectioned, and specific lesions were photographed. Selected areas of tissue were taken for further microscopic analyses.

Microscopy

For light microscopy, tissues were dehydrated in graded alcohols and embedded in hydroxylthylglycol merthacrolate (Polysciences Inc, Warrington, Pa). Sections 2 to 3 μm thick were cut on an ultramicrotome (LKB Ultrotome III; Bromma). These were stained with Richardson's stain.

For transmission electron microscopy (TEM), tissues were postfixed in osmium tetroxide, dehydrated in graded alcohols, and embedded in vinylcyclohexenedioxide-DER 736 (Spurr's) resin (Electron Microscopy Services, Fort Washington, Pa). Sections were cut at a thickness of 75 nm, placed on 3-mm 150 mesh copper grids, stained with lead citrate, and examined in a transmission electron microscope (Phillips 410).

For scanning electron microscopy, tissues were dehydrated in graded alcohols, critical point dried, and placed on aluminum mounts with colloidal silver paste (Peleo; Ted Pella Inc, Redding, Calif). These were sputter coated with gold and examined in a scanning electron microscope (Phillips 501).

RESULTS

Clinical Results

Eleven subretinal hemorrhages were evaluated from 25 minutes through 14 days. Minimal anterior segment inflammation was noted on postoperative day 1, which resolved within 24 hours. Because of bleeding from the pars plana sclerotomy site, three eyes had mild vitreous hemorrhage that settled inferiorly within the 1st hour postoperatively. No vitreous organization was observed over the subretinal lesions. There was no evidence of subretinal blood leaking through the pipette track in any lesion.

The 11 subretinal hemorrhages were initially convex, with a uniform, dense red appearance (Fig 1). They developed a gravity-oriented serum-erythrocyte layering within 3 to 6 hours after hemorrhage. In all lesions, there was a central area of organized coagulum that did not settle into the inferior erythrocyte layer (Fig 2). Once formed, the inferior rim of erythrocytes and the central organized lesion did not shift even with a change of head position for several hours. During the first 3 days, indirect ophthalmoscopy revealed retinal thickening, wrinkling, and increased opacification observed over the central clot. At 7 and

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24 days, the wrinkling was less prominent and the retina appeared less edematous over the lesion.

Two additional hemorrhages were created into blebs containing tPA. Clinically these appeared similar to the study hemorrhages. They did not enlarge or spread during the 1 hour of observation before enucleation.

Three eyes from the control group (B: bleb with no hemorrhage) were followed up for 1 hour, 7 days, or 14 days. Mild inflammation was noted in the anterior segment on postoperative day 1. This resolved within 24 hours. The subretinal blebs were resorbed over 24 hours, leaving unremarkable clinical findings except for a small tan mark identifying the previous needle entry site. The hematologic and coagulation studies for all cats were within the normal ranges for our laboratory.

Histopathologic Results

In all subretinal hemorrhages, fibrin within the organized clot demonstrated dense sites of interdigitation with photoreceptor outer segments (POSs). This occurred in a central area that corresponded to the area of organized coagulum seen clinically. Outside of this central clot, there was scattered fibrin without strand formation. In six of the clots more than 1 hour old, the fibrin strands caused focal traction on POSs with resultant mechanical retinal damage. Throughout the subretinal lesions, fibrin was identified morphologically and by its approximately 22.5-nm banding pattern[12] seen with TEM.

Within 25 minutes, fibrin was observed to interdigitate with the overlying photoreceptor layer (Fig 3). By 1 hour, sheets of photoreceptor outer and inner segments were torn away from the overlying retina at regions of fibrin-POS interdigitation (Figs 4 and 5). Neutrophils were prominent along the torn margins of the outer and inner segments. Except for this fibrin-associated damage, the retina overlying the blood at 1 hour appeared essentially normal by light and electron microscopic examination. Within the subretinal space, the erythrocytes were packed centrally and bordered by the fibrin, platelets, leukocytes, and a rim of serous fluid.

At 1 day, the retina from the outer nuclear layer inward showed minimal degenerative changes over the blood. A gravity-oriented differentiation of the subretinal clot was noted, with serous fluid present between the erythrocytes and the retina superiorly, while less fibrin and a denser packing of erythrocytes was seen inferiorly.

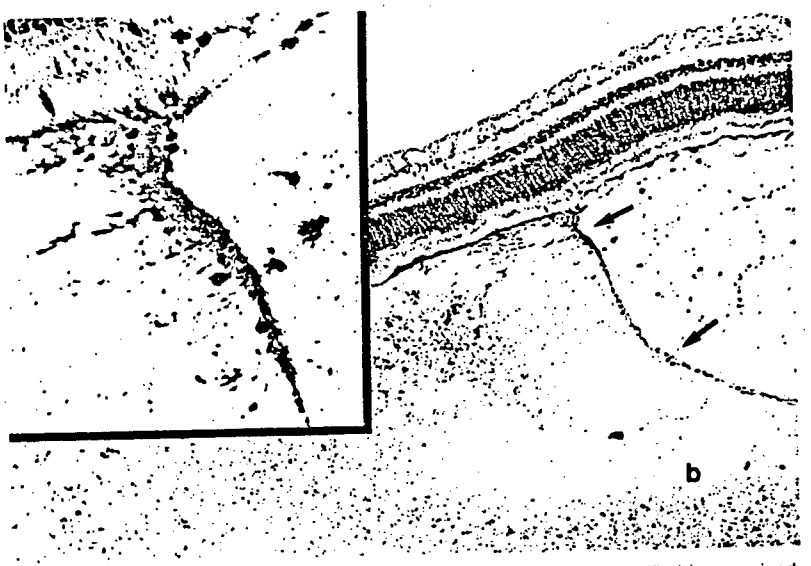


Fig 3.—Fibrin (arrows) in a 25-minute-old subretinal hemorrhage is identified in organized strands extending through the subretinal blood (b) to end in a dense attachment at the photoreceptor outer segments (Richardson's stain, original magnification ×125). Inset. Note the area of attachment of the fibrin strand to the photoreceptor outer segments and the presence of neutrophils along the organized fibrin strand (Richardson's stain, original magnification ×500).

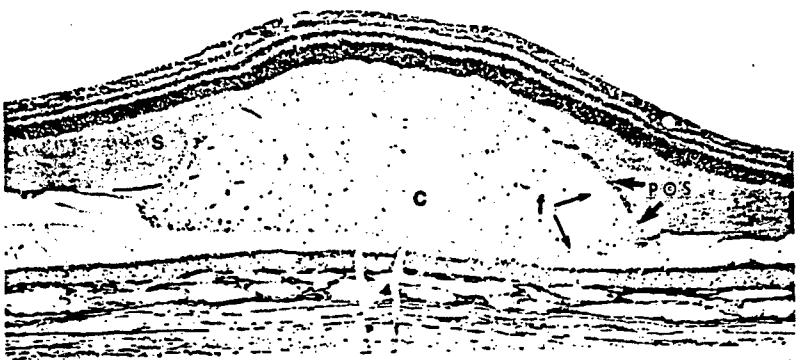


Fig 4.—Clot organization in a 1-hour-old subretinal hemorrhage. The clot (c) is densely packed centrally with criscrossing fibrin strands (f). Marginated over the central clot is a dense layer of fibrin with torn photoreceptor outer segments (POSs) adherent in a sheet to the clot's surface. The serum component (s) separates this from the remaining retinal layers (Richardson's stain, original magnification ×50).

The clot appeared well organized, with macrophages, many leukocytes, and fibrin marginated over the central area of erythrocytes. Channels of fibrin were identified crisscrossing the central areas of blood, and torn sheets of POSs were seen displaced toward the center of the packed erythrocytes. A neutrophil response appeared prominent over the torn sections of POSs and along the remaining disrupted margins of the vacuolating inner segments. By TEM, the photoreceptors appeared minimally vacuolated in areas where they remained intact. The inner retinal layers appeared morphologically minimally affected by the underlying blood.

The lesions on days 2 and 3 demonstrated notable organization of the clot. This continued to appear gravity oriented, with serum located superiorly and erythrocytes packed inferiorly. The fibrin and leukocytic response was prominent at the erythrocyte-serum interface, or at the superior margin of the erythrocytes when the serum had resorbed. In the 2-day lesion, in which the serous component had resorbed, large retinal folds were adherent to

Fig 5.—Transmission electron micrograph of a 1-hour-old subretinal hemorrhage. This area of the clot demonstrates erythrocytes (e) bordered by a layer of lymphocytes, platelets, and fibrin. Note the fibrin (small arrows) interdigitating with the photoreceptor outer segments. Large arrows indicate torn surfaces where photoreceptor inner and outer segments separated from the remaining retinal layers (R) (original magnification × 1400).

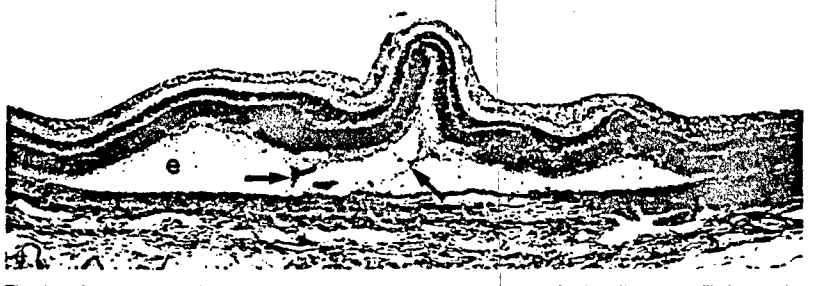
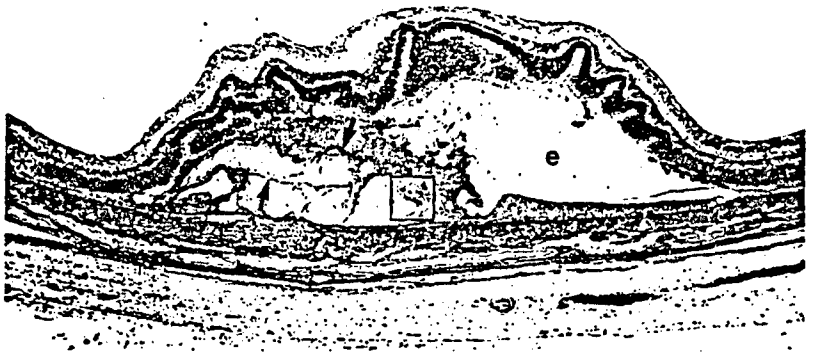


Fig 6.—A 2-day-old subretinal hemorrhage demonstrating retinal folds adherent to fibrin bands (arrows) that crisscross the packed erythrocytes (e) (Richardson's stain, original magnification × 50).

Fig 7.—A 3-day-old subretinal hemorrhage demonstrating the gravity-oriented clot organization and sheets of torn, displaced photoreceptor outer segments. Asterisk indicates the superior margin of the lesion. In this area, the serum has resorbed. The densest area of fibrin organization (arrows) is located at the erythrocyte-serum interface. More inferiorly in this lesion, packed erythrocytes (e) with less fibrin correspond to the settled erythrocytes as seen in a typical clinical picture (Fig 2). Boxed area is enlarged in Fig 8 (Richardson's stain, original magnification × 50).



fibrin bands, which may have been instrumental in their formation (Fig 6). Throughout this lesion, the photoreceptors were intact, without any notable areas of torn POSs. Only few small foci of fibrin associated individual photoreceptor tearing could be found on serial sectioning. The intact photoreceptors showed vacuolization and degeneration. Both 3-day lesions demonstrated large sheets of torn POSs adherent to fibrin bands in the organized area of clot as seen in Fig 7. Both hemorrhages showed some residual plasma superiorly, and one had prominent retinal folding. Phagocytic cells with ingested erythrocytes and POS debris were present in all three lesions in fibrin areas and along the entire outer retinal surface. The neutrophil response was less prominent than at 1 day, particularly inferiorly in the lesions. Vacuolization of attached photoreceptor outer and inner segments was notable. The torn POSs were more significantly deteriorated, with degenerating POSs interspersed in areas of material no longer characteristic of POSs (Fig 8). There was minimal inner retinal vacuolization.

At 7 days, two separate retinal lesions in one eye demonstrated significant destruction of outer retinal elements over the blood. The erythrocytes remained densely packed, with fibrin throughout. The POSs were torn from the retina over the entire surface of the clot in the larger lesion, with more severe degeneration of photoreceptor outer and inner segments into an amorphous band of electron-lucent material (Fig 9). Minimal outer nuclear layer pyknosis and loss occurred centrally. The inner retina demonstrated some increasing vacuolization. Macrophages were identified with ingested degenerated POSs and erythrocytes. A fibrous-neovascular membrane was identified originating from the choroidal stab site within one clot. In both lesions, fibrous cells appeared to organize at the retina and clot margins. Retinal pigment epithelial (RPE) cells showed shortened apical microvilli, mitochondrial distortion, rounding of the apical surface, and reduplication centrally.

By day 14, there was extensive severe destruction of the outer retinal layers over significant portions of the hemorrhagic detachment (Fig 10). Increasing numbers of phagocytic cells were observed in the dense erythrocyte layer and immediately overlying the RPE layer. In peripheral areas over the hemorrhage, photoreceptor outer and inner segments showed mild to severe vacuolization and shortening.

Fig 8.—Transmission electron micrograph of a section of torn photoreceptor outer segments (open arrow) that were displaced centrally in the 3-day-old clot (Fig 7, open box). The segments still interdigitate with a meshwork of fibrin (solid arrow). Note the severe degeneration of photoreceptor outer segments with loss of identifiable cellular elements (original magnification ×2650).

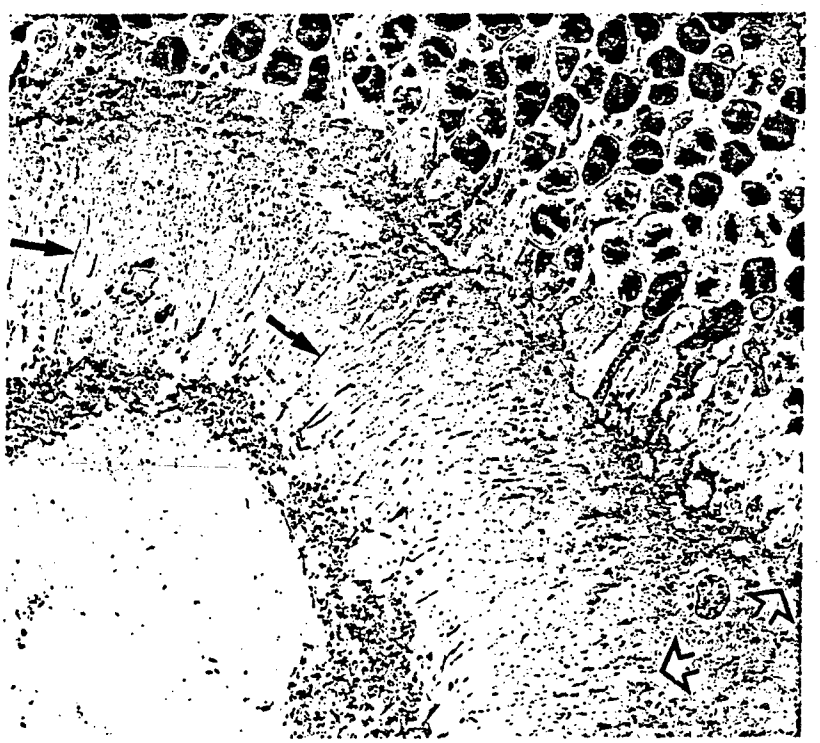


Fig 9.—Transmission electron micrograph of an area of 7-day-old subretinal hemorrhage. Fibrin (solid arrows) is identified interdigitating with amorphous material that resembles degenerated outer segment material (seen also in Fig 7). The photoreceptor outer segments are separated in several areas from the overlying retinal layers (open arrows). The photoreceptor inner segments also show degeneration (original magnification × 1300).

COMMENT

This study demonstrates a model of hemorrhage beneath the holangiotic retina of the domestic cat. Identified within this model is a sequence of clot organization beneath the retina, associated retinal damage, and sites of progressive retinal degeneration during the first weeks after injury. This progressive, focally severe injury occurred over 7 to 14 days after hemorrhage.

Previous studies of subretinal hemorrhage have either used a merangiotic (partially vascularized) retinal model or have not investigated the progression of clot organization in the first 14 days. Koshibu[7] described the degra-

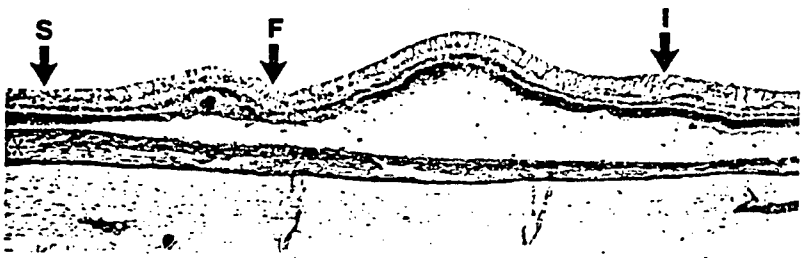


Fig 10.—A 14-day-old subretinal hemorrhage with focal retinal degeneration in areas overlying the resorbing central clot. Asterisk indicates the superior portion of the lesion. The entire lesion is not in the photograph. Minimal retinal damage is seen superiorly (S) where the serum has resorbed and inferiorly (I) over the packed erythrocytes. The retina overlying the serum-erythrocyte interface (F) demonstrates loss and disorganization of outer retinal layers (Richardson's stain, original magnification × 50).

dation and resorption of erythrocytes over 6 months in the rat eye after the subretinal injection of a saline-blood-heparin mixture. He observed POS disruption after 2 days and inner segment degeneration with pyknotic nuclei at 20 days after blood injection. Because of heparinization, this study did not address the issue of the organization of blood elements and the effects of clot formation on survival of the overlying retina. Glatt and Machemer[10] reported irreversible retinal degeneration in their rabbit model of subretinal hemorrhage within 24 hours. A notable finding was that the degeneration was more marked in nonvascularized retinal areas over the hemorrhage. Their examination of subretinal blood in a single cat at 3 days suggested less retinal degeneration than in the rabbit.

In developing the technique to create subretinal hemorrhage in this study, we selected the transscleral technique of Glatt and Machemer and combined it with classic subretinal bleb formation to ensure minimal retinal damage in creation of these lesions. With the transscleral hemorrhage technique, a significant autologous hemorrhage could be consistently produced. Maintaining a low intraocular pressure at the time of choroidal perforation was an important factor in ensuring significant subretinal hemorrhages. The pipette technique, with a low flow of volumetric pumping, induced no significant mechanical injury to the neurosensory retina or RPE during bleb formation. Only focal injury along the pipette track was identified. This was verified by light microscopy, TEM, and scanning electron microscopy of fresh subretinal blebs. With this technique, there was no leakage of subretinal blood through the retinal pipette hole. This was consistent with the observation of Marmor et al,[11] who demonstrated that with a micropipette tip of 40 to 50 μm, one or more pipette tracks had no influence on outflow of materials from a subretinal bleb.

This study was undertaken to determine the early events associated with subretinal hemorrhage in a holangiotic model. The most striking finding in the 1st hour after hemorrhage was rapid formation of a fibrin clot. The density of the fibrin meshwork over and throughout the clot, although variable, was consistently associated with fibrin interdigitation with the photoreceptors. The fibrin appeared to cause the most significant early abnormality due to mechanical shearing of the photoreceptors in six of nine hemorrhages more than 1 hour old. In lesions where mechanical damage to photoreceptors did not occur, striking fibrin attachments were seen between clot and the overlying retina (Figs 3 and 6). The fibrin attachments and associated mechanical damage were absent in the relatively fibrin-free hemorrhages in which tPA was present at formation.

By comparison, in the rabbit model of hemorrhage beneath merangiotic retina, Glatt and Machemer[10] noted fibrin within the subretinal blood but only minimal retinal changes (edematous photoreceptors) 1 hour after hemorrhage. Perhaps the cat inflammatory or platelet response is significantly different from that of the rabbit, or the holangiotic retina responds more rapidly to induce significant fibrin organization.

Fibrin effects also appeared to direct the areas of degeneration in lesions after the 1st day. The area of the most intense degeneration at days 3 through 14 was over the coagulum at the erythrocyte-serum interface. This corresponded to the area of most abundant fibrin and most frequent tearing of POSs. Less retinal degeneration appeared above and below the fibrin-dense area. Indeed, the retina overlying densely packed erythrocytes in the inferior areas of the hemorrhage demonstrated relatively minimal histopathologic changes. This suggests that it is not the mere presence of erythrocytes as a barrier that causes the early severe degeneration, but rather fibrin and perhaps other inflammatory products at the clot interface that are responsible for retinal injury.

One could interpret our findings as showing that the acute fibrin organization with the sequela of photoreceptor disruption induced the later focally severe retinal degeneration. Another interpretation could consider the degeneration secondary only to the inflammatory response in this area. The inflammation, however, might be induced by cell disruption from the mechanical damage.

There is no evidence of significant direct retinal injury from creation of the hemorrhages, control blebs, or the nonfibrin tPA hemorrhages. In all lesions, only the 40- to 50-μm pipette track could be identified, along with a focal site of choroid and RPE disruption corresponding to the penetration site. All histopathologic examination of early tissue demonstrated no significant injury to the retina. Delayed degeneration was in an area much larger than the small perforation site.

An interesting finding is the growth of fibrocytic cells in all of the 14-day eyes and a single larger, organized fibrovascular membrane after a 14-day period. Ryan[11] created many transscleral subretinal hemorrhages while trying to produce subretinal neovascular membranes. The neovascular membranes were not consistently produced in his model.

This model of hemorrhage in the holangiotic retina could be useful for further studies of the cellular events involved in the degeneration of the retina over a clot. The results suggest that fibrin involvement and retinal damage should be more critically examined in the early interval after subretinal hemorrhage and that fibrinolysis may be an important consideration in planning treatment or removal of a subretinal hemorrhage.

Early damage from subretinal hemorrhage with fibrin formation might be a consideration in a human patient with an acute intraoperative subretinal hemorrhage. Removal of the blood before the establishment of a fibrin scaffold may be an important aspect of visual recovery. The fibrin adhesion identified throughout the first 7 d

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the cat model suggest that surgical removal of such a subretinal clot may be inadvisable without the preceding use of a fibrinolytic agent.

In this study, careful clinical examination of the retina over clots revealed no difference in the appearance of lesions that had photoreceptor tearing when compared with those in which no tearing occurred. In a patient it may be similarly difficult to identify whether photoreceptor tearing has occurred or how extensive this may be. If large photoreceptor areas should be torn in a human patient, the retina may do poorly despite fibrinolytic injection or blood removal. The ability to reverse the pathologic sequelae of subretinal hemorrhages with fibrinolytic agents is currently being studied in this laboratory. The time for any attempted subretinal surgery would appear to be before the onset of irreversible damage, which would be at least before 7 days in the domestic cat, or even possibly before the tearing of photoreceptors occurs at 1 hour in this model.

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Ultramicrosurgical Removal of Subretinal Hemorrhage in Cats

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Subretinal hemorrhages are associated with progressive degeneration of the outer retina and a corresponding poor visual prognosis. Mixed results have been reported in previous attempts to remove such subretinal hemorrhages. We developed an ultramicrosurgical system that used the control of a stereotactic micromanipulator to direct a micropipette tip through a small retinotomy into the subretinal space in three cat eyes. Low-dose recombinant tissue plasminogen activator was then introduced into the subretinal space around the subretinal hemorrhage via a controlled microinfusion system. The recombinant tissue plasminogen activator solution facilitated clot lysis and subsequent removal through the micropipette. Light- and electron-microscopic analysis of histopathologic specimens disclosed good preservation of retinal architecture in the three cat eyes in which experimental subretinal hemorrhages were removed. This was in contrast to the retinal degeneration observed in similar but untreated experimental subretinal hemorrhages.

MASSIVE SUBRETINAL HEMORRHAGES are associated with a poor visual prognosis[1] that is most likely caused by a degeneration of the outer retina.[2,3] Accordingly, various surgical approaches have been tried to extract these subretinal clots. These approaches have involved either the use of 20- to 30-gauge cannulas to wash out or evacuate the subretinal blood or forceps to grasp and remove the solid clot or semiliquid blood.[4-10] With any of these techniques, a retinotomy that is at least 310 μm in size is required (30-gauge cannula). A retinal hole of this size typically requires some form of retinopexy and tamponade with intraocular gas or silicone oil to ensure reattachment of the retina. A retinotomy of this size also increases the risk of retinal redetachment and proliferative vitreoretinopathy.[10,11]

We developed an ultramicrosurgical system that uses the control of a stereotactic micromanipulator to direct a micropipette with a 50- to 65-μm outer-diameter tip across the vitreous, through the retina, and into the subretinal space in cats. With this system it is possible to infuse or evacuate various fluids from the subretinal space in a controlled fashion. Using this approach, we infused recombinant tissue plasminogen activator beneath the retina to lyse and remove one- and two-day-old subretinal clots in cats.

Methods

We used 3- to 6-month-old, domestic shorthair cats according to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Laboratory studies including a normal complete blood cell count with differential, platelet estimate, bleeding time, prothrombin time, and partial thromboplastin time were required for entry into the study. Fundus photography was performed pre- and postoperatively.

Creation of subretinal hemorrhages—A subret-

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inal hemorrhage was created in one eye of each cat by first making a subretinal bleb of balanced saline solution with a micropipette and then passing a 20-gauge needle through the posterior sclera and choroid and into the bleb and withdrawing the needle to allow choroidal bleeding into the subretinal space as previously described.[2] Routine veterinary and ophthalmic care was provided pre- and postoperatively as reported previously.[2]

Micropipette fabrication—The glass micropipettes were created from 1.5-mm, thin-walled capillary tubes that were cleaned with 1 M hydrochloric acid, rinsed with distilled water, and dried. The capillary tubes were then pulled into two micropipettes (micropipette puller, Kopf Instruments, Tujunga, California) and ground to the desired size on an air-driven grinding wheel[12] using wet 3-μm abrasive paper. The finished micropipettes were cleaned by suctioning acetone through them. The tips were inspected and measured with a standard light-microscope reticule, before placement in a micropipette storage tray for gas sterilization.

Ultramicrosurgical apparatus—The ultramicrosurgical system consisted of micropipettes connected to a syringe pump apparatus and positioned by a stereotactic micromanipulator. A glass micropipette with an outer diameter of 50 to 65 μm at the beveled tip was placed in an electrode coupler that was mounted in a modified Kreiger and Straatsma vitreoretinal stereotactic micromanipulator (Fig. 1).[13] The stereotactic manipulator provided precision adjustments along two arcs of movement on the side arms and the x, y, and z axes. Standard intravenous tubing was used to connect the electrode coupler to a syringe pump (Harvard Apparatus, South Natick, Massachusetts). The syringe pump was set at 5 μl/min for infusion and between 5 and 200 μl/min for aspiration. Intraoperatively, the cat's head was taped to a fitted foam rubber cushion to stabilize it during the procedure, which was conducted with the cat under general anesthesia achieved by intu-

Subretinal hemorrhage removal—Three subretinal hemorrhages were removed with this system with each cat under general anesthesia achieved with halothane. A pars plana infusion line with balanced saline solution was placed inferotemporally and a pars plana incision was made. A posterior vitrectomy was performed with an automated vitrectomy system over the area of subretinal hemorrhage. With the micromanipulator system, the micropipette was positioned within the vitreous cavity immediately over the superior margin of the subretinal hemorrhage. The micropipette was then advanced through the neurosensory retina and into the clot with the microadvancement drive. Recombinant tissue plasminogen activator solution was infused at a rate of 5 μl/min with the syringe pump to prevent occlusion of the tip and to help define the entry into the subretinal space. A solution of either 10-μg/ml recombinant tissue plasminogen activator (one cat) or 200-μg/ml recombinant tissue plasminogen activator (two cats) in balanced saline solution was pumped into the subretinal space around the hemorrhage for 60 seconds (Fig. 2). Thus, the total dose of recombinant tissue plasminogen activator injected into the subretinal space ranged from 0.5 to 5 μg. The micropipette was removed from the eye; no fluid egressed or backflowed out of the bleb back into the vitre-

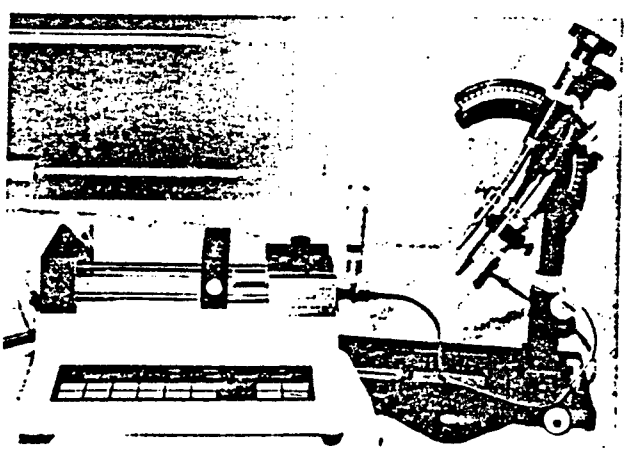

Fig. 1 (Toth and associates). The ultramicrosurgical apparatus. Basic elements include a syringe pump, intravenous connector tubing, an electrode coupler, and a glass micropipette. The electrode coupler and micropipette are held by a stereotactic manipulator. Inset, The difference in size between a glass micropipette with a 60-μm tip and the tip of a 20-gauge needle is shown.

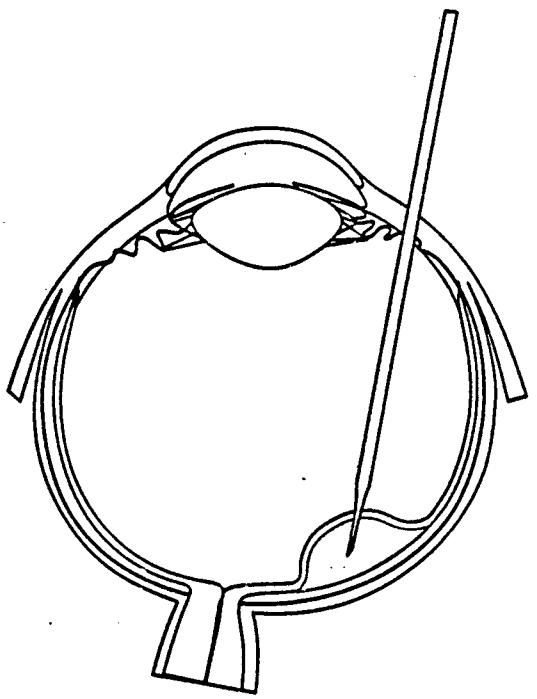

Fig. 2 (Toth and associates). Diagram of the approach to subretinal hemorrhage removal with a glass micropipette. The glass micropipette is inserted transvitreally through a sclerotomy and stereotactically manipulated through the subretinal space. Recombinant tissue plasminogen activator is injected through the micropipette and the subretinal hemorrhage is then aspirated through micropipette after fibrinolysis has occurred.

ous. Within 20 minutes, the clot became gelatinous as it dissolved. A new micropipette with a 65- to 300-μm outer-diameter tip was similarly introduced into the subretinal space either through the previous pipette site or at a new margin of the lesion while infusing a balanced saline solution at a rate of 5 μl/min. The pump was switched to the aspirate mode and the admixture of blood, recombinant tissue plasminogen activator solution, and balanced saline solution was removed from the subretinal space. A thin film of blood often remained beneath the flattened retina. The micropipette system was removed from the eye, flushed free of blood, and reintroduced into the eye. To remove the remaining subretinal blood, the micropipette was advanced into the subretinal space while infusing balanced saline solution at a rate of 5 μl/min to stir up the remaining erythrocytes. This mixture was then evacuated from the subretinal space through the micropipette as before. This procedure was repeated up to three times during a given operation. Despite these efforts, shallow pockets of subretinal blood remained at the end of the operation. In one eye an air-fluid exchange was performed. The micropipette was removed from the eye and the sclerotomy and lateral canthotomy were closed. Standard ophthalmic and veterinary care was provided as previously described.[2] Postoperatively, the cats were monitored with ophthalmoscopy daily and fundus photography when possible for three days and then every two to three days. The cats were killed 14 days after subretinal hemorrhage removal with an intramuscular injection of ketamine hydrochloride, and an intravenous injection of pentobarbital. The eyes were immediately enucleated. The tissues were processed for light-microscopic analysis as previously described.[2]

Subretinal blood removal with metal needles—The previously described technique was also used with several types of metal cannulas instead of the glass micropipettes to attempt to remove subretinal hemorrhages in two eyes. Blunt cannulas and beveled sharp-tipped 25- to 33-gauge needles (210- to 520-μm outer diameter) were used. In all cases it was impossible to remove the subretinal blood through the metal needles, although we were able to inject the recombinant tissue plasminogen activator into the hemorrhages. The cats were killed and the eyes were enucleated.

Results

Clinical results—Before hemorrhage removal, the one- and two-day-old subretinal hemorrhages had the typically formed central coagulum with wrinkling of the overlying retina and an inferior collection of layered-out erythrocytes as reported in our subretinal hemorrhage study (Fig. 3).[2]

No apparent difference in the efficacy of fibrinolysis with either the 10 μg/ml or the 200 μg/ml of recombinant tissue plasminogen activator was observed. In all cases, the formed coagulum dispersed into a reddish mixture within five to 15 minutes after adding the recombinant tissue plasminogen activator solution. Leakage from the pipette entry site in the retina was not evident. No bleeding complications associated with the use of subretinal re-

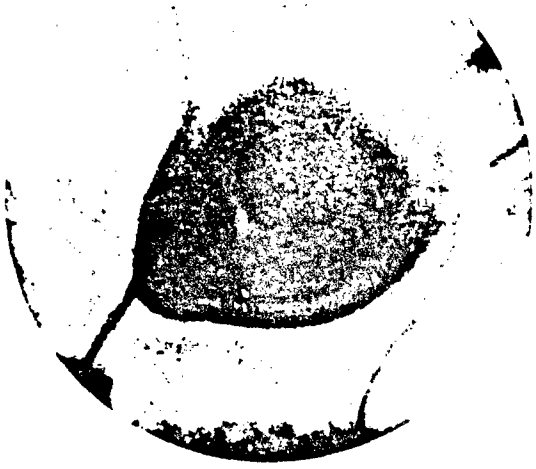

Fig. 3 (Toth and associates). Fundus photograph of a 30-minute-old experimental subretinal hemorrhage in a cat.

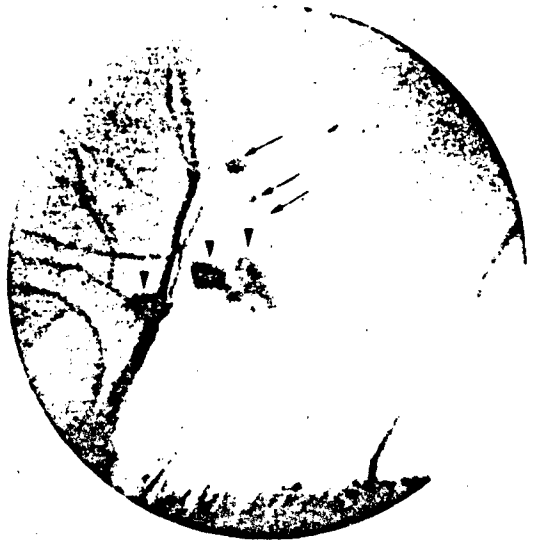

Fig. 4 (Toth and associates). Fundus photograph of the same eye shown in Figure 2, 14 days after removal of the experimental subretinal hemorrhage with recombinant tissue plasminogen activator. The black marks in the area of the previous subretinal hemorrhages are caused by changes in the tapetum from the transchoroidal puncture (arrows) and the micropipette contact (small arrows).

combinant tissue plasminogen activator were observed.

Using this ultramicrosurgical micropipette infusion and aspiration system, subretinal clots were successfully lysed and removed in three cat eyes. The lysed clot and saline mixture was readily aspirated through the micropipette system. Sometimes the blood–recombinant tissue plasminogen activator admixture occluded the glass micropipettes with tips with outer diameters of < 75 μm, but not with tips ≥ 75 μm in diameter. This may have been caused by microscopic bits of clot that occluded the micropipette tips, but were not grossly visible. Micropipette tips 60 to 100 μm in diameter readily passed through the retina into the clot, whereas larger tips (greater than 120 μm in diameter) tended to push the retina away from the pipette before passing through the retina. In preliminary in vitro and in vivo tests, the 30- to 33-gauge (inner diameter, 110 μm) 2-inch-long metal needles occluded with all attempts to withdraw lysed clots.

A small focus of subretinal blood remained after the initial withdrawal in all cases because of a loss of communication through the subretinal space as the retina flattened around the micropipette tip. Redetaching the retina over the entire lysed clot area using slow infusion of balanced saline solution through the micropipette system allowed further access to these areas and a more complete removal of the remaining hemorrhagic foci.

Clinically, the retina was completely reattached over the area of previous subretinal hemorrhage within 36 hours of treatment (Fig. 4). Dark tapetal marks were observed at the sites of previous choroidal perforations that had created the initial hemorrhage. Even finer tapetal marks were seen at the site of micropipette retinotomies. Twenty-four to 36 hours postoperatively, mild wrinkling of the retina was visible, which resolved over the next seven to 12 days. In the eye with a thin residual rim of blood, wrinkling cleared after an additional four days. By Day 8 to Day 12 it was difficult to discern the area involved with the previous subretinal hemorrhage from the adjacent normal retina except for the tapetal marks previously noted. During the two weeks after hemorrhage removal, no rebleeding or vitreous hemorrhage developed.

Histopathologic results—Tissue sections from the area involved with the original subretinal hemorrhage were examined with light and electron microscopy 14 days after subretinal hemorrhage removal. In all three eyes the retinal architecture was intact except for foci with

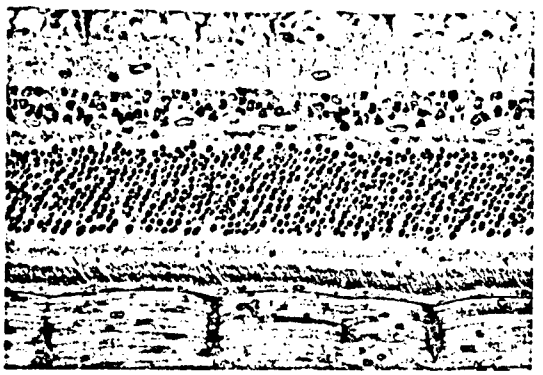

Fig. 5 (Toth and associates). A histologic section of a retina 14 days after removal of an experimental subretinal hemorrhage with recombinant tissue plasminogen activator. The photograph illustrates a full-thickness section of a retina taken from a region that was previously overlying an experimental subretinal hemorrhage that was removed after two days. The cellular architecture is intact except for enlargement of some of the retinal pigment epithelium and a decreased length of the photoreceptor outer segments.

minor changes involving the retinal pigment epithelium–photoreceptor complex (Fig. 5). In these foci the photoreceptor outer segments were short and irregularly aligned as compared to the unoperated-on control sections. Infrequently, a 20- to 40-μm focus of red blood cells or amorphous debris was observed within the subretinal space. With electron microscopy, the photoreceptor outer segments varied from a normal appearance in most areas, to a few sites where they were abnormally short, with some vacuolization and mild distortion (Fig. 6). These changes were accentuated over the foci of residual subretinal material. The retinal pigment epithelial cells were mildly distended, but appeared to be normal otherwise. The photoreceptor inner segments and the remaining inner retinal architecture were ultrastructurally intact.

Fig. 6 (Toth and associates). Electron micrograph of a retina 14 days after removal of an experimental subretinal hemorrhage with recombinant tissue plasminogen activator. The photograph shows preservation of the retinal pigment epithelium, photoreceptor outer segments, and photoreceptor inner segments from a region that was previously detached over a two-day-old experimental subretinal hemorrhage (× 3,300).

Discussion

Evacuation of an experimental subretinal hemorrhage with minimal trauma to the retina was demonstrated using an ultramicrosurgical system to inject recombinant tissue plasminogen activator solution under the retina and to remove the lysed clot. Differences between this approach and those previously reported include the following: (1) injection of low-dose recombinant tissue plasminogen activator solution and evacuation of lysed clot through a micropipette with a small tip that produced a self-sealing approximately 200- to 300-μm retinal hole; (2) use of a micromanipulator to minimize retinal and retinal pigment epitheli-

um trauma while in the subretinal space; and (3) a controlled microinfusion system to minimize trauma from rapid irrigation.

We previously demonstrated that recombinant tissue plasminogen activator solution at a concentration of 1,000 μg/ml is toxic in the subretinal space of cats, whereas doses ≤ 200 μg/ml are nontoxic.[14] Intravitreally administered recombinant tissue plasminogen activator has been used in humans in doses up to 25 μg to lyse large fibrin clots[15,16] and in animal experimental models of subretinal treatment without hemorrhage removal.[17,18] Although recombinant tissue plasminogen activator solution was continuously pumped while we manipulated the micropipette within the vitreous cavity (never longer than 15 minutes with a maximal intravitreal dose of 15 μg), no abnormal intraoperative or postoperative bleeding was observed. Additionally, recombinant tissue plasminogen activator toxicity to the retina involved with the subretinal hemorrhage or to the uninvolved retina was not histopathologically evident.

Features of the glass micropipette that make it ideal for this procedure include the following: (1) its ability to be drawn into a small tip, (2) a low resistance to fluid flow, and (3) a transparent wall. The micropipette tip can be ground to any size ranging from 3 to 300 μm with a microgrinding system. The outer diameter of the micropipette tips that we created was about one-fifth the size of the smallest available metal cannula tip. This small micropipette tip slipped easily through the retina without any need for a preliminary retinotomy. Marmor and associates[19] have demonstrated that retinal holes from micropipettes 50 μm or smaller in diameter do not result in a discernible loss of subretinal solution. Work in our laboratory suggests that tips with an outer diameter as large as 200 μm will also produce self-sealing holes in the retinas of cats (unpublished data). The smaller retinal hole created by the guided micropipette prevented loss of the recombinant tissue plasminogen activator solution from the subretinal space, maximizing its duration of contact with the clot. Application of low-dose recombinant tissue plasminogen activator solution may not be as effective with other techniques that use a larger-bore cannula and larger retinotomy sites because greater amounts of the recombinant tissue plasminogen activator solution may be lost around the cannula at the retinotomy site.

The smallest metal needles that are currently available are 33 gauge with a 210-μm outer-diameter tip. The beveled metal tips of these needles are long and can easily enter the choroid before the inner lumen of the needle has completely penetrated the retina. A 33-gauge cannula with a blunt metal tip deflected the retina ahead of the needle into the choroid before penetrating the retina with excessive injury to both tissues. Once in the subretinal space, the 210-μm blunt tip was, however, effective for subretinal infusion.

Another benefit of the glass micropipette was that it seldom clogged while aspirating the subretinal blood (when > 75 μm in outer diameter). In part, this was caused by the contours of the micropipettes. Despite the small lumen at the tip, the resistance to fluid flow is low because of the short length of this narrowed lumen (< 1 mm) and the rapid increase in the inner diameter to a size of almost 1,400 μm just beyond it. In contrast, the metal needles occluded in all attempts to withdraw blood both in vitro and in vivo.

The transparent glass also allowed for excellent visualization while removing subretinal blood. The metal system provided no visualization to identify movement of blood or sites of occlusion in the lumen.

One drawback to the glass micropipette is the large (1.5-mm) outer diameter of the hub that requires a larger-than-standard sclerotomy. Another drawback is the potential for the glass micropipette to break inside of the eye. One micropipette broke at the point of attachment to the electrode holder and was removed from the eye without complication. The micropipette tip might also fracture while entering the sclerotomy site. The risk of broken glass within the eye is a concern in any potential operation. Accordingly, a micropipette cover might be beneficial in protecting the glass system during entry and removal from the eye.

We found the degree of precision and fine control offered by the micromanipulator to be improved over the standard surgical techniques. This equipment provided controlled advancement and retraction of the surgical tip by micrometers rather than millimeters, resulting in less trauma to the eye. Conversely, free-hand surgical manipulation allows more rapid and flexible movement in approaching the retina, but may tear the retina and injure subretinal structures during lengthy subretinal procedures as a result of tremor.

The added benefit of the microinfusion and microwithdrawal pump in this system was the precise flow control it provided. This allowed a

steadier rate of subretinal infusion and prevented an uncontrolled rapid detachment of the retina. This may be important in preventing the loss of retinal pigment epithelial cells by vigorous subretinal infusion. Vigorous subretinal infusion has been used experimentally to remove the retinal pigment epithelium from Bruch's membrane in preparation for retinal pigment epithelial transplantation.[20] It would seem prudent, therefore, to maintain a gentle flow of solutions beneath the retina to avoid such retinal pigment epithelial damage. This may be responsible, in part, for the relative histopathologic preservation of the three eyes of our cats at 14 days.

This ultramicrosurgical system removed subretinal hemorrhages with a minimum of retinal pathologic sequelae. The relatively intact retinal architecture of the three eyes in which the experimental subretinal hemorrhage was removed was in sharp contrast to the retinal degeneration observed over similar, but untreated experimental subretinal hemorrhages[2] and to the poor retinal preservation in other experimental subretinal hemorrhage treatment studies.[17,18] The areas of mild photoreceptor outer segment changes that we observed after hemorrhage removal were similar to those observed in other models of experimental retinal detachment.[21,22] These changes probably represented reattachment and regeneration changes that might be expected to normalize over a greater time. However, even if these photoreceptor outer segment abnormalities would not improve with time, the condition of the outer retina was substantially better than that of untreated 14-day-old subretinal hemorrhages. Identically produced subretinal hemorrhages resulted in severe outer retinal degeneration with loss of photoreceptor outer segments, photoreceptor inner segments, and part of the outer nuclear layer by Day 14.[2] Although the three eyes in this study fared better than those that were untreated in our previous work,[2] a larger study will be necessary for a true histopathologic picture. We are currently evacuating experimental subretinal hemorrhages at later time intervals to define the window of opportunity during which this surgical intervention might be useful.

Although experimental at this time, some features of this approach might eventually be applicable to subretinal operations. Studies of human subretinal membrane and hemorrhage removal have had mixed results.[4,10,11] Advancing beyond the currently available instrumentation to an ultramicroscopic subretinal surgical system may help to improve the visual outcome in these cases.

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OPHTHALMIC MINIATURE

Knowing he was coming, Peggy had decided against sunglasses, a sign of trust to leave them off. Her wall-eyes are naked to him, her face has this helpless look, turned full toward him while both eyes seem fascinated by something in the corners of the ceiling. He knows only one eye is bad but he never can bring himself to figure out which. And all around her eyes this net of white wrinkles the sunglasses usually conceal.

John Updike, *Rabbit Redux*
New York, Fawcett Crest Books, 1971, p. 99