An Experimental Model of Preretinal Neovascularization in the Rabbit

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Although progressive retinal neovascularization is a potentially blinding complication of several diseases, there are no good animal models. The authors developed a consistent model of preretinal neovascularization in the rabbit by partially digesting the posterior vitreous with repeated injection and aspiration of 1 IU of hyaluronidase before injection of 250,000 homologous dermal fibroblasts. The evolution of the new preretinal vessels was monitored by indirect ophthalmoscopy, fundus photography, and fluorescein angiography. A grading system was devised using fundus photographs and fluorescein angiograms to describe the progression of new vessel growth and the extent of fluorescein leakage. Ninety-five percent of the eyes had vascular engorgement and hyperemia but no fluorescein leakage by day 1. Fifteen percent of the eyes had clinically evident new preretinal vessels, and 32% had severe fluorescein leakage by day 7. Ninety-five percent of the eyes had definite neovascularization by day 14. Severe fluorescein leakage peaked at day 14 (55% of the eyes) and decreased thereafter. Involution or atrophy of the vessels occurred in all eyes by day 42. This model will be useful for studying the pathogenesis of preretinal neovascularization and evaluating potential treatments for its prevention. Invest Ophthalmol Vis Sci 32:46–52, 1991

Progressive retinal neovascularization (NV) is responsible for severe visual loss in numerous disease processes. The most prominent are diabetes mellitus, retinal vein occlusion, and retinopathy of prematurity. Despite various modalities of therapy (panretinal photocoagulation, cryopexy, and vitrectomy), progressive retinal NV continues to occur resulting in severe visual loss.1–7

Many investigators have attempted to induce retinal NV experimentally by intravitreal injection of dermal fibroblasts,8–12 intravitreal implants impregnated with potential angiogenic stimulators (eg, Interleukin-1),13 laser photocoagulation of retinal veins,14–16 hyperoxygenation,17–19 and intravitreal tumor cell transplantation.20 Normotensive and spontaneously hypertensive Royal College of Surgeon rats also develop retinovitreal NV.21,22 Unfortunately, none of these models consistently yield a high rate of NV.

Our goal was to develop such a model so that mechanisms governing the development of preretinal NV and new modalities of therapy could be evaluated. We also devised a grading system of preretinal new vessel growth and fluorescein leakage (FL) to ensure that the results of subsequent studies using the model can be compared.

Materials and Methods

Preparation of Cultured Fibroblasts

Homologous dermal fibroblasts (grown from explants of rabbit rump skin) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, antibiotics, and antimycotics (penicillin sodium, streptomycin sulfate, and amphotericin B) and maintained in a humidified atmosphere of 5% carbon dioxide in air.8,9 The cells were allowed to reach confluence and split 3 days before injection to ensure maximum proliferative activity. Cells were harvested by incubating them with 4 ml of 0.25% trypsin and 0.02% edetic acid for 4 min and collecting the cells in stop media (Dulbecco’s modified Eagle’s medium with 10% calf serum plus antibiotics and antimycotics). The dispersed cells were centrifuged at 900 rpm for 10 min and resuspended in 4 ml of phosphate-buffered saline (PBS). An aliquot was re-
moved for counting and viability testing by trypan blue exclusion which always resulted in greater than 95% viability. The cells were then centrifuged again, the media discarded, and the cells resuspended in PBS to reach a final concentration of 250,000 cells per 0.1 ml.

Preparation of Hyaluronidase

Lyophilized bovine testicular hyaluronidase (Wyeth; Wyeth, Philadelphia, PA) was reconstituted with 0.9% sodium chloride to obtain a final concentration of 1 IU per 0.1 ml of saline.

Intravitreal Injections of Hyaluronidase and Fibroblasts

Twenty pigmented Dutch belted rabbits weighing 1.4–2.5 kg were anesthetized with ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5 mg/kg), and their pupils were dilated with topical phenylephrine 0.5% and tropicamide 0.25%. All procedures were in accord with the ARVO Resolution on the Use of Animals in Research. The eye was propapsed, and a 25-gauge needle was inserted through the sclera and retina 4 mm posterior to the corneoscleral junction in the superotemporal quadrant under stereomicroscopic control. With the bevel of the needle directed downward, 0.1 ml of 0.9% saline containing 1 IU of hyaluronidase was injected over the nasal vascularized wing and disc. The injection was done under direct visualization using an irrigating contact lens. The needle was then swept over the vascularized wings and optic disc. The sweeps were made as close to the retinal surface as possible to disrupt mechanically the vitreal attachments in these areas. A hyaluronidase-vitreous mixture was aspirated and reinjected several times until the vitreous was partially mechanically destroyed and liquefied. Liquefied vitreous (0.2 ml) was then aspirated into the syringe, and the needle was removed. A separate 25-gauge needle was passed through the previous puncture site, and 250,000 fibroblasts suspended in 0.1 ml of PBS (pH 7.0) were injected with a sweeping motion as close as possible over the vascularized wings and disc. The rabbit was then placed on its back for 30–60 min to allow the cells to settle onto the vascularized wings and disc.

Clinical Examination

All animals were examined preoperatively and postoperatively using indirect ophthalmoscopy, fundus photography (Topcon TRC 50 VT Paramus, NJ) and fluorescein angiography. All 20 rabbits were examined on days 1, 3, 7, 14, and 28, and detailed fundus drawings were made. Ten rabbits were also examined on days 21, 42, 56, and 84 to follow the natural progression of the new vessel growth.

Grading of NV

Fundus photographs and fluorescein angiograms were arranged in a temporal sequence so that the grader could evaluate the progression of the new vessels over time. The following grading system was developed (Fig. 1). Grade 0 showed no vascular abnormalities. Grade 1 had engorged, tortuous, hyperemic preretinal vessels. Grade 2 had microvascular abnormalities. The contact of a strand or clump of cells with vascularized retina caused the appearance of a red vascular ring around either the base of the strand or the clump of cells. We knew from previous light- and electron-microscopic studies of similar areas that new capillary buds were present, but we could not identify them clinically. To describe this situation adequately (where the observer was highly suspicious of new vessel growth but could not clinically identify it), we used the term microvascular abnormalities. Grade 3 had easily identifiable individual capillary loops growing into a strand, a corona of vessels with a fine brush border or a glomerular tuft. The new vessels usually had a bright red appearance with minimal associated white fibrous tissue. Grade 4 had a combination of actively growing vessels and vessels undergoing involution. The involutorial changes usually included a combination of the following findings: (1) a decrease in the redness of the corona or brush border, (2) a decrease in the density of the capillaries comprising the brush border, (3) an increase in the ability to identify individual capillary loops, (4) an increase in capillary diameter, or (5) the formation of large capillary loops. These changes were often associated with an increase in white fibrous proliferation. Grade 5 had fine vessels replaced by large vascular loops or atrophic-appearing fibrous tissue. At this point new vessels were sometimes difficult to identify or were barely visible.

Grading of FL

The extent of FL was graded by comparing early phases of the angiogram (usually less than 15 sec) with late phases (usually greater than 180 sec). Grade 0 showed no evidence of leakage (Fig. 2). Grade 1 showed mild to moderate leakage not obscuring the vascular pattern of NV (Fig. 3). Grade 2 showed severe leakage obscuring the individual capillaries (Fig. 4). The outline of the vascular pattern of the new vessels but not the fine detail of the individual capillaries could be distinguished.
Fig. 1. (A) Grade 0 neovascularization. Preoperative photo of rabbit fundus showing normal appearance of preretinal vessels. All subsequent figures are of the same eye over time (Rabbit 1029 OD). (B) Grade 1 neovascularization. Day 1 post-fibroblast injection. Hyperemia (arrow) and vascular engorgement. (C) Grade 2 neovascularization. Day 3. Note ring of redness (arrow) surrounding cells that have settled on the vascularized retina. (D) Grade 3 neovascularization. Day 14. Clinically evident new vessels in the proximal and distal aspects of the strand. (E) Grade 4 neovascularization. Day 28. Areas of actively growing vessels combined with involuting vessels. Note the decreased hyperemia and density of new vessels. Large capillary loops are present along with increased white fibrous tissue. (F) Grade 5 neovascularization. Day 57. Few vessels remain and they have an atrophic appearance.
Grading of Proliferative Vitreoretinopathy

The progression of proliferative vitreoretinopathy (PVR) was graded using the staging system originally described by Fastenberg et al. Grade 0 showed no abnormalities in the fundus. Grade 1 showed intravitreal membranes, strands, cellular debris, and/or a small pucker(s). Grade 2 showed focal retinal traction, a large pucker(s) causing retinal traction, and distortion or peripapillary traction. Grade 3 showed localized retinal detachment of one or both medullary ray(s) with an open-funnel appearance. Grade 4 showed extensive retinal detachment usually involving both medullary rays, peripapillary retina and a portion of the avascular retina; there was a closed-funnel appearance with the disc no longer visible. Grade 5 showed total retinal detachment. At this stage the retina had been drawn anteriorly and centrally forming a tight stalk which originated at the optic disc and continued anteriorly along the strand of fibroblasts to the entrance site. With the contraction of the strand the retina was eventually drawn into a retrolenticular location.

Results

NV

The progression of NV, FL, and PVR is summarized in Figures 5-7. On day 1, 19 of 20 eyes (95%) had hyperemia and vascular engorgement (Grade 1 NV) (Fig. 5). The remaining eye had microvascular abnormalities (Grade 2 NV). New preretinal vessels (Grade 3 NV) were first noted clinically on day 7 in 15% of the eyes. The new vessels tended to grow into, around, and along the strands of fibroblasts which had attached to the vascularized retinal wing. Occasionally, new vessels were seen to grow into the peripheral aspect of the strand from the entrance wound. By day 14, 19 of 20 eyes (95%) developed Grade 3 NV.

Involutional changes of the new vessels were first noted on day 21 when 20% progressed to Grade 4 NV (Fig. 5). The remaining eyes (80%) had Grade 3 NV. By day 28 and 42 the number of eyes with Grades 4 or 5 NV increased to 85% and 100%, respectively.

FL

On day 1, 19 of 20 eyes (95%) had no evidence of FL (Fig. 6). Its severity markedly increased between days 1-7 and peaked on day 14. Nine of 20 eyes (45%) displayed mild to moderate (Grade 1) FL, and
11 of 20 eyes (55%) exhibited severe (Grade 2) FL on day 14. By day 28 most eyes had Grade 1 FL.

**PVR**

The progression of PVR was rapid and moderately severe (Fig. 7). On day 1, 17 of 20 eyes (85%) had strand formation alone or combined with focal traction (Grade 1 or 2 PVR; Fig. 1B). Three of 20 eyes (15%) had extensive retinal detachments (Grade 4 PVR). Many of the eyes had large amounts of white debris in the inferior vitreous cavity. By day 3, 16 of 20 eyes (80%) had some degree of retinal detachment (Grade 3 or worse PVR). In addition to the epiretinal debris seen in many eyes with detachments, there were subretinal gray-white balls located inferiorly. The remaining four eyes had Grade 1 or 2 PVR. All 20 eyes (100%) had total retinal detachments (Grade 5 PVR) by day 14. No eyes spontaneously reattached their retinas. The height of the detachment progressed until the retina was drawn to a retrolenticular location (Figs. 1D–F). The detachments at this point had tightly closed funnels with a central white stalk and very thin attenuated peripheral retina. Most of the time the traction was so severe that holes were torn in the retina. Occasionally the traction resulted in detachment of the pigmented epithelium overlying the ciliary processes and drawing in of the processes toward the posterior center of the lens.

**Discussion**

Although many investigators have attempted to develop reliable and reproducible models of retinal...
NV, most of those available do not consistently result in NV. For instance, Sugita et al.\(^8\) observed new vessels in only 20% of rabbit eyes after intravitreal injections of autologous dermal fibroblasts. Tano et al.\(^9\) increased the NV to 58% by improving culture techniques. Ophir et al.\(^10\) observed NV rates of 45% and 88% using autologous and homologous cells, respectively, but they did not specify whether these rates were based solely on clinical observations or a combination of clinical and histologic observations. In contrast to the varying rates of NV observed in these studies, we were able to induce preretinal vessel growth consistently in all rabbit eyes using intracocular injections of hyaluronidase and homologous tissue-cultured dermal fibroblasts.

The severity and rapidity of retinal detachments in previous fibroblast injection models was proportional to the number of fibroblasts injected\(^25\) and whether or not the vitreous was intact.\(^27\) These models either resulted in retinal detachments so severe that further studies were not possible or required a prolonged follow-up time. They established, however, that the presence of a retinal detachment was necessary for the development of clinically visible NV. Our goal was to produce rapidly developing retinal detachments of intermediate severity. In addition, we wanted a model that was simple and easy to use. We found that repeated injection and aspiration of hyaluronidase enabled us to disrupt the vitreous mechanically and chemically.\(^23\) This resulted in the formation of a pocket of partially liquified vitreous in the posterior vitreous cavity. This maneuver also seemed to increase surfaces to which the injected fibroblasts could adhere. The dispersion of cells only in the posterior vitreous cavity over the vascularized retinal wings maximized the potential for the rapid development of retinal detachments and subsequent NV. We showed in a previous study that 1 IU of hyaluronidase is not toxic to the rabbit retina.\(^23\)

The growth of preretinal vessels proceeded in a predictable fashion. Within 24 hr of injecting the fibroblasts 95% of the eyes showed evidence of vascular irritation such as hyperemia and vascular engorgement. Based on previous histologic studies,\(^11\)\(^12\)\(^24\)\(^26\) we attributed these early vascular changes to the presence of inflammatory mediators. In particular, polymorphonuclear leukocytes and lymphocytes have been noted in the vitreous of rabbits injected with homologous fibroblasts as early as 1 day after cell injection.\(^24\) Monkeys sensitized to insulin or bovine serum albumin, followed by intravitreal injection of the same antigen, develop optic nerve head NV, also implicating inflammation in the pathogenesis of intracocular NV.\(^28\)

The new vessel growth rate peaked by day 14 with 95% of eyes exhibiting Grade 3 NV. Thereafter the growth of the new vessels declined, evidenced by increased fibrous tissue, decreased hyperemia, loss of the fine capillary brush border appearance, increased diameter of residual capillaries, and the formation of large vessel loops. This decline in vascular growth has been noted at the cellular level in previous models using autoradiography.\(^24\) These studies showed that tritiated thymidine labeling decreased after 14 days. In addition, electron microscopy of 14- and 28-day specimens showed mature-appearing vessels with thin walls, wide lumens, and a well-defined basal lamina over the distal portions of the developing vessels. In our model, new vessel growth continued to decline, although at a slower rate after day 28, with 100% of the eyes exhibiting vascular involution or atrophy by day 42.

The orderly progression of NV we noted correlated with the changes in FL and the extent of PVR (Figs. 5–7). The severity of FL increased as NV developed and declined as new vessels began to involute. At the peak of new vessel growth (days 14 and 21), there was a corresponding peak in vascular leakage (45% and 50% Grade 2 FL, respectively). During this time we noted a marked increase in the severity of PVR with 100% of the eyes exhibiting Grade 5 PVR by day 14. We suggest that our preliminary observation that a tractional retinal detachment was a prerequisite to the consistent development of clinically evident new preretinal vessels was true.

We must be careful in generalizing our observations of angiogenesis in the epiretinal vessels of the rabbit to angiogenesis of intraretinal vessels in other species. Lowry et al.\(^29\)\(^30\) demonstrated that the retina of the rabbit, because of its lack of vascularity, depends primarily on the glycolytic pathway for its me-
tabolism. By contrast, the vascularized retina of the monkey has substantially higher activities of the oxidative pathways of glucose metabolism (eg, the tricarboxylic acid cycle and pentose shunt). These marked metabolic differences between vascularized and avascular retinas could produce crucial differences in the angiogenic responses of vessels in or near those retinas.

In summary, we developed a reliable and reproducible technique for the rapid induction of new preretinal vessels in the rabbit using intravitreal injections of hyaluronidase and homologous tissue-cultured dermal fibroblasts. This model will be useful in further studies of the pathogenesis of preretinal NV and its prevention. For example, we recently showed that NV can be prevented in this model by intravitreal injection of triamcinolone acetonide 24 hr before fibroblast injection.31

Key words: fluorescein angiography, hyaluronidase, neovascularization, retina, vitreous

References