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Neovascularization in Experimental Retinal Venous Obstruction in Rabbits

Masato Tamura

Department of Ophthalmology, Hirosaki University School of Medicine, Hirosaki, Aomori, Japan

Purpose: To investigate the later pathohistological changes in experimental retinal venous obstruction in rabbits.

Methods: Experimental retinal venous obstruction was produced in retinal blood vessels of rabbits by trans-adventitial dropping of thrombin. Fundus observation was performed 3 months and 1 year thereafter. Retinal histological examinations were performed by fluorescein microscopy, light microscopy, and transmission electron microscopy.

Results: Vessels with a rete mirabile and arteriovenous anastomosis were observed after 3 months. On a flat gelatin-fluorescein preparation, regions with no perfusion, thought to be areas of vascular occlusion in the periphery, were observed extensively. Clear leakage of gelatin-added fluorescein was noted from vessels in the periphery, and minute neovascularization with a rete mirabile from these vessels was confirmed. Newly formed retinal vessels were *also* observed by transmission electron microscopy. The endothelial cells of these newly formed vessels had a large nucleus, a number of ribosomes, and thin basement membrane. Proliferative changes included glial cells that had penetrated into the basement membrane of ghost vessels.

Conclusion: Our long-term observations confirmed that proliferative changes and neovascularization occurred in this model of retinal venous obstruction. **Jpn J Ophthalmol 2001;45:144–150**

Key Words: Experimental retinal venous obstruction, long-term observation, newly formed vessels, proliferative change, thrombin.

Introduction

Central retinal vein occlusion is an ocular circulation disorder that is encountered frequently in clinical practice. If left untreated it can cause neovascularization and proliferative changes in the terminal stage. A reliable treatment for this disease, as first described by Michel[1] in 1878, has not been entirely established. One of the reasons for this lack of clarification is the lack of an animal model that is useful and suitable for studying the disease. As a result, sufficient pathohistological investigations have not been performed. To date, the methods for inducing retinal vein thrombosis in experiments have used a laser beam [2–9], diathermy,[10] or physical means of impairing the entire vascular wall, such as mechanical ligation.[11] These methods, however, may be unsuitable for studying the mechanism of the development of retinal bleeding or absorption as a major initial symptom of the disease.

Based on the experimented findings that thromboplastin in the perivascular connective tissue is involved in the triggering of the extrinsic coagulation mechanism, Sakuraba[12] and Matsumoto[13] have successfully created retinal vein obstruction by dropping thrombin on the wall of the rabbit retinal vein, with no mechanical damage to the vessel or tissue (called the Hirosaki model). In this study, the author has performed pathohistological and electron microscopic examinations over an extended period of time to ascertain whether proliferative changes and neovascularization occur in this Hirosaki model as late lesions, similar to those in a human eye.

Received: April 14, 2000

Correspondence and reprint requests to: Masato TAMURA, MD, Department of Ophthalmology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki-shi, Aomori-ken 036-8562, Japan

Published by Elsevier Science Inc.

0021-5155/01/$–see front matter
PII S0021-5155(00)00353-1

Materials and Methods

In the experiment, we used 53 eyes of 29 albino rabbits weighing 2.5 kg each, about 5 months of age. As confirmed by funduscopy, no abnormalities were present. Mydriasis was first performed through instillation of tropicamide and phenylephrine hydrochloride, and the animals were anesthetized by intravenous injection of 25 mg per kg of body weight of sodium pentobarbital through the auricular vein. A Goldmann-type contact lens for fundus observation was then placed on the cornea. While the ocular fundus was observed through an operating microscope, a 27-G needle was injected into the vitreous cavity through the pars plana, and under direct vision, 0.01 mL (5 units) of a thrombin solution was dropped on a retinal vessel. The thrombin solution was prepared with 5,000 units of a thrombin lyophilizing agent (Mochida, Tokyo) dissolved in 10 mL of physiological saline solution for the experiment.

Two additional rabbits were used as controls; they were treated with only 0.01 mL of physiological saline solution.

Fundus observation was performed 24 hours after thrombin instillation, and incidence of retinal venous obstruction was investigated. Fundus photography of 10 rabbits, by which retinal venous obstruction was successfully observed, was performed before the thrombin was dropped and after 24 hours, 1 week, 1 month, 3 months, and 1 year. A series of histological examinations were performed at 3 months in 10 eyes of 5 animals and at one year in 7 eyes of 5 animals, using a method to fix and extend the preparation with an intravenous injection of gelatin-added fluorescein (intravenously injected gelatin-fluorescein preparation technique).[14] Briefly, a mixture of 4 mL of 10% gelatin solution and 1 mL of 10% fluorescein sodium solution was intravenously injected, and penetration into the eyes was confirmed by the yellow change in color of the conjunctiva. Then, sodium pentobarbital was administered in an overdose, and

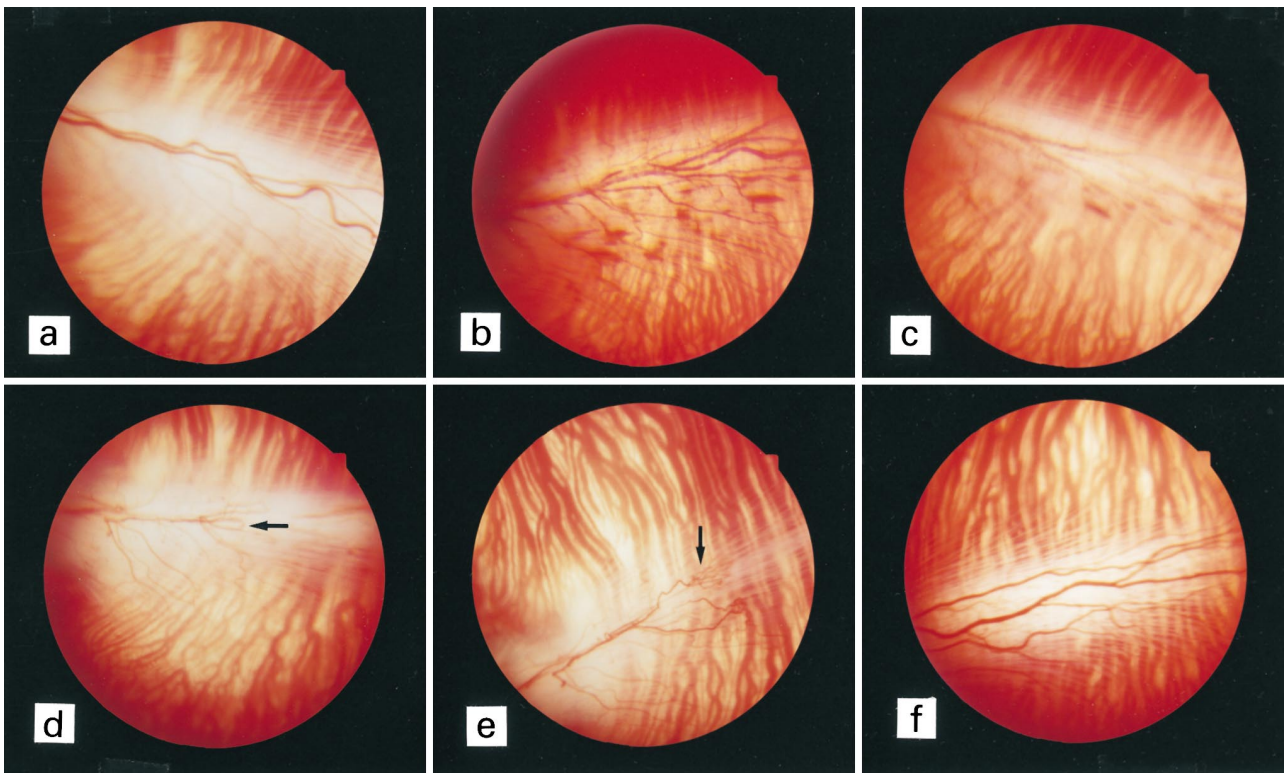

Figure 1. Ophthalmoscopic findings in experimental retinal venous obstruction in rabbits. (**a**) Before dropping thrombin. (**b**) Twenty-four hours after dropping thrombin; dilated venules and retinal hemorrhage are observed. (**c**) One week after treatment; retinal hemorrhage is decreased. (**d**) One month after treatment; retinal hemorrhage has completely disappeared and venules have contracted. Loop-like arteriovenous anastomosis (arrow) gradually develops. (**e**) Three months after treatment; vessels with rete mirabile (arrow) are confirmed in peripheral margin of vascular wing. (**f**) Three months after dropping physiological saline solution: no abnormal findings are noted in control eye.

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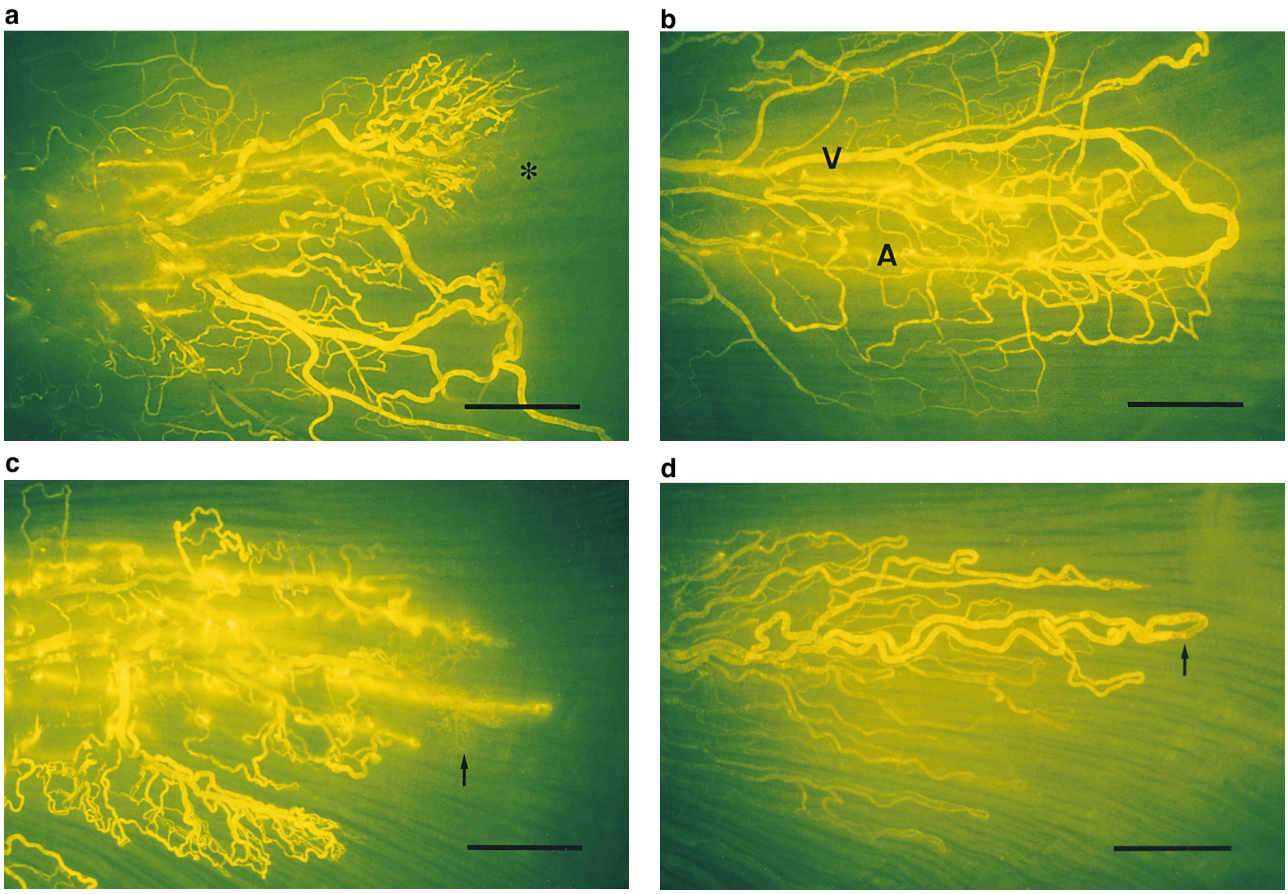

Figure 2. Findings in preparations fixed and extended with intravenous injection of gelatin-added fluorescein. (**a**) Three months after dropping thrombin; regions with no perfusion, regarded as areas of vascular occlusion in periphery (asterisk), are extensively observed, and abnormal blood vessels, including arteriovenous anastomoses, are observed around them. Bar = 500 μm. (**b**) Three months after treatment; diameter of venules (V) have already recovered to state close to normal, but marked narrowing still exists in companion arterioles (**A**). Bar = 500 μm. (**c**) Three months after treatment; minute neovascularization with rete mirabile (arrow) is confirmed. Bar = 500 μm. (**d**) One year after treatment; extended capillaries (ar-

the eyeballs were removed immediately after cardiac arrest. The eyes were divided into two groups; the posterior half was immersed in 2% glutaraldehyde and the choroid was removed. Only the retina was sliced and observed by fluorescence microscopy. Next, immersion and fixation with 2% glutaraldehyde was performed, and postfixation with 2% osmium tetroxide was carried out. After dehydration through an alcohol series and immersion with propylene oxide, the specimens were embedded in epoxy resin. Semi-ultrathin sections of these specimens were prepared, stained with toluidine blue, and observed by light microscopy. Then, the ultrathin sections were double-stained with uranium and lead, and observed by transmission electron microscopy.

All animal experiments in this paper followed the Guideline for Animal Experimentation, Hirosaki University, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Reseach.

Results

Incidence of Retinal Venous Obstruction

Retinal venous obstruction was confirmed by ophthalmoscopy in 23 of 53 eyes (43%) 24 hours after treatment.

Ophthalmoscopic Findings

Twenty-four hours after dropping the thrombin, major venules above the targeted site had clearly been dilated, compared with those before treatment (Figure 1a). A punctate and linear frame-shaped hemorrhage along the vascular wing on the same side was

confirmed (Figure 1b). This retinal hemorrhage gradually decreased after 2 or 3 days and was completely absorbed after 1 month. The extension of the major venules disappeared after 1 week (Figure 1c), and the venules contracted after 1 month (Figure 1d). After an additional month, a loop-like arteriovenous anastomosis that appeared to be collateral circulation, developed gradually, and the diameter of the contracted venules simultaneously began to recover. After 3 months, the venous blood column had nearly returned to the pretreatment state (Figure 1e). During this period, vessels with a rete mirabile in the peripheral margin of the vascular wing were confirmed in 6 of 10 eyes (Figure 1e). Vessels with a rete mirabile were also observed in 4 of 7 eyes after 1 year. Their size and form were close to those observed at 3 months. No abnormal findings were noted in the control eyes throughout the study (Figure 1f).

Findings in the Flat Gelatin-Fluorescein Preparations

Three months after the thrombin was dropped, regions with no perfusion, thought to be areas of vascular occlusion in the periphery, were observed extensively. Also, abnormal blood vessels, including arteriovenous anastomoses that are not normally present, were observed around them (Figure 2a). The diameter of the venules had recovered to a state close to that of the controls, but marked narrowing still existed in the companion arterioles (Figure 2b). Clear leakage of gelatin-added fluorescein was noted from vessels in the periphery, and minute neovascularization with a rete mirabile from these vessels was confirmed (Figure 2c). No vessel with a rete mirabile was observed in the extended capillaries in the periphery, even after 1 year (Figure 2d). No abnormal findings were noted in the controls.

Light and Electron Microscopic Findings

In the region where the gelatin-added fluorescein leaked, detachment of endothelial cells from the basement membrane and ghost vessels without endothelial cells were frequently observed. In addition, arterioles with an obstructed lumen were often seen proximal to these vessels (Figure 3a). Vessels with a relatively wide lumen that penetrated deeply into the fibrous layer of the retinal nerve from the side of the vitreous body were observed in the retina around the leaking vessels (Figure 3b). Also confirmed was proliferative tissue from the side of the retina toward the vitreous body (Figure 3c).

Observation with electron microscopy revealed

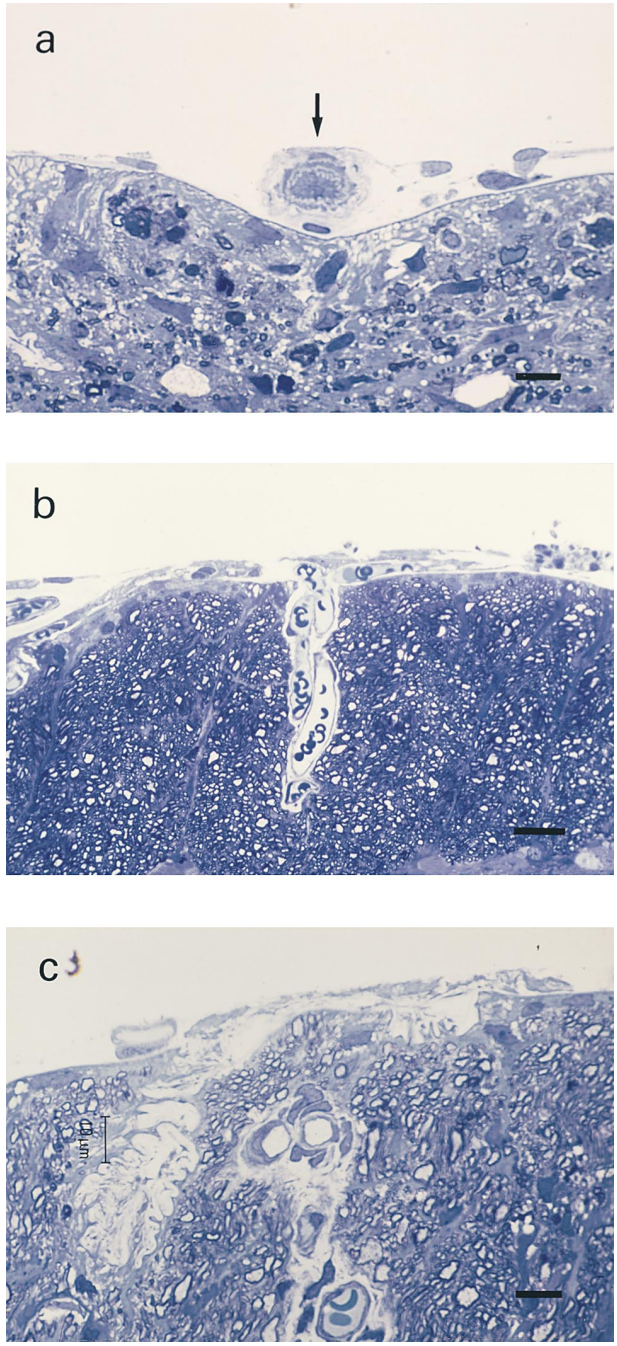

Figure 3. Light microscopic findings (3 months after dropping thrombin). (**a**) Obstructive change indicating fibrin mass (arrow) still existing in arteriole is observed. Bar = 10 μm. (**b**) Vessels with relatively wide lumen penetrate deeply into fibrous layer of retinal nerve from side of vitreous body. Bar = 20 μm. (**c**) Proliferative tissue from side of retina toward vitreous body is confirmed.

vessels with degranulated platelet aggregates in their lumen (Figure 4) and ghost vessels without endothelial cells in the region of leaked fluorescence. In some of the ghost vessels, glial cells ruptured the re-

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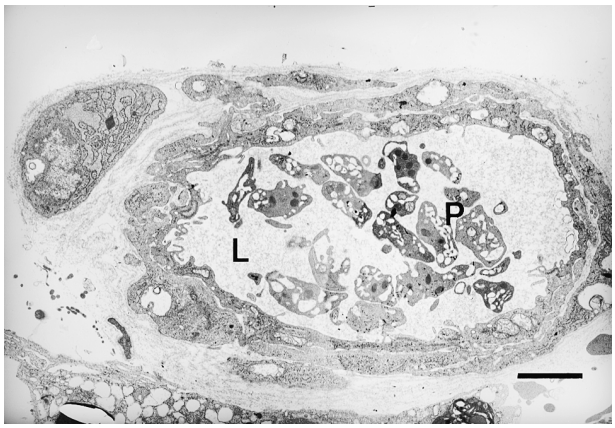

Figure 4. Electron microscopic findings 3 months after dropping thrombin: degranulated platelet (P) aggregates are observed in lumen (L) of obstructed vessels. Bar = 2 μm.

sidual basement membrane and penetrated into the vascular lumen (Figure 5). Endothelial cells of the vessels with a rete mirabile (Figure 2c) had a very high ratio of cytoplasm to nucleus, many rough endoplasmic reticula in the cytoplasm, a very thin basement membrane, and some distinct discontinuous regions (Figure 6). In another vessel, glial cells ruptured the inner limiting membrane and proliferated into the vitreous cavity (Figure 7).

Discussion

This experimental system has been an excellent model to simulate the development of retinal vein obstruction and its clinical course in the human eye.

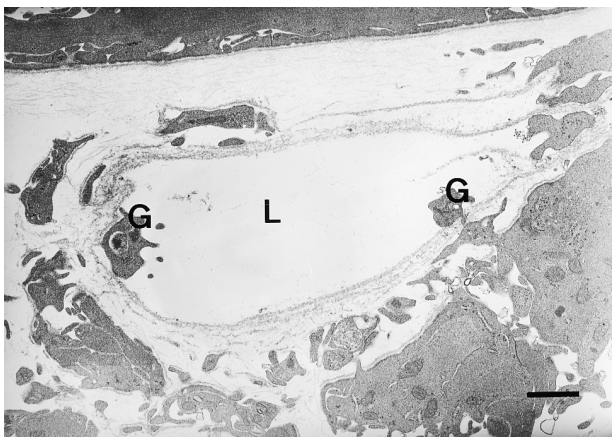

Figure 5. Electron microscopic findings 3 months after dropping thrombin: glial cells (G) rupture residual basement membrane and penetrate into vascular lumen (L) in ghost vessel without endothelial cells. Bar = 1 μm.

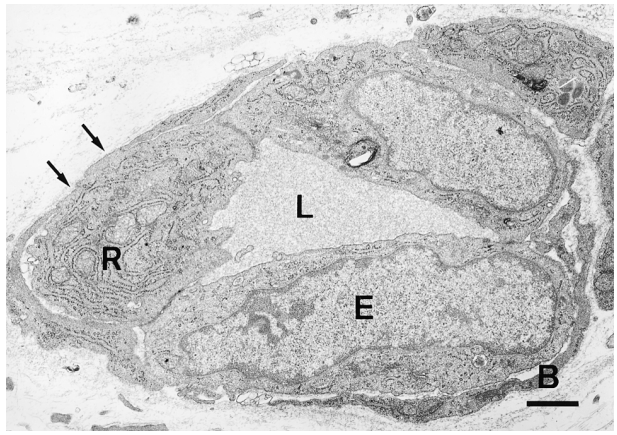

Figure 6. Electron microscopic findings of rete mirabile, shown in Figure 2c: endothelial cells (E) have very high ratio of cytoplasm to nucleus, many rough endoplasmic reticula (R) in cytoplasm, very thin basement membrane (B), and some distinct discontinuous regions (arrows). L: lumen. Bar = 1 μm.

As described, the model produced thrombotic obstruction in the retinal veins of rabbits without damage to the vascular wall or surrounding tissues, and invariably caused bleeding. Major findings in the late lesion in the current experimental venous obstruction were so-called ghost vessels with no endothelium and only a basement membrane, and vessels with a basement membrane that had noticeably detached from the endothelium. The coexistence of both types of abnormal vessels indicated that obstructed and ne-

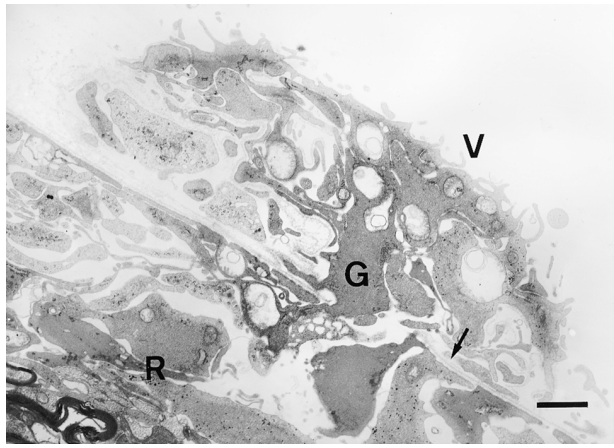

Figure 7. Electron microscopic findings in retina where vein is obstructed (3 months after dropping thrombin): glial cells (G) proliferate in fibrous layer of retinal nerve (R), rupturing inner limiting membrane (arrow), and proliferating into vitreous cavity (V). Bar = 1 μm.

crotized major venules reconstructed their lumen and were recanalized—a finding that suggested unresolved disease conditions even after 3 months.

In addition to this finding, which may be characterized as a healing mechanism, a vascular lesion was observed where glial cells invaded into the lumen of ghost vessels. This lesion had proliferated in the examination at 3 months and at 1 year. A similar finding was reported by Hockley et al[5] in a case 5 weeks after vascular obstruction due to laser irradiation. This finding, which is considered a final stage of a vascular occlusive lesion,[15] was also confirmed by Bloodworth and Molitor[16] at the final stage of diabetic retinopathy with vascular occlusive lesions. They considered this an indication of the phagocytosis of glial cells in capillary walls that had degenerated. Results obtained in this study suggested that complete healing of obstructed vessels cannot be expected in the natural course, even though partial recanalization has been observed.

Formation of collateral circulation, which is also considered a healing mechanism, has been recognized in most experimental models of this disease.[3,6–8,17] This formation originates from preexisting vessels and has been shown to be a new pathway from the area around the occlusion to the ischemic area. As is frequently observed, it has been generally difficult to discriminate via ophthalmoscopy between collateral vessels in a central retinal vein occlusion and neovascularization with tortuosity. In this study, fluorescence fundus photography was useful for discrimination because marked leakage of fluorescence occurred in the junctional cleft of endothelial cells at the tip of the neovascularization, while such leakage from the collateral circulation does not occur.[18] However, since pigment in the retinal pigment epithelium was absent and background fluorescence in the choroid was very intense in the albino rabbits used for this experiment, it was difficult to render a judgment with only this evidence. Intravenously injected gelatin-fluorescein preparation technique was useful in discriminating the structures and examining the detailed configuration of blood vessels. In this experiment, no leakage of gelatin-added fluorescein was observed in the region where collateral vessels were suggested by their configuration, and judgment was relatively easy. As described, the most marked leakage was observed in vessels in the periphery of the vascular wing that formed a new vessel with a rete mirabile and slight leakage.

In addition to proliferative changes, a new and significant finding was neovascularization. It has been confirmed that processes of glial cells proliferate into the vitreous cavity through the inner limiting membrane on the surface of the ischemic retina in animals used in experiments.[5,16,19] It was also clinically significant that a lesion, which is similar to the proliferative lesion when retinal vein obstruction is found, was confirmed in the experimental system. Archer[19] stated that the morphological features of neovascularity included juvenileness of constituent cells, large endothelia, a nucleus with a large and distinct nucleolus in the cytoplasm, an abundance of polysomes (aggregates of ribosomes), well-developed cell organelles, and thinness and discontinuity of the basement membrane. He also stated that at the growing tip of the capillary, the endothelial cells grew larger, the lumen became narrower, and the cells had more Golgi complexes, mitochondria, and lysosomal bodies. These findings were compatible with those obtained by electron microscopy in the current experiment.

This neovascularization, which is clinically well known, has rarely been reported in experiments with animals. In the reports by Hamilton et al[4] and Virdi and Hayreh[8] that confirmed neovascularization histologically, a laser was used to irradiate the vein several times until it was permanently obstructed. It was clear that the reactive factors due to burns cannot be excluded as a cause of the neovascularization. In this respect, trans-adventitial dropping of thrombin caused bleeding purely derived from venous obstruction with no mechanical operation of vessels. To our knowledge, this may be the first study to confirm neovascularization by long-term observation; and the development of neovascularization may be quite similar to that of central retinal vein occlusion in the human eye. This suggests that the presence or absence of neovascularization may be used as an indication for judging effectiveness in future experiments regarding treatment.

As described, such neovascularization was observed in half of the cases in the experiments. The remaining half showed only the extension of small vessels and no definite neovascularity in the periphery. A similar finding was reported by Shilling and Kohner[20] who followed up central retinal branch vein occlusion in human eyes for 2 to 4 years. The authors stated that the cases they observed over the long-term were divided into two types: permanent extension and obstruction of capillaries. No neovascularization was noted in the former, and it was in cases of permanent obstruction observed only at a frequency of 62%. Consequently, the extension of capillaries observed in the current experiment may indicate a tendency toward healing.

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The author wishes to dedicate this paper to the late Professor Emeritus Shuichi Matsuyama of Hirosaki University. The author also wishes to thank Ms. Miwako Kikuchi and Mr. Eiji Kawamura for their devoted assistance during the experiment. This article was published in the *Nippon Ganka Gakkai Zasshi* (*J Jpn Ophthalmol Soc*) 1994;98:175–82. It appears here in a modified form after peer review and editing for the *Japanese Journal of Ophthalmology.*

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