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SCANNING ELECTRON MICROSCOPY OF MICROCORROSION CASTS: APPLICATIONS IN OPHTHALMOLOGIC RESEARCH

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Abstract

In light of the complicated nature of the ocular vasculature, it has been difficult to define the normal ocular anatomy by reference to two-dimensional tissue sections. Since it provides three-dimensional replicas, scanning electron microscopy (SEM) of vascular corrosion casts has therefore been an invaluable addition to the study of ocular vasculature. This technique also often permits identification of a normal vessel's arterial, venous, or capillary nature by its surface features. In addition, this technique is finding increased use in defining anatomical features of human vascular disease and is especially well suited for the study of experimental neovascularization as it relates to the eye. This paper reviews the application of SEM of microscopic casts to the study of normal and diseased ocular vessels, as well as the contribution of this method to studies of experimental ocular neovascularization.

Key Words: Scanning electron microscopy, vascular casts, neovascularization, ophthalmology.

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Introduction

In light of the curvilinear shape of the eye and the complexity of its vasculature, it is difficult to reconstruct its normal and pathologic vascular anatomy from tissue sections. Scanning electron microscopy (SEM) of microcorrosion casts has therefore been a valuable addition to ophthalmologic research since it provides a three dimensional-image of the ocular vessels and their connections. In addition, it provides a method to identify the arterial, venous, or capillary nature of the vessels studied by means of their surface features.

Historical Background

A precursor to the scanning electron microscopy of microcorrosion casts was the dissecting microscopic study of corrosion casts using a neoprene latex (4-6). From such studies came much valuable information about the three dimensional architecture of the ocular vasculature such as that of the choroid and aqueous drainage system. The development of plastic material which could withstand the effects of the electron beam was a major advance and permitted casting of blood vessels and their examination with the higher resolution afforded by SEM. Two years after this technique was first applied to the study of the vasculature of the liver and kidney (40), it was employed to examine the blood vessels of the eye (33). Since that time, with improvements in the casting material, the technique has become widely used in ophthalmological research as is discussed in this report.

Preparation of the Casts

The preparation of the casts from the eye and other tissues of experimental animals has been discussed in detail elsewhere (9,10,16,17,31). In brief, our procedure for the eyes of the rat or mouse is to cannulate the left ventricle, or for larger animals such as the cat, the internal carotid artery, and flush blood from the ocular vessels with a Ringer's lactate solution. For human specimens, the internal carotid artery or ophthalmic artery is cannulated and perfused with heparinized normal saline (12-14). A mixture of methyl methacrylate (Mercox) and catalyst is then infused under hand pressure. After thirty min, the eye can be excised and, for the next four days, the soft tissues are removed by maceration and digestion with 5% sodium hydroxide in water. The tissue-free cast is coated with gold-palladium and examined by SEM. There are multiple variations of this method in regard to

the composition of the pre-casting perfusate, the pressure of injection, type of casting material, and methods for removing the surrounding soft tissues (31). Unfortunately, little is recorded about the physical properties of the various casting compounds and ways to modify these for specific structures.

Accuracy of Microcorrosion Casts

Since the tissue is removed prior to SEM, only the casts themselves remain and it is not possible in a given specimen to corroborate the accuracy with which the casts reflect the state of the vascular anatomy under study. In particular, some vessels may not have filled and others may be narrower or wider than in vivo. In addition, it seems unlikely a priori that blind-ending channels without blood flow such as vascular sprouts would fill. In addition, the contributions to the vascular anatomy by changes in composition of the casting medium, the pressure of injection, and shrinkage of the plastic, need to be studied more fully (31). Thus, there will probably always remain some uncertainty about the extent to which a cast depicts accurately the full extent of the vascular network under study.

Nevertheless, multiple observations comparing structure as seen by the casts with that studied in vivo (10,55), in tissue whole mounts (7), micro-cinematography (41), in tissue sections, or by fluorescein angiography (8) suggest that corrosion casts of the eye provide a generally accurate representation of a vasculature, and that defects in the filling of normal vessels are usually apparent at the time of SEM. The definition of vascular anatomy by the study of casted vessels in pathological states can be more difficult. Comparison of the pericorneal vessels responding to experimental corneal injury as seen in vivo and in casts reveals, however, that neovascular sprouts are incompletely filled (10). Similar observations have been noted in experimental subretinal neovascularization (42). The precise relationships between the diameter of vessels in vivo and in the casts of varying perfusion pressure have not been defined. However, comparison of whole sections of the inflamed pericorneal vessels with vascular casts of similar vessels under the same experimental conditions suggests a close correlation in size (7). In addition, in vivo slit-lamp photographs of the pericorneal vessels proliferating in response to chemical cautery of the cornea correlate extremely well with the anatomy as seen in the casts (10). As has been described in more detail previously (9,10), extravasation of contrast material is not infrequent, especially from inflamed and proliferating vessels. This occurs in the form of beads or columns of plastic that usually are readily distinguished from plastic confined to the lumens of blood vessels.

Applications of Microcorrosion Casts to Ophthalmologic Research

Normal Vascular Anatomy

General Comments. As is described below for specific vascular units, SEM of vascular casts provide an excellent three-dimensional overview of the complicated vasculature of the eye and of the interconnections between vessels of different type and caliber. In addition, the surface features of the vessels, when considered with information about the caliber, often permit identification of the vessel's specific nature, i.e. capillary, artery, or vein (7-10,14,18,22,23,27,31,35-38,42,48,49,52,54,55,62). Arteries are recognized by their general uniform or gradually tapering caliber, the presence of short longitudinal grooves, and impressions of endothelial cell nuclei (Figure 1). The latter are generally elliptical and aligned along the long axis of the blood vessel. In studies of the pericorneal vessels of rats, the endothelial nuclear impressions are not symmetric, being somewhat blunter on their upstream end and thus appearing to "point" in the direction of blood flow (7). It is not clear whether this asymmetry is natural or is induced by the casting procedure. If natural, it would suggest that the direction of blood flow in certain vessels can be inferred from surface features of the casts. Veins, on the other hand, are more irregular in caliber, more prone to anastomosis, lack the short longitudinal grooves, and casts of them contain endothelial nuclear impressions which are less oval and less precisely aligned to the long axis of the vessel. Leaflets of values can be seen in some vascular systems (22). Branches of small arteries frequently begin with a prominent constriction suggesting a vascular sphincter (7,53,61,62). In the retina, as has been noted also in whole mount tissue sections (20), the nuclei of the endothelial cells in this groove are sometimes arranged about the constrictions in an annular pattern (54). In our studies of the pericorneal vessels, such an arrangement of endothelial cell nuclei was not seen (7), suggesting that not all of these constrictions are similar, and result of a muscular sphincter but some could represent endothelial cell cushions. In the ciliary body, furthermore, arteriolar constriction with possible blood flow regulatory importance are not at branch points and are not sharply defined (38). Capillaries are noted generally for their multiple anastomoses and small caliber. Their endothelial cell nuclei are rounder than those of arteries and therefore resemble those of the venules, and are not precisely aligned to the long axis of the vessel.

Optic nerve head. Microcorrosion casts have been utilized to define the controversial nature of the angio-architecture in and around the optic nerve head. Such studies in humans (12), monkeys (3,34,47, 56), and guinea pigs (43) have disclosed similarities and differences between species. Most of the vascular supply to the prelaminar region of the optic nerve comes from the short posterior ciliary arteries. This is achieved mainly from branches that arise directly from peripapillary arterioles but also indirectly from the peripapillary choroidal arterioles. A direct communication has not been demonstrated between the peripapillary choriocapillaris and the capillary network of the optic nerve in the guinea pig or monkey by some investigators (43), while others working on rabbits and Japanese monkeys (Macaca fuscata) claim that the peripapillary arteriolar network and choriocapillaris anastomose with the prelaminar capillaries of the optic nerve (34). The general observation that capillaries in the region of the optic disc anterior to the lamina cribrosa are spared in glaucomatous cupping, whereas the overlying capillaries of the disc proper are not, has been correlated with an observation established by vascular casts that the pre-laminar vessels are derived from the posterior ciliary arteries whereas the optic disc capillaries are in continuity with those of the

retina (1).

That portion of the human optic nerve that lies within the lamina cribrosa of the sclera is encircled by the Zinn-Haller arterial circle into which branches of the short posterior ciliary arteries drain. While not forming a complete ring, this peripapillary arterial circle usually sends two or three branches into the optic nerve of guinea pigs (43). In the monkey, which apparently lacks a circle of Zinn, branches of the posterior ciliary arteries pierce the optic nerve directly (43).

Choroid. Although the general architecture of the choroidal vasculature was determined in general detail many years ago by the use of neoprene casts and fluorescein angiography, or direct visualization and fluorescein angiography, the determination of the complex three dimensional nature of this vascular network awaited the SEM study of corrosion casts. Rats (30,36,64), kittens (32,35), guinea pigs (35), cows (27), subhuman primates (2,24-26,34,52,53,58), rabbits (24,34), ducks (22), and man (49,62), have been studied. This vasculature is supplied in large part by branches of the short ciliary arteries and, more anteriorly, by recurrent branches of the long posterior ciliary arteries. SEM of corrosion casts has made apparent the heterogeneity of the choriocapillaris which arises from the choroidal arteries. Thus, in primates in the submacular region and surrounding area, the choriocapillaris is a flat sheet with a high ratio of vascular lumen to cross sectional area. At such sites, the casts appear as those of densely packed vessels whose interstices are seen as only small holes in an otherwise solid network. From the retinal side, it is not possible to determine the site of entrance and exit of arterioles and venules. More peripherally, however, the choriocapillaris has a distinctive lobular appearance, and supplying arteries and draining venules are readily apparent when viewed from either the external (choroidal) or internal (retinal) face (Figure 2) (52). Such a difference between central and peripheral choroid has not been described in rats (36). The validity of casts in the studies of choroidal vessels is supported by fluorescein angiographic studies which demonstrate functional subunits of the choriocapillaris similar to the anatomic units seen in casts (19). The identification of anatomic subunits by the presence and type of endothelial cell nuclei has been emphasized in the study of the human choriocapillaris (62). In the peripapillary region, the vascular casts disclose a defined ring-like anastomotic system supplied by branches of the short posterior ciliary arteries. These vessels branch and deliver blood in a centrifugal fashion to the surrounding (peripapillary) choriocapillaris (2).

Ciliary body. Vascular casting has revealed different morphologies for the vessels in the ciliary muscle and the ciliary processes (28,29,33,38,39,50, 58,61). The capillaries of the muscle have a more random distribution whereas those of the ciliary processes conform to the three-dimensional structure of the processes. In the latter, arterioles arise largely from the great arterial circle and pass posteriorly to supply each process in a fan-like fashion. The capillaries extend to the surface of each ciliary process where they merge with venules which pass then posteriorly to exit from ciliary processes. The venules drain towards the pars plana from which they extend posteriorly to merge with the adjacent venules and enter the vortex veins. The value of corrosion casts in defining the anastomosing network of veins has been well demonstrated in studies of the venous system of the pars plana (49). In subhuman primates, man, rabbits, kittens, and guinea pigs the ciliary body is supplied with arterial blood given the major arterial circle (28,38,39,60,62). In monkeys (Macaca fascicularis), both the anterior and long posterior ciliary arteries contribute to the major arterial circle (MAC) from which arterioles arise to supply the ciliary processes (38,39), whereas no contribution to the MAC has been noted in rabbits (28) or in humans (60,62).

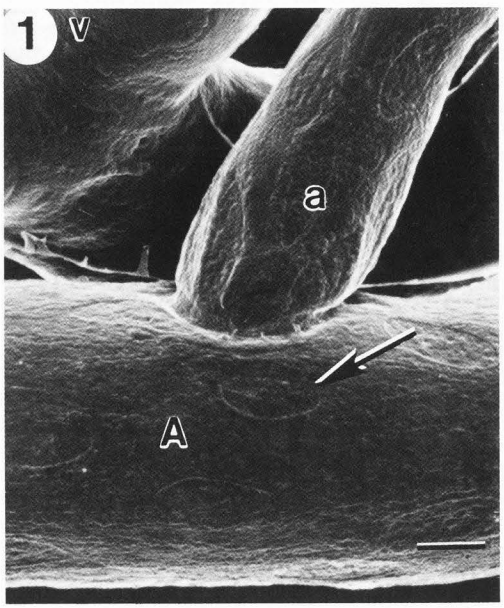
Iris. The iris is supplied by vessels from the major arterial circle and these extend in a radial fashion toward the pupil. A capillary network lies in the anterior stroma of the iris and almost reaches the edge of the pupil. Another capillary bed is found in the posterior surface (44). Venous drainage passes centrifugally, merging with the veins of the ciliary body, ultimately leaving the eye through the vortex veins (60).

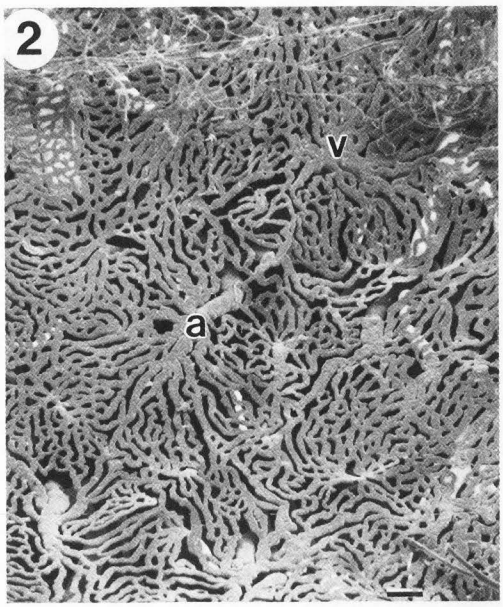
Retina. The distribution of the retinal "arteries" and veins are well understood on the basis of funduscopic observations. Prior to the advent of SEM of corrosion casts, the location and pattern of capillaries within the retina was controversial. Some investigators suggested that these vessels were randomly distributed throughout the layers of the retina and others that they were present in a distinctive laminated pattern (52). Scanning corrosion of the monkey (Macaca irus) retina makes it clear that, at least in this species, the vessels are not randomly distributed, but are present as a three-layered pattern with intercommunications (52).

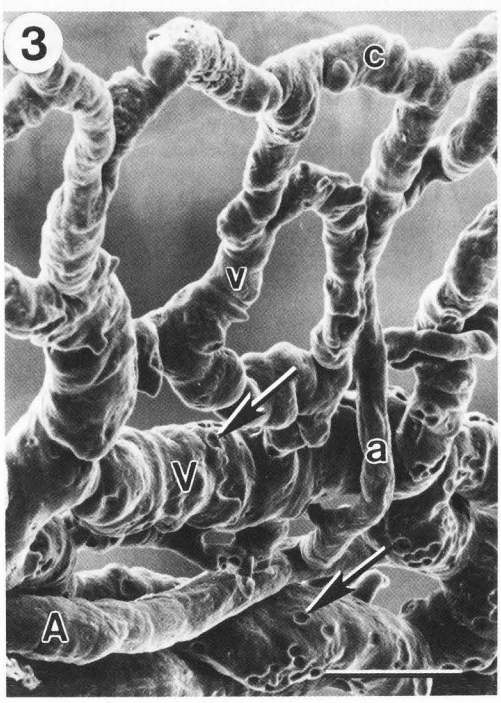
Pericorneal vessels. The vessels that surround the normally avascular cornea are supplied by the anterior ciliary arteries and are noteworthy for a circumferential single artery that, in the rat, is flanked by two well defined veins (7). The pericorneal vascular plexus is supplied by small arteries or arterioles which begin with a short small constriction from the artery and pass centrifugally to enter the capillaries at the most medial portion of this plexus (7). The connections between these vessels and the capillaries can be observed readily as they merge with venules, which in turn enter the pericorneal veins.

Vessels of aqueous drainage. The three-dimensional appearance of the human canal of Schlemm and the distal pathway of aqueous drainage through the ciliary veins was initially defined by light microscopy of neoprene casts produced by injecting the latex into the canal (4-6). However, despite the fact that the drainage of aqueous from the anterior chamber appears to require active transport across cells, these channels have been filled in cynomolgus monkeys (Macaca fascicularis) (57) and dogs (59) by injection of a plastic casting mixture of Mercox CL2R and methyl methacrylate or Batson's corrosion compound number 17 into the anterior chamber and into the episcleral veins. These studies will illustrate the lack of a single well defined circumferential channel in the dog that would correspond to the Canal of Schlemm in primates.

Hyaloid vascular system. The anatomy of the hyaloid system of the rat eye has been observed postnatally from day 1 to day 20, and the initial system and its regression to completion by day 16







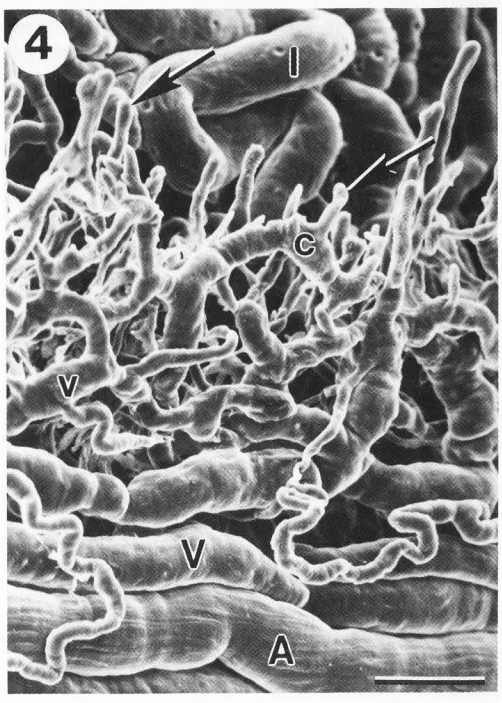


Figure 1. As here in a cast of normal pericorneal vessels of a rat, arteries (A) and arterioles (a) can be identified by the prominent indentations from endothelial cell nuclei (arrow). Note the absence of these impressions in the wall of an adjacent venule (v). Note also the prominent sphincter-like constriction at the origin of the arteriole. Bar = 10 μm.

Figure 2. A vascular cast of the normal human choroid near the equator discloses the lobularity produced by the domains of capillaries supplied by arterioles (a) and drained by venules (v). Bar = 100 μm.

Figure 3. Vascular casts of the pericorneal vessels responding to chemical cautery disclose prominent impressions from adherent leukocytes that are most conspicuous in the walls of veins (V) and venules (v) (arrows). No such impressions are seen in the pericorneal artery (A) or arteriole (a). (c) capillary. Bar = 100 μm.

Figure 4. In corneal neovascularization induced by chemical cautery, vascular casts display the origin of neovascular sprouts (arrows) from the walls of venules (v), and to a lesser extent, capillaries (c). Note the absence of sprouts from the pericorneal artery (A). (I) iridial vessels; (V) pericorneal vein. Bar = 50 μm.

has been documented by examination of vascular casts (21).

Pathologic Vascular Anatomy

Although most studies of ocular vasculature using SEM of microcorrosion casts have focused on normal anatomy, the technique is being used with increasing frequency to study experimentally induced vascular changes in animals as well as disease states in humans.

Corneal Neovascularization: SEM of corrosion casts has been an extremely valuable adjunct to light and transmission electron microscopic (TEM) studies of corneal neovascularization as induced by chemical cautery (7). The pericorneal vessels are readily studied *in vivo* by slit-lamp examination and fluorescein angiography because of their superficial position in a transparent medium (8). Following chemical cautery to the rat cornea, SEM of vascular casts has revealed a sequence of events beginning with vasodilatation and the margination of leukocytes, the latter apparent as hemispherical defects on the surface of the casts (7). As would be expected from many previous light and electron microscopic studies, these inflammatory cells were seen largely on venules and to a lesser extent capillaries (Figure 3). The arteries and arterioles were not affected. After 27 h after injury and thereafter, the casts revealed the first evidence of vascular buds which arose largely from venules and were never seen arising from arteries or arterioles (Figure 4). With time, these capillary sprouts began to branch and made contact and anastomoses with adjacent branches. By means of the casts, the evolution of the newly-forming vascular plexus was evident as a continual branching of the newly formed vessels which established contact with adjacent branches and resulted in circulation. As the new vessels extended toward the injury site, it was evident that branches were prominent, however, neovascularization had ceased. Further removed from the cautery site the anastomoses and branches were undergoing atrophy.

A striking feature of the casts at eight or more days after injury was the observation of two types of new vessels. Most had the surface features of venules, that is, they were somewhat irregular in character and had a smooth contour without impressions of endothelial cell nuclei. Other vessels had the small grooves and prominent impressions of endothelial nuclei consistent with arteries or arterioles. Both kinds of vessels were traced back peripherally to the pericorneal vascular plexus where, as was especially well seen with stereomicroscopy, the latter connected with the periocorneal arteries, whereas the new vessels with venular surface features drained into the pericorneal veins. Studies in the rabbit using fluorescein angiography confirmed that some newly-formed vessels in the cornea, which filled early with fluorescein dye, brought blood to the site of injury whereas other vessels drained the lesion and filled the later in the angiogram. By TEM the vessels supplying the lesion contained myocytes whereas the draining vessels did not (8).

Retinal Neovascularization: After autotransplantation of skin fibroblasts into the vitreous, neovascular fans develop from the retinal vessels and have been studied by direct observation by fundus biomicroscopy and photography. Such retinal vascularization has also been studied by SEM of corrosion casts. When casted, these loops have similar features to those in the cornea, including a predominant origin from venules (55).

Retinopathy of Prematurity: SEM of vascular casts has been applied to the neovascularization of retinopathy of prematurity (ROP) (13,14) and an experimental approach to this disease (11,18,63). The observations on human tissues are discussed below in the section on SEM of vascular casts as applied to human disease. While the precise mechanisms of ROP are unclear, it is presumed to be related to high levels of ambient oxygen to which the infants were exposed. The development of the retinal vasculature in newborn kittens is retarded by high levels of ambient oxygen, and when such animals are removed from this environment, a retinal vasoproliferative state ensues. Although the retinopathy in kittens does not progress in the characteristic manner of the human disease, it is clearly associated with neovascularization that is presumably related to ROP. This experimental model has been studied in vascular casts which have documented well the inhibitory effect of high levels of circulating oxygen on normal retinal vascularization (11,18,63). The vascular casts also illustrate vividly in three dimensions the vasoproliferative response by new vessels that grow toward the vitreous in the form of tufts and canopies overlying those of the retina. The exact sequence of events has not been defined yet as clearly for this model as it has for vasoproliferative states of the cornea.

Iris Neovascularization: As has been discussed above for corneal and retinal neovascularization, vascular casts have been useful in documenting the venular origin of new iridial vessels in ROP (13,14). Proliferation of iridial vessels have also been studied by casting following the induction of glaucoma by intra-ocular injection of α-chymotrypsin (1).

Retinal Photocoagulation: The neovascularization, and other responses, following photocoagulation of the retina have been the subject of multiple studies (41,42,45,51). Prior to the use of vascular casts, the proximity of the larger choroidal vessels, the choriocapillaris, and the retinal vessels had made it difficult to establish the three dimensional aspects of subretinal vascular proliferation. However, the application of casts to the study of this response to laser photocoagulation has resolved many of these issues, by demonstrating the three-dimensional aspects of this neovascularization with utilization of the stereo mode. As in the other systems described above, the source of new vessels was venules (42). In studies of vascular repair of the choriocapillaris following argon laser retinal photocoagulation, SEM of vascular casts has been useful in establishing a relationship between intensity of the coagulation and the size of the lesions and types of vessels involved, and the time of repair (45). A similar study utilizing casts has followed the response of the choriocapillaris to xenon photocoagulation (51). These studies disclosed that the defects in the choriocapillaris following the injury were gradually filled in by small neovascular sprouts from the surrounding vessels. A study following laser coagulation revealed the utility of combining micro-cinematography of choroidal vessels through a sclera window with SEM of vascular casts. This indicates that, in common with fluorescein angiography, the casting procedure can be preceded by other methods to provide

functional assessment of the normal and injured vessels (41).

Phototoxic Retinopathy: The utility of SEM of corrosion casts to determine the origin and interconnections of vessels is apparent in studies of phototoxic rat retinopathy. In this setting abnormal vessels develop next to the retinal pigment epithelium and SEM of the casts shows clearly that the abnormal fenestrated vessels are of retinal, rather than choroidal, origin (54).

Changes in hypertensive animals: The angioarchitecture of the ciliary bodies of rats with spontaneous hypertension has been studied by casting, as well as whole mount sections and TEM. The results suggest that the low intraocular pressure in these animals is due to a reduction in the number of filled vessels, as well as of their fenestrations, and to perivascular fibrosis (15).

Optic Nerve Transection: Vascular casts have been used to quantitate the density and size of capillaries in the normal optic nerve head and in the nerve head of cynomolgus monkeys made pale by previous optic nerve transection (46). These studies revealed that the percentage of tissue volume occupied by capillaries was reduced even though fluorescein angiography was normal. The change in this volume was, however, insufficient in itself to explain such optic nerve pallor, and Quigley et al. (46) suggested that the pallor was due more to change in the composition and transparency in the tissue than changes in the amount of hemoglobin present.

Retinopathy of prematurity in humans: Vascular casts in retinopathy of prematurity have disclosed extensive intraocular neovascularization from the retina, iris, and ciliary body. As in other ocular sites, the new vessels originated predominantly from the venules (13,14).

Other Conditions in humans: SEM of vascular casts have been used to visualize at high resolution the vascular changes of human ocular disease such as microaneurysms in diabetic retinopathy (Figure 5) and arteriolar/venular crossing defects in hypertension (Figure 6) (14).

Conclusions

In light of the complexity of the ocular vasculature, SEM of vascular corrosion casts has been invaluable in defining normal ocular anatomy, vascular changes in spontaneous human or animal disease, and the means by which new vessels emerge in response to experimental injury. The method, as applied to normal anatomy, has permitted careful analysis of each of the eye's many circulatory subunits and given preliminary details about the considerable interspecies differences. The studies of spontaneous human disease have been limited in number but have developed methods by which studies can be extended to other disease states. In light of the many ocular diseases associated with vascular proliferation, SEM of vascular casts is especially valuable in defining the details of new vessel growth and providing a bridge between observations in vivo and light and transmission electron microscopy of tissue sections.

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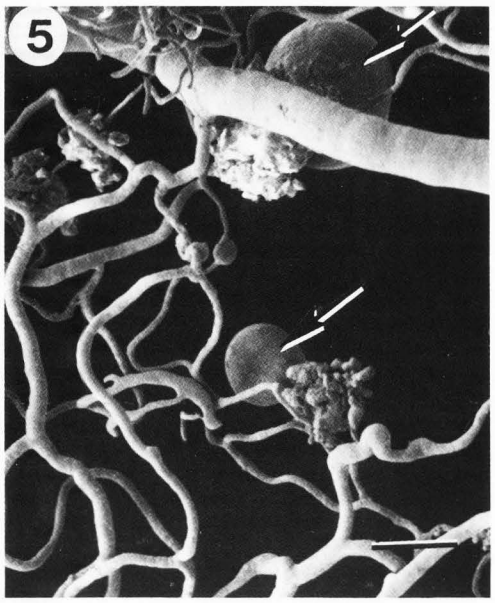

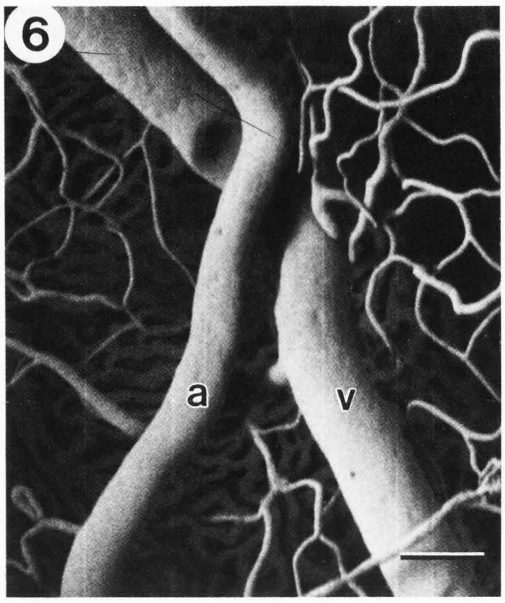

Figure 5. Retinal microaneurysms (arrows) from a 60-year-old man with diabetes and hypertension are seen here by scanning electron microscopy of a vascular cast. Bar = 100 μm.

Figure 6. As in this 60-year-old man noted in Fig. 5, crossing defects are produced as a retinal arteriole (a), which produces a groove in the underlying venule (v). Bar = 10 μm.

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Discussion with Reviewers

Reviewer III: How reliable do you find the endothelial nuclear imprint patterns and differentiating arteries from veins?

Authors: Arteries can be distinguished from veins by a number of features including generally more regular smoother profile for arteries, short longitudinal grooves, and the more prominent impressions of endothelial nuclear imprint patterns. The nuclear imprints of the arteries are generally somewhat more narrow, more pointed, and more precisely aligned along the long axis of the vessels. Those of veins are usually broader and more randomly positioned in regard to the axis of the vessel. Generally, the imprints of endothelial cells of arteries are more conspicuous and may be seen where it is difficult to find imprints in the walls of the veins. Occasionally, however, individual nuclear imprints in veins are similar in orientation and shape to those of arteries so that this feature is in itself not an absolute diagnostic criterion but is nevertheless a generally useful criteria, particularly when considered with other features.

Reviewer III: When using this technique to study vascular pathology, what precaution should be taken to maximize the possibility that your findings represent actual vascular abnormalities and not simply artifacts of vascular filling?

Authors: Since the technique is a process by which the vessels themselves are digested prior to SEM study, there is no absolute criteria to insure that the changes are not artifactual. However, the certainty with which the changes can be viewed as genuine rests on the repeated finding of similar abnormalities under similar experimental conditions. Correlations of casts with light and electron microscopic studies of vessels under similar conditions and comparison of changes in light of the experimental procedure and the time following injury, if applicable, are also relevant. With these considerations, it is apparent that artifactual findings usually include either a failure of the casting material to fully perfuse a vessel, or the extravasation of casting material. The failure to perfuse a vessel is usually evident in normal vessels by a sudden inappropriate termination of a vessel usually with a hemispherical profile. Often in normal vascular anatomy, these terminations are opposed by a similar termination in a somewhat distal segment of the vessel producing an obvious gap. In proliferating vessels, the detection of incomplete filling is more difficult since the vascular sprouts are blind-ended. We have illustrated this phenomenon by comparing in vivo photographs of vessels proliferating in response to chemical cautery with a cast of the same eye (J. Scan. Elect. Microsc. Tech. 1, 341-348., 1984). This clearly demonstrates that, as expected, blind-ended proliferating sprouts are not filled.