J. Anat. (1996), 188, pp. 693–703, with 9 figures Printed in Great Britain

Precapillary patterns and perivascular cells in the retinal microvasculature. A scanning electron microscope study

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(*Accepted 25 January 1996*)

ABSTRACT

The microvasculature of the rat retina was studied in male Wistar rats in order to examine the features of the precapillary vascular pattern and structure that could affect blood flow regulation. Vascular corrosion casts and partially digested tissue specimens were observed by scanning electron microscopy. Side branching rather than bifurcation was the predominant microvascular pattern in the arterial tree. Two types of precapillary arteriole were present, one with the characteristic pattern of a preferential channel; the other gave off capillaries as terminal branches. At the origin of arteriolar side branches, smooth muscle cells appeared to buckle the endothelial nuclei into the vascular lumen. It is concluded that the rat retinal microvasculature appears to be characterised by 2 distinctive features: (1) side branching of arterioles which allows preferential flow in the most superficial layers of the retina; (2) peculiar luminal restrictions of arterioles and capillaries which permit fine regulation of blood flow.

Key words: Retinal vessels; vascular corrosion casts.

INTRODUCTION

An important functional characteristic of the retinal microcirculation is autoregulation. It has been demonstrated that the retinal microvascular bed, although lacking innervation, tends to compensate for changes in perfusion pressure (Riva & Loebl, 1977; Riva et al. 1986). Lack of autoregulation of retinal blood flow could be an important factor in the development of certain retinal lesions, for example as has been shown in patients with glaucoma (Grunwald et al. 1984). However, very limited data are available in the literature concerning the morphology of vessels involved in blood flow regulation in the retinal microvascular bed (Kuwabara & Cogan, 1960; Hogan et al. 1961; Friedman et al. 1964; Henkind & De Oliveira, 1968). All of these data were based on light microscopy. More recently, other investigators (Shimizu & Ujiie, 1978; Shiraki et al. 1980; Seki, 1987; Motti & Niemeyer, 1983; Schaepdrijver et al. 1989; Fryzkowsky, 1992) employed a newer technique, vascular corrosion casts observed by scanning electron microscopy (SEM), to study the 3-dimensional arrangement of the retinal microvasculature in the monkey (Shimizu & Ujiie, 1978), diabetic human (Fryzkowsky, 1992), cat (Motti & Niemeyer, 1983) and rat (Shiraki et al. 1980; Seki, 1987; De Schaepdrijver et al. 1989). Even these later studies did not provide information concerning the arrangement of precapillary vessels and the presence of specific sites of blood flow regulation. This was probably due to the fact that, especially at the posterior pole, the casts of capillaries of the nerve fibre layer obscure the arterioles, which lie further away from the vitreous.

In a previous corrosion cast study of ours (Pannarale et al. 1991) by SEM on the microvasculature of the rat retina, a holangiotic (Leber, 1903) or euangiotic (Johnson, 1901; Leeson, 1979) retina, provided observations on its arterial, capillary and venous components. In the present study we have investigated the microvasculature of rat retina by SEM of corrosion casts and digested tissue specimens, and by light microscopy (LM). We chose corrosion

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casts because the observation of such casts by SEM makes it possible to follow 3-dimensionally any type of branching pattern of a microvascular tree (Murakami, 1971; Miodonski & Jasinski, 1979), to observe endothelial imprints, which make venous and arterial vessels readily recognisable (Hodde et al. 1977), and to observe the imprints of some perivascular cells, the disposition of which can be deduced (Gaudio et al. 1990). With 'traditional' techniques, vessels cannot be adequately followed in different focal planes or the 3-dimensional relationship of vessels analysed as it is lost because of trypsinization (Shimizu & Ujiie, 1978).

Finally, partially digested tissue specimens provide information on the perivascular cells and their placement along the microvascular branches (Mazanet & Franzini-Armstrong, 1982). We devised a technique for obtaining partially digested specimens in order to study a large number of vessels per specimen and to maintain the 3-dimensional pattern of the microvascular bed.

MATERIALS AND METHODS

Twenty male Wistar rats, weighing 200–250 g were used for the study. All experiments conformed to the ARVO Resolution on the Use of Animals in Research.

The vascular bed of the head of each rat was injected with low viscosity methylmethacrylate based resins (Fahrenbach et al. 1988; Lametschwandtner et al. 1990). Two different types of specimens were obtained from the excised eyes: (1) microvascular casts of retina completely freed from tissue remnants; (2) specimens digested with NaOH to different extents in which the cast was still surrounded by perivascular cells and, to varying degrees, by retinal tissue.

Casting procedure

All rats were anaesthetised with an ether/air blend. Heparin (4000 I.U./kg) and acetylcholine (4 mg/kg) were administered intravenously before the casting procedure (Pannarale et al. 1991; Gaudio et al. 1993*a*, *b*). The descending aorta was cannulated in a retrograde manner with an Inpharven – Int cannula (Inphardial, Mantova, Italy) (outer diameter 1.4 mm; inner diameter 1.1 mm) inserted through the abdominal aorta. After the right atrium had been incised and the ascending aorta ligated, modified Batson 17 resin (Fahrenbach et al. 1988) or diluted Mercox (Vilene, Tokyo, Japan) (4 parts of Mercox CL 2: 1 part of monomethyl methacrylate) was injected until polymerization was observed (around 40 cc of resin per animal were injected). The pressure in the cannula was monitored (Conel electronic manometer, Rome, Italy) via its side port. This pressure was between 200 and 250 mmHg. This value was obtained by increasing injection pressures until consistent and satisfactory filling of the vessels was achieved. Due to the viscosity and time of polymerization of the resin, lower injection pressures did not provide complete casts of the microvasculature. Higher injection pressures caused either extravasation or excessive filling of the choroid and unsatisfactory filling of the retinal microvasculature.

Processing of samples

Two different procedures were followed thereafter for obtaining either a corrosion cast (microvascular cast freed of tissue remnants) or a specimen with only incomplete digestion of retinal tissue around the methylmethacrylate-filled retinal vessels (partially digested specimens).

1. *Corrosion cast preparations.* The animal cadavers were left for 24 h at room temperature to allow complete solidification of the injected resin. The eye globes were enucleated and left in a water bath for 24 h. The eyes were then macerated in a 15% NaOH solution at room temperature for 24 h and rinsed in distilled water. Subsequently, the casts were placed in 5% trichloroacetic acid (CCl_3COOH) solution to free them from tissue remnants. Finally, they were frozen in distilled water and freeze dried (Gaudio et al. 1984; Lametschwandtner et al. 1984). The anterior segment casts were dissected away from the rest of the specimen.

2. *Digested tissue (semicorroded) specimens.* Immediately after polymerization of the injected resin, the eyes were enucleated, the anterior segment excised, the vitreous peeled off, and the remaining part of the globe immersed in fixative solution (2.5% glutaraldehyde in cacodylate buffer, pH 7.4). After 48 h, the vitreous chamber was filled with a 20% aqueous NaOH solution and incubated at 37 °C for 1 h, after which the NaOH was washed out. Following post-fixation in an OsO_4 solution, the samples were dehydrated in alcohol and critical point dried (Gaudio et al. 1990).

SEM observations

For the observations, both types of sample were glued onto stubs by 'Silver Dag' and covered with gold

using an Edwards sputterer. The prepared casts were examined with a Hitachi S 4000 field emission (FE) SEM operating at 7–10 kV (Gaudio et al. 1993*a*).

Morphometry and quantitation

Each measurement was performed on 3 randomised photographic fields in 10 specimens.

Light microscopy

Four rats were perfusion fixed with 10% buffered formalin. Eyes were taken out, the anterior segments were excised and the vitreous body peeled off. Posterior segments were immediately immersed in 10% buffered formalin. Normal procedures for paraffin embedding followed. Frontal serial sections 5 μm were stained with haematoxylin-eosin and observed by light microscopy.

OBSERVATIONS

SEM observations

At the level of the papilla, the central artery (CA) gives rise to 4–8 branches that run towards the periphery mostly with a straight course. The arterial branches near their origins overlie the branches of the central vein and regularly intermix with them towards the periphery (Fig. 1). All arterial vessels run in the most superficial (vitreal) layer of the retinal microvasculature. Smaller venous vessels always take origin from the deeper layers. Arterial and venous vessels are clearly recognisable by different endothelial imprints on their surfaces (Fig. 2).

Collateral arterioles arise from the arterial branches of the central artery. In the casts, the origin of these vessels constantly showed a reduction in diameter and the point of origin on the parent vessel showed a circumferential oval depression around the origin of the collateral (Fig. 3). On LM, this appearance was seen to correspond to the presence of smooth muscle cells buckling endothelial nuclei into the lumen (see LM observations). In digested tissue specimens, both the branches of the CA and their collaterals showed a layer of circularly arranged smooth muscle cells. At branching points, the smooth muscle cells of the parent vessel changed orientation and became arranged in the direction of the vascular stem (Fig. 3*b*).

The collaterals of the CA branches divided dichotomously once or twice, giving rise to precapillary

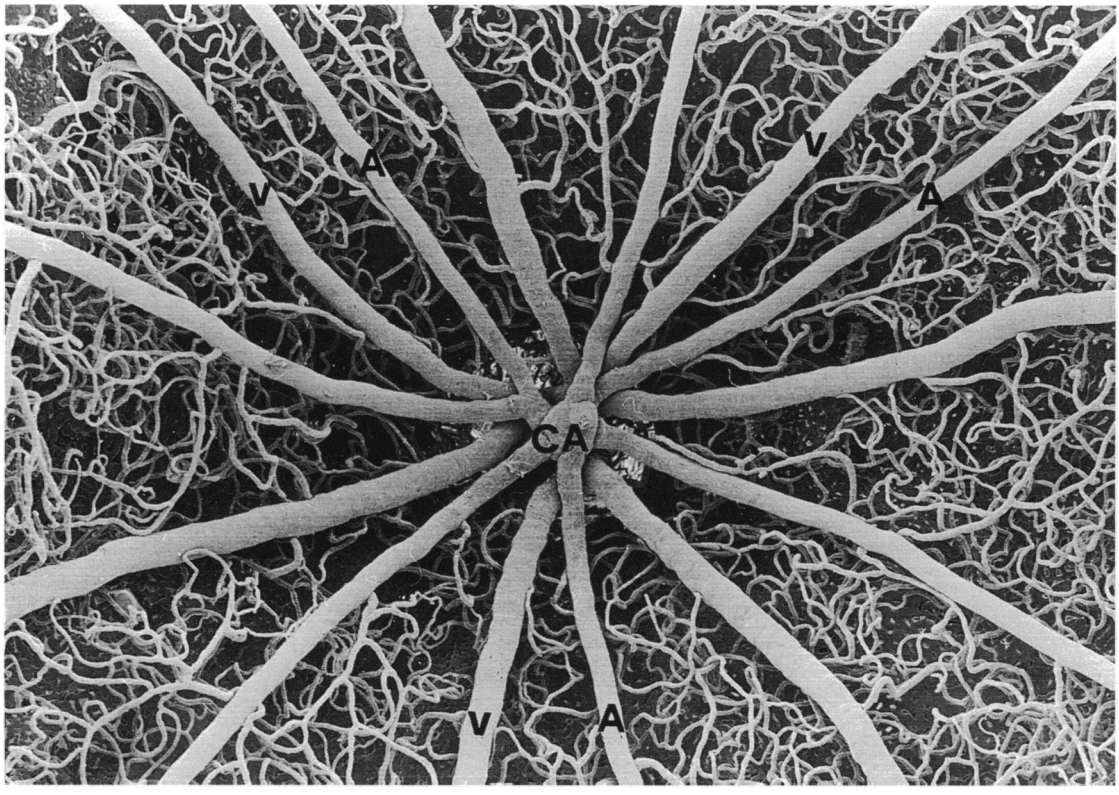

Fig. 1. Scanning electron micrograph of microvascular cast of the papilla and posterior pole of the retina. Arterial branches overlie venous branches. CA, central artery; A, arterial branches; V, venous branches. ×65.

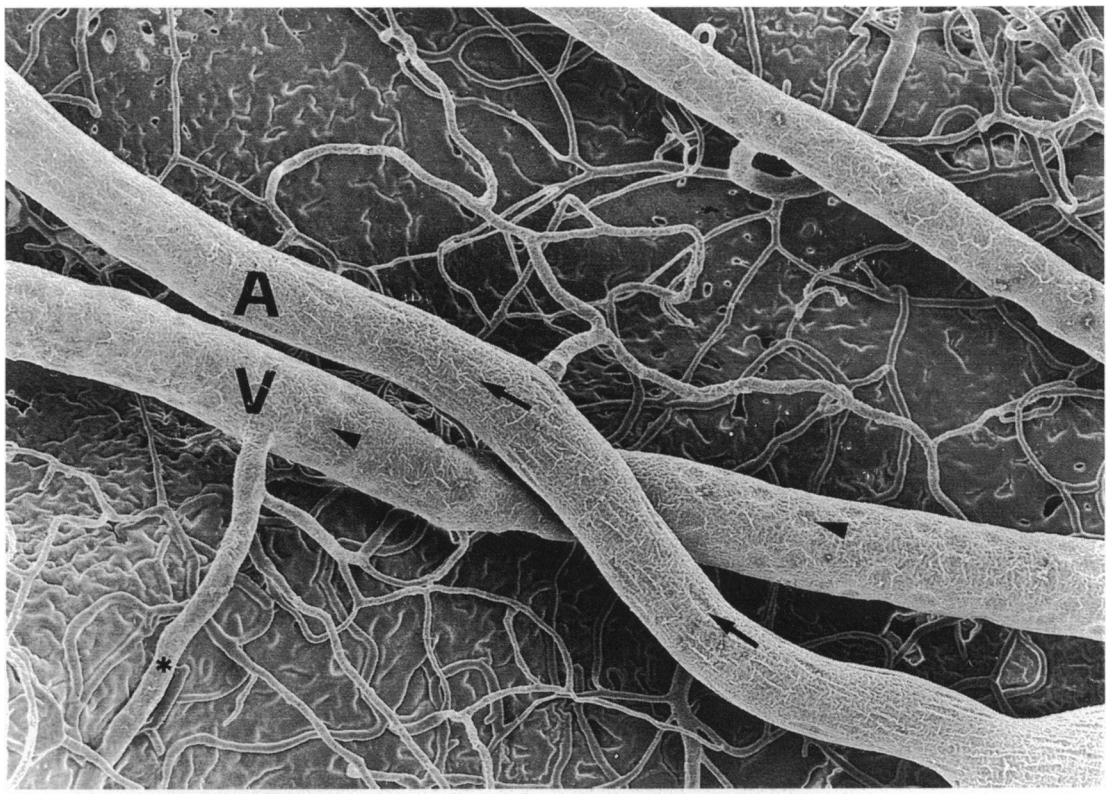

Fig. 2. Scanning electron micrograph of microvascular cast. Arteriovenous crossing of 2 main branches. Note different endothelial nuclear imprints (arrows). Imprints are spindle-shaped on the arterial cast (arrows) and round on the venous cast (arrowheads). A, artery; V, vein, *postcapillary venule. × 180.

arterioles most often as collateral but also as terminal branches (Fig. 4). Within 750 μm from the papilla, precapillaries always arose directly from CA branches (Fig. 5). In digested tissue specimens also, most of these vessels showed a lining of circularly arranged cells similar to the muscle cells of larger vessels.

At the origin of arterioles, an evident circumferential imprint around the cast was present if the diameter of the parent vessel was larger than 11.91 ± 1.45 μm (Fig. 3). If the diameter of the parent vessel was larger than 35.01 ± 2.81 μm it also showed an oval depression around the origin of the collateral. This aspect corresponds to the presence of a circularly arranged perivascular/smooth muscle cell in the partially digested specimens and was similar to that found at the origin of collaterals of CA branches. No smooth muscle cells bulging from the outer surface of the vessel were observed. Only arterial vessels lined by unveiled smooth muscle cells were considered for observation.

Precapillary arterioles gave rise to capillaries following 2 branching patterns: (1) capillaries taking origin as terminal branches of the arterioles after a dichotomous division (Fig. 6); (2) capillaries taking origin as collaterals of an arteriole that ran towards a postcapillary venule and, finally, acquiring the diameter of a capillary. This pattern was the only one present within 750 μm from the papilla. In this pattern, capillaries originated from the arteriole at a right angle or even at acute angles and their arterial end constantly showed a circumferential imprint (Fig. 7).

Beyond a distance of 750 μm from the papilla, the ratio between side branching precapillaries and bifurcating precapillaries was 1.778. However, even at this level most of the capillary bed originated from side branching precapillaries, because the average number of capillaries arising from collateral branching precapillaries all over the retina was 5.57 ± 0.82 and those originating from bifurcating capillaries was 2.

In semicorroded specimens, pericytes were randomly scattered along the capillary bed (Figs 8, 9). At the level of the origin of capillaries given off as collaterals of precapillary vessels (preferential channels) large processes of pericytes were almost constantly present, either along or around the offspring (Fig. 8). A further site where pericyte bodies and larger processes were found more frequently was at

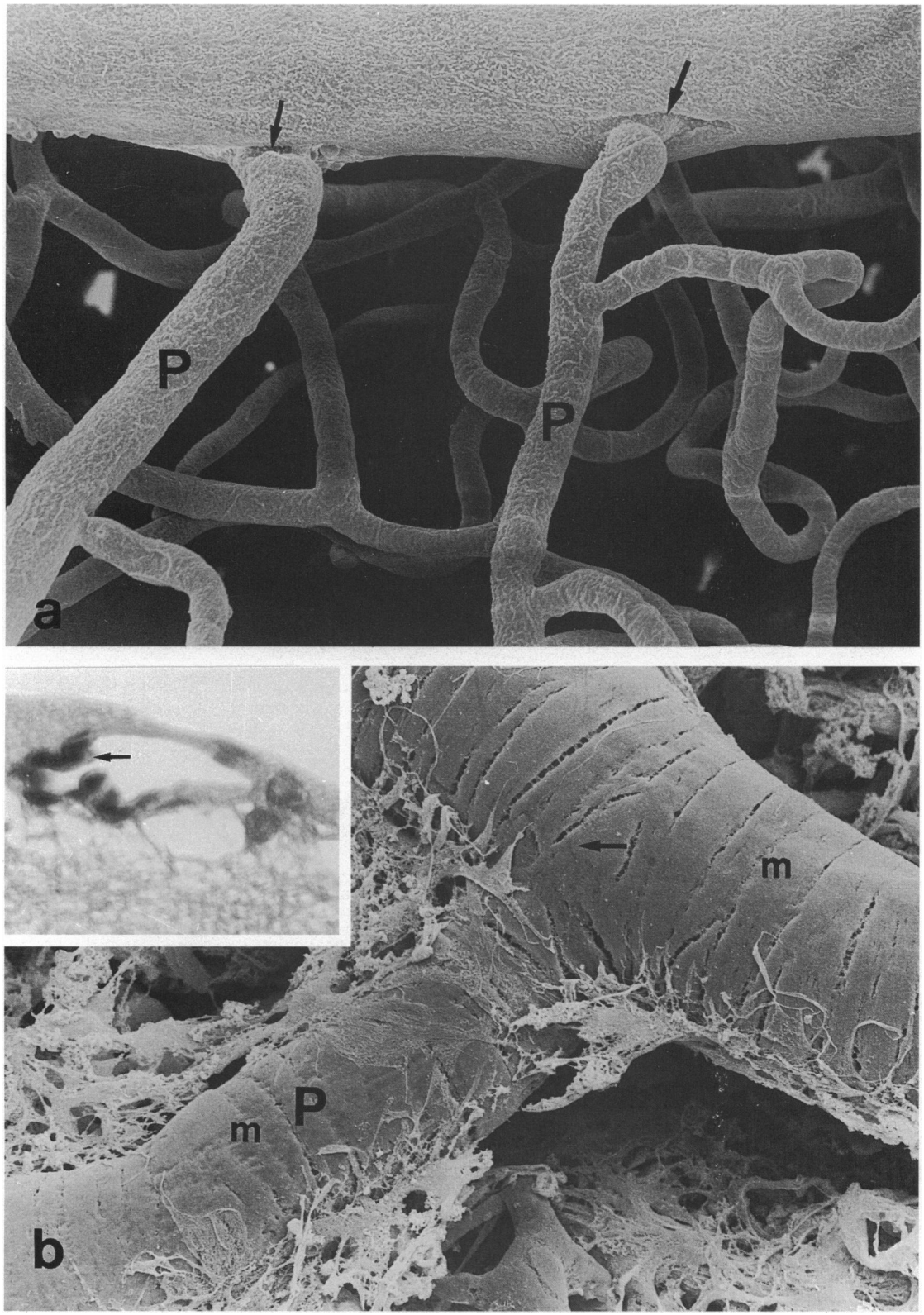

Fig. 3. Corrosion cast, semicorroded specimen and LM observation on the origin of a precapillary (P) from an arterial branch. (*a*) SEM of vascular corrosion cast; note the reduction in diameter of the collateral vessel and the oval depression on the parent vessel (arrows). × 400. (b) SEM of digested tissue specimen; note the varied orientation of smooth muscle cells (m) of the parent vessel at the branching point (arrow). × 720. Inset: LM; note the buckled endothelial nuclei (arrows) at a corresponding branching point. × 300.

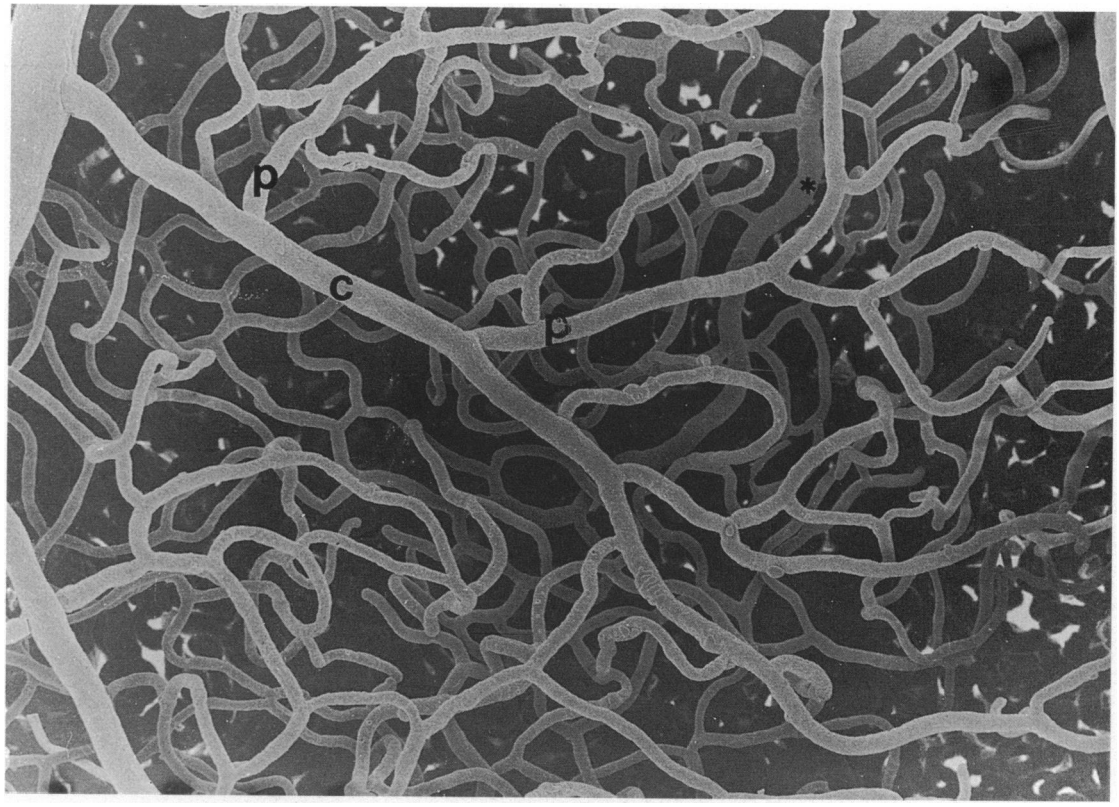

Fig. 4. Scanning electron micrograph of microvascular cast. A collateral vessel of a CA branch divides dichotomously once or twice, giving rise to precapillary arterioles both as collateral and terminal branches. C, collateral of CA branch; P, precapillary; *postcapillary venule. × 180.

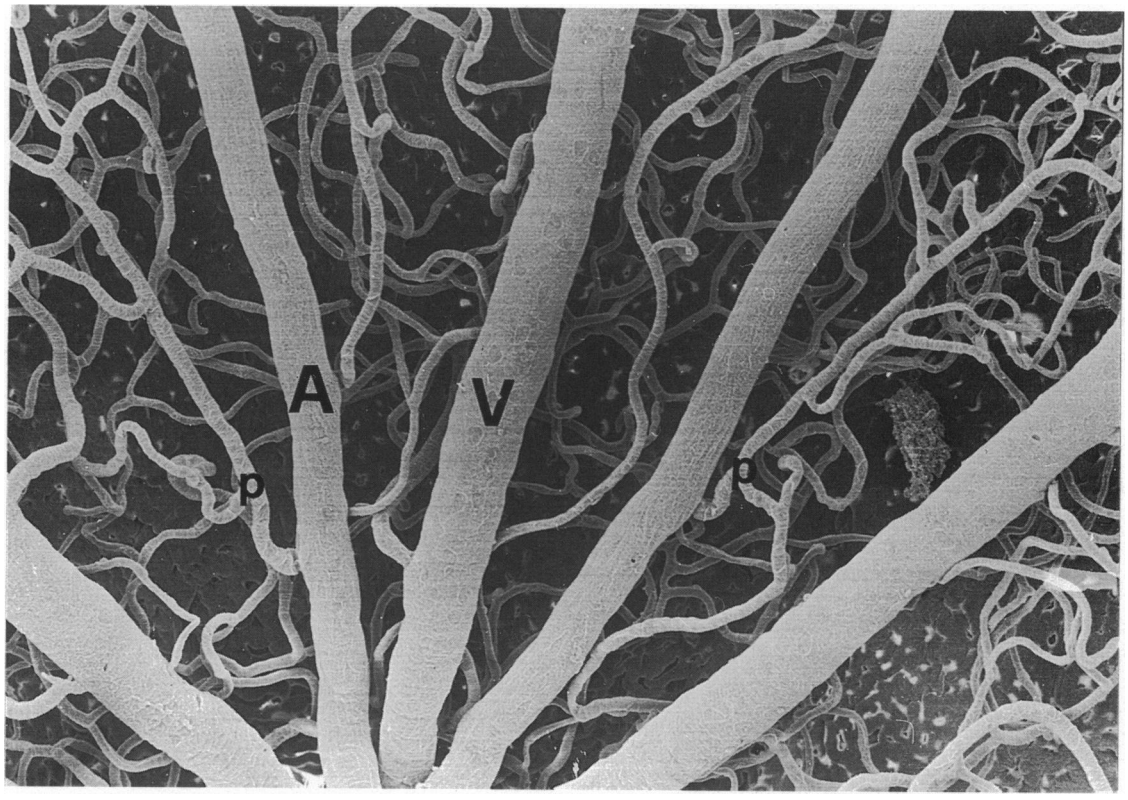

Fig. 5. Scanning electron micrograph of a microvascular cast. Precapillary vessels arise directly from a CA branch near the papilla; A, CA branch; P, precapillary; V, branch of the central vein. × 160.

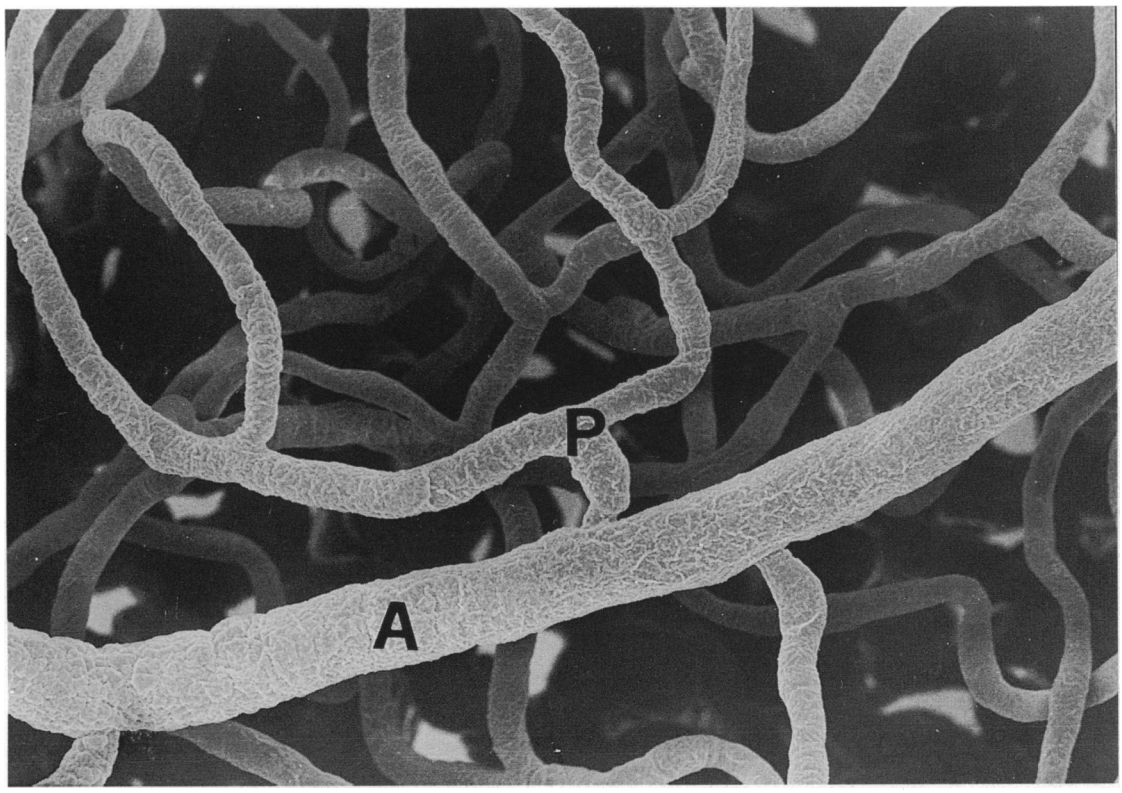

Fig. 6. Scanning electron micrograph of a microvascular cast. Precapillaries give origin to capillaries as terminal branches after dichotomous division. A, CA branch; P, precapillary. ×480.

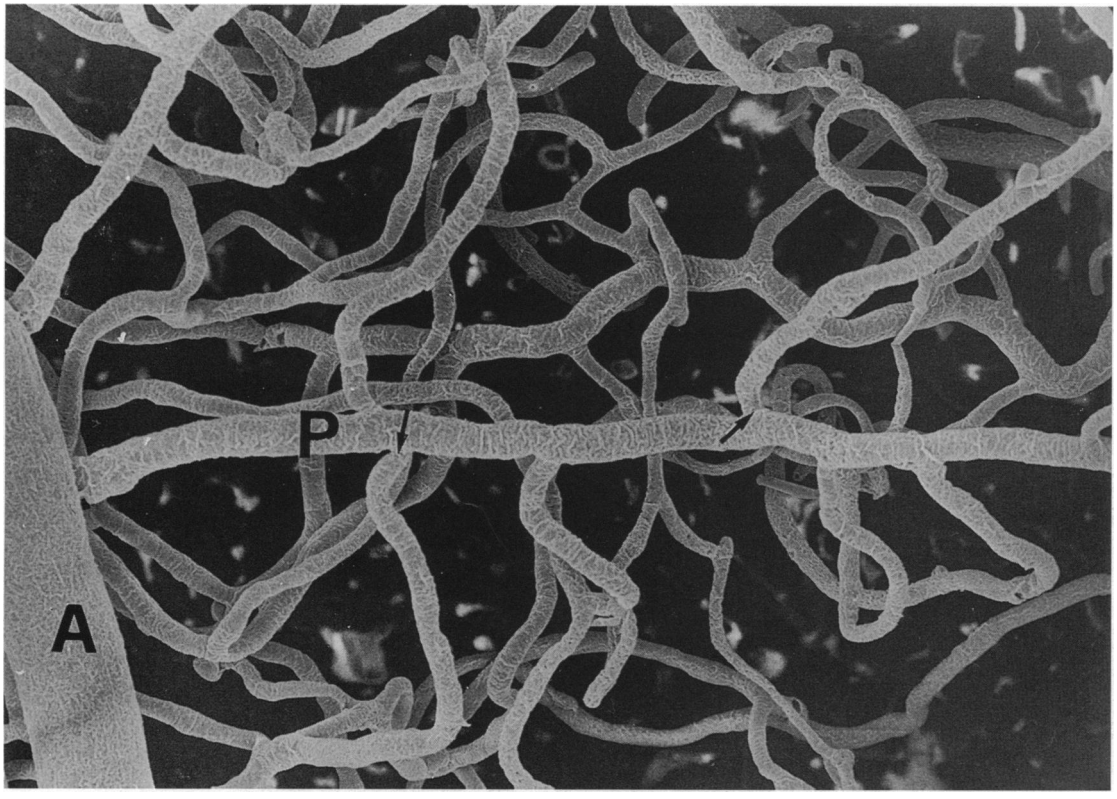

Fig. 7. Scanning electron micrograph of a microvascular cast showing a precapillary vessel (P) arising directly from a CA branch (A). Capillaries originate at right or acute angles. Note restriction in diameter at capillary origins (arrows).

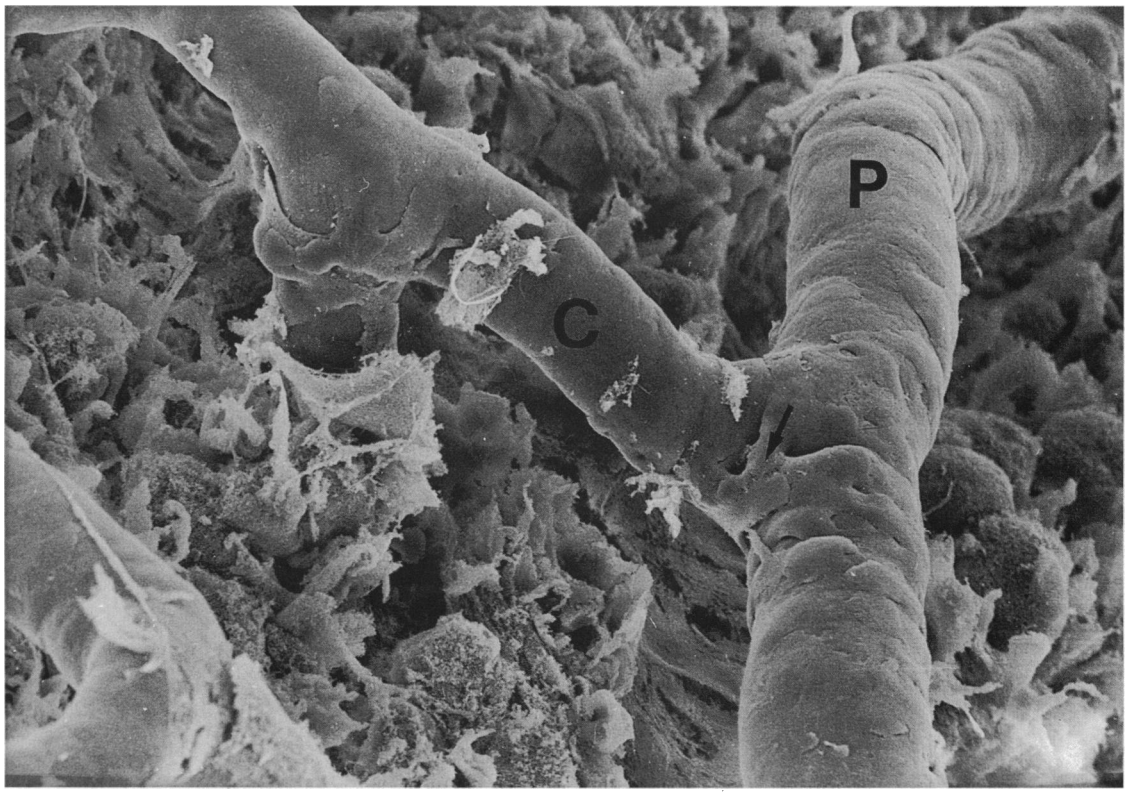

Fig. 8. Scanning electron micrograph of a semicorroded tissue specimen showing the terminal part of a side branching precapillary (P) and one of its offshoots (C). Note pericyte process around the origin of the capillary (arrow).

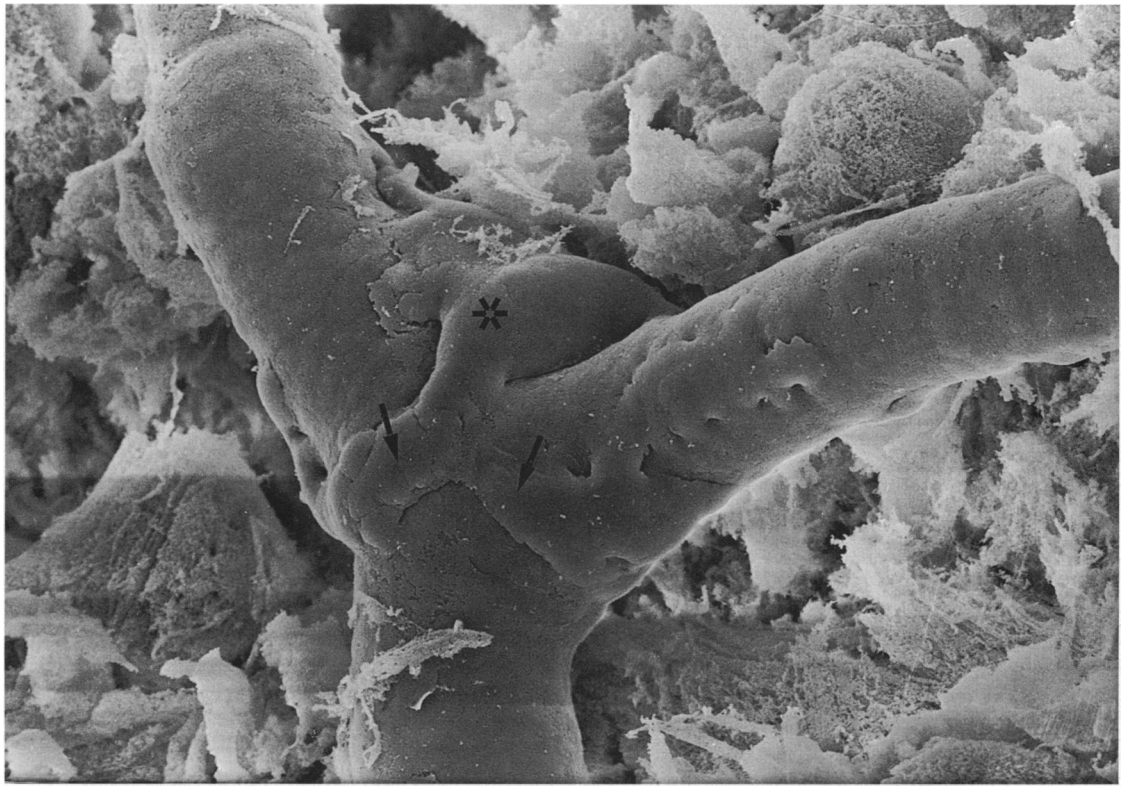

Fig. 9. Scanning electron micrograph of a semicorroded tissue specimen showing a pericyte at a branching point, origin of a capillary extending deeper into the retina. Asterisk, pericyte body; arrows, circumferential processes. × 3000.

the origin of capillaries extending deeper into the retina (Fig. 9).

LM observations

On light microscopy, branches of the central artery were readily recognised by their rounder cross section and thicker wall, as compared with the central vein branches, and for their giving rise to collaterals which could be followed along the ganglion cell layer. Branches of the central artery showed a bilayered lining of smooth muscle cells. Collaterals of CV branches, on the other hand, had a monolayered lining of smooth muscle cells.

The origins of collateral arterioles of CA branches were characterised by a reduction of the lumen and a special orientation of the monolayer of smooth muscle cells. Smooth muscle cells at collateral branching points appeared to extend deeper and to buckle the endothelium into the vascular lumen (Fig. 3), as demonstrated by serial sectioning. No clear bistratification of smooth muscle cells was detected at these branching points.

DISCUSSION

Vascular corrosion casts made it possible to follow in 3 dimensions the microvascular patterns in the rat retina and to trace exactly the course of precapillary vessels and their offshoots. Due to the extended depth of field of the SEM and the absence of retinal tissue, all the vessels in a given portion of the retina, even lying in different planes, could be visualised at the same time. Moreover, in the rat, no capillaries overlie arterial vessels (Pannarale et al. 1991) which, in turn, are very easily observed. Another advantage of corrosion casts is that arterial and venous vessels are readily recognised on SEM without the need to trace them back to the parent vessel, because they are characterised by differing endothelial imprints on their surface (Hodde et al. 1977).

Semicorrosion of retinas perfused with casting media was applied to retinal tissue by our group in 1992 (Pannarale et al. 1992). It has proved to be more successful in maintaining 3-dimensional arrangement of vessels than other similar techniques such as 'microdissection by ultrasonication' (Hilliard et al. 1987). We believe that this technique can complement others in demonstrating the exact placement of perivascular cells. The well maintained 3-dimensional pattern of vessels and smooth muscle cell morphology made studying arterioles very easy by comparing semicorroded specimens and vascular corrosion casts.

In most instances capillaries take origin as collaterals (side branches) of an arteriole that runs towards a postcapillary venule. Blood flow through side branching capillaries has been thoroughly studied by Fung (1981). He demonstrated that red blood cell flow through the smaller side branch can be lower than that through the large, main branch for 3 reasons. (1) *Entry condition into the capillary*. In vitro experiments have established that side branching at right angles reduces the numbers of red blood cells in the branch. In this branching pattern, the capillaries of the retina arise at right angles, or even have a course almost opposite to the direction of blood flow in the arteriole. (2) *Plasma skimming effect*. The haematocrit in the arteriole near the vessel wall is lower than in the rest of the vessel. A side branching capillary receives blood with a lower haematocrit than in the rest of the parent vessel. This general rule can be easily applied to the side branching capillaries in the retina, at least to those stemming from larger precapillary arterioles. (3) *Equation* $H_T = H_F V_m / V_c$ (Fung, 1981). The haematocrit in the side branching capillary (H_T) is equal to the product of the haematocrit in the larger precapillary (H_F) and the ratio between the mean velocity of whole blood through the capillary (V_m) and red cell velocity through the capillary (V_c). Red cells move faster than the plasma in the capillary. For this reason, $V_m/V_c < 1$. This means that H_T is always a fraction of H_F (Fung, 1981). These rules appear to favour blood flow through the arteriole as compared with that in its collateral capillaries (Fung, 1973; Yen & Fung, 1978).

The constrictions at the origins of these capillaries indicate the presence of peculiarly arranged processes of pericytes that could permit fine regulation of flow through capillaries (Gaudio et al. 1985, 1989) by varying the entry conditions for red blood cell into the capillary. The presence of vessels with this branching pattern, typical of a preferential channel, could explain situations in which an increase of flow in retinal arterioles does not correspond to a similar increase in retinal capillaries (Zeimer et al. 1992*a*, *b*). Caution should therefore be taken about attributing measurements made at arterial level directly to capillaries. As arterioles lie in the most superficial layer of the retinal microvasculature, the ganglion cell and nerve fibre layer appears to be preferentially perfused. We found no evidence of precapillary sphincters, but true circularly stratified contractile structures are not required to prevent blood cell flow in capillaries. Pericytes are able to buckle the vascular lumen and block cell flow in the retina (Tilton et al. 1979; Wallow & Burnside, 1980; Das et al. 1988). The

type of imprints we found almost constantly at the origin of capillaries originating at side branches of an arteriole is compatible with the presence of a pericyte (Gaudio et al. 1985, 1989, 1990). Semicorroded specimens, specifically prepared for demonstrating pericytes, show them precisely at those sites (Gaudio et al. 1993). It must be noted that preferential flow can take place at a precapillary level simply due to the microvascular pattern and independent of any vasomotor activity. If active contractility were demonstrated at the level of capillary origin this would allow for a superfine regulation of blood flow through capillaries (Wallow & Burnside, 1980).

Variability in the number of capillaries perfused by blood cells is often denied on the basis of the qualitative in vivo observations in Friedman et al. (1964) on choroid-deprived retina through a scleral window in the cat. They observed that 'most of the capillaries showed circulating blood cells and plasma most of the time' but also intermittency of flow 'at some capillary junctions'. This extremely high rate of capillary perfusion may be explicable in terms of their experimental model, a choroid-deprived retina. In fact, other in vivo observations (Thuràsky, 1957), performed through the vitreal cavity with the retina and choroid in situ, showed that only 30% of retinal capillaries were perfused at a given time. This suggests variability in perfusion through certain populations of capillaries. Side branching precapillaries are the most adaptable structures for the redistribution of blood flow through capillaries.

The preferential channels could act as a by-pass around nonperfused capillaries. In fact, the retinal vessels most susceptible to increased intraocular pressure are peripapillary radial capillaries (Henkind, 1967; Henkind & De Oliveira, 1967) which constitute a constant flow layer (Zeimer et al. 1992) with anastomoses (Shimizu & Ujiie, 1978) but without arterioles. Vessels with the same branching characteristics as those of the preferential channels can be observed in flat mount preparations of human retinas (Lutty & McLeod, 1992) as the spared vessels of an hypoperfused retina. Moreover, there is a predominance of side branching over bifurcations in the whole arterial bed in the rat. This makes the rat a more interesting model of retinal microcirculation, because sidearm branching prevails over dichotomous branching in the arterial vessels of the posterior human retina (Kuwabara & Cogan, 1960).

As a general rule in the rat, it was possible to see a circumferential constriction on the cast of the vascular lumen at the origin of every arterial side branch. These imprints are artifacts as they are never observed on the surface of venous casts and the filling of the vessel downstream and upstream from the constriction point is equal. Many authors (Kuwabara & Cogan, 1960; Henkind & De Oliveira, 1967) have reported observations on arteriolar annuli, nonmuscular and in some cases acellular structures, lining these branching points. On the other hand, our semicorroded samples showed that the outer surface of the vessel does not appear buckled. This observation excludes the influence of any structure such as the annuli in the development of imprints on the cast surface. Light microscopy has shown that at the origin of collateral branching arterioles smooth muscle cells can cause buckling of the endothelium towards the lumen. However, no clear evidence of a true sphincter structure at such level was present.

We can explain these observations by the presence of discrete sites of vasomotility with a specific cellular arrangement in the arterial wall. At these sites, specially oriented smooth muscle fibres are able to buckle endothelial nuclei, slowing down blood cell flow. We do not consider these structures to be true sphincters. In fact, we did not observe a true multilayered circumferential arrangement of smooth muscle cells capable of closing the vessel lumen completely. On the other hand, blood cells can stop at those branching points, as observed by Friedman (1964). This is in agreement with Shiraki et al. (1980) who interpreted the same imprints on corrosion casts as due to 'endothelial cell impressions' in the rat. Uije (1976) observed similar constrictions of the cast at the site of arteriolar branching in the monkey.

In conclusion, in the retinal microvasculature, side branching and the presence of sites of a peculiar arrangement of smooth muscle cells and pericytes should be considered as the structural basis for a very fine and selective regulation of blood flow.

ACKNOWLEDGEMENTS

This study was supported by grants from Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (40% and 60% funds), the Italian Consiglio Nazionale delle Ricerche and PhD funds for the physiopathology of the ocular microcirculation.

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