The role of the pericytes of the adventitial microcirculation in the arterial intimal thickening

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Summary. Segments of rat femoral arteries, with one collateral each, occluded between ligatures and dissected from surrounding tissue, developed intimal thickening, with or without ligation of their collaterals. Numerous newly-formed capillaries from the surrounding arterial microcirculation growing into the adventitia, tunica media and intimal thickening were demonstrated by means of serial longitudinal sections, predominantly in the ostium of the collateral. When the ligatures were applied without damaging the microcirculation surrounding the artery and the normal continuity of the adventitial vessels was unchanged, earlier presence of intimal thickening was observed. When the fibrous layers of the adventitia were removed at the moment of the arterial ligation, the continuity between newly-formed vessels of the neoadventitia and those growing into the media and neointima was much more evident. It was then noted that the pericytes constituted a major component of the intimal thickening. The introduction of contrast material in microcirculation confirmed the connections between newly-formed adventitial and intimal vessels. At the beginning of the experiment, autoradiographic studies showed an increased DNA synthesis in the cells of preformed postcapillary venules and capillaries of surrounding arterial microcirculation and later in those of the newly-formed vessels growing into the arterial wall. These results indicate that newly-formed capillaries derived from surrounding arterial microcirculation penetrate the wall of the occluded arterial segments and contribute to the intimal thickening formation. It is likely that the pericytes and endothelial cells (EC) of these ingrowing vessels are sources of myointimal cells at the intimal thickening and of endothelium at the luminal surface, respectively.

Key words: Pericyte, Endothelial cell, Intimal thickening, Smooth muscle cells, Angiogenesis

Introduction

The origin of myointimal cells that appear as a response to various types of arterial injury is still not clear. Recently, it has been suggested that intimal thickening seems to originate in cells from the vasa-vasorum, in particular the pericytes (Díaz-Flores and Domínguez, 1985). Also, using porous synthetic grafts inserted into the aortiliac circulation, it has been demonstrated that some neointimal cells are derived from the pericyte population (Clowes and Kirkman, 1986). Therefore, this new possibility must be added to the generally accepted hypothesis that intimal cells are derived from medial smooth muscle cells (SMC) (Hassler, 1970; Stemerman and Ross, 1972; Benditt and Benditt, 1973; Ross and Glomset, 1973, 1976 ; Fishman et al., 1975; Mustard and Packham, 1975; Benditt, 1977; Bhawan et al., 1977; Guyton and Karnovsky, 1979; Thomas et al., 1979; Karnovsky, 1981; Ross, 1981; Thomas, 1983), and to the other, less convincing hypotheses, that attribute the origin of the cells of the intimal thickening to arterial endothelial cells (Mehrota, 1953; Reddy and Cliff, 1979; Gebrane et al., 1982), multipotential mesenchymal cells of the intima (Ghani and Tibbs, 1962; Sparagen et al., 1962; Zollinger, 1967; Irey and Norris, 1973; Feigl et al., 1985), or transformed mononuclear cells of the circulating blood (Ghani and Tibbs, 1962; Stump et al., 1963; Feigl et al., 1985).

This study was undertaken to characterize and compare the formation of intimal thickening in a segment of the femoral artery between two ligatures under varying conditions which modify the continuity of its surrounding microcirculation and the structure of the adventitia. The results, obtained by light and electron microscopy (L.M. and E.M.), perfusion of intravascular

contrast medium, and observation of DNA-synthesis activity, provide evidence that pericytes of the arterial surrounding microcirculation are a source of the myointimal cells when the arterial circulation has been interrupted.

Materials and methods

Experimental animals

Adult Sprague-Dawley rats with an average weight of 300 g were used in accordance with the guidelines of the Animal Care Advisory Committee of the University of La Laguna. The rats were fed standard rat chow and water unrestrictedly and maintained in pathogen - free conditions.

Microsurgical performance in the femoral artery

The rats were anesthetized with Ketamine (150 mg/ Kg. i.p.). Using a surgical microscope, a 1.5 cm segment of a femoral artery and one of its collateral branches, were exposed. Ligatures were applied to both the proximal and distal ends of the dissected femoral arterial segment, with or without damaging the microcirculation surrounding it. The collateral was either ligated or not.

Tritiated thymidine ³H labelling

To assess DNA synthesis, an isotonic saline solution containing tritiated-thymidine (^{3}H - thymidine-methil; Nuclear Ibérica, Madrid, Spain) at a dose of 1 C/g of body weight was injected into the vena cava 90 minutes before some animals were to be killed.

Vascular perfusion

Before the arteries were removed, some rats were perfused with an aqueous barium solution (micropaque) as a contrast medium, to facilitate detection of vascular sprouts derived from the microcirculation surrounding the arterial segment.

Tissue processing

Specimens for L.M. were fixed in formalin, embedded in paraffin and cut into serial longitudinal sections. These were stained with hematoxylin-eosin, Mallory trichrome and orcein. Specimens for E.M. were fixed in a glutaraldehyde solution, diluted to 2% with sodium cacodylate buffer, pH 7.4, for six hours at 4° C, washed in the same buffer, postfixed for 2 hours in 1% osmium tetroxide, dehydrated through a graded acetone series and embedded in epoxy resin. 1.5 nm thick sections were cut, mounted on acid-cleaned slides and stained with 1% toluidine blue. From selected areas, thin sections were obtained, double stained with uranyl acetate and lead citrate, and examined under an electron microscope.

Autoradiography

The slides destined for autoradiographic studies were dipped in Ilford L-4 photographic emulsion (Ilford, Mobberley, England), dried, and stored in the dark at 5° C for 15-25 days. The autoradiograms were developed in Kodak D 19 solution (Buffalo, New York), fixed in acid fixer, washed, dried and stained as described above.

Experimental design

To study the formation of intimal thickening in occluded arteries, in a total of 100 cases, ligatures were applied in both femoral arteries. The collateral was either ligated or not. To compare the intensity, time of appearance and characteristics of the intimal thickening after varying conditions of the surrounding arterial microcirculation, in 20 of these cases, ligatures were applied after the arterial segments were dissected free of surrounding tissue. In 20 cases the adventitia was also removed. In another 20 cases, the ligatures were applied without damaging the microcirculation surrounding the artery. 20 cases were processed for autoradiographic study, and in 20 cases vascular perfusion with barium solution was undertaken. The animals were sacrificed at daily postsurgery intervals, from day 1 to 20.
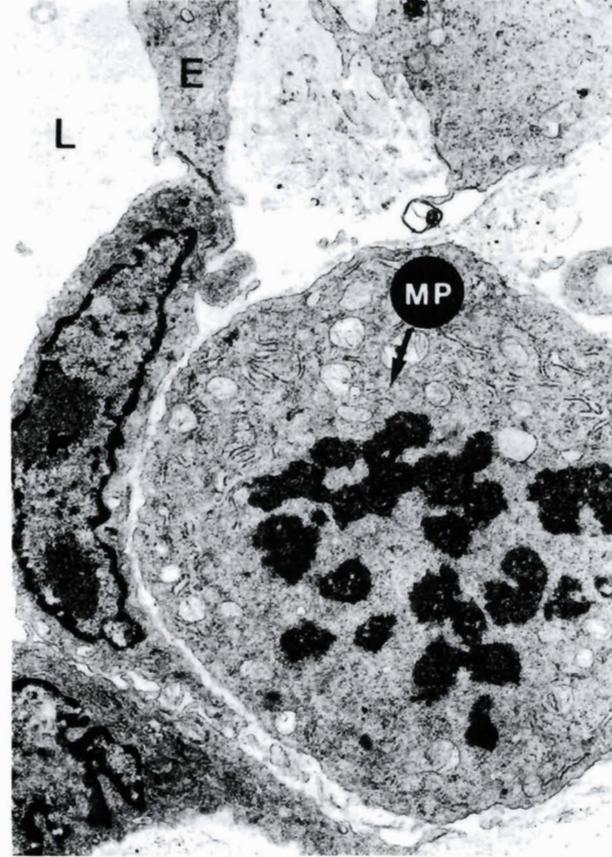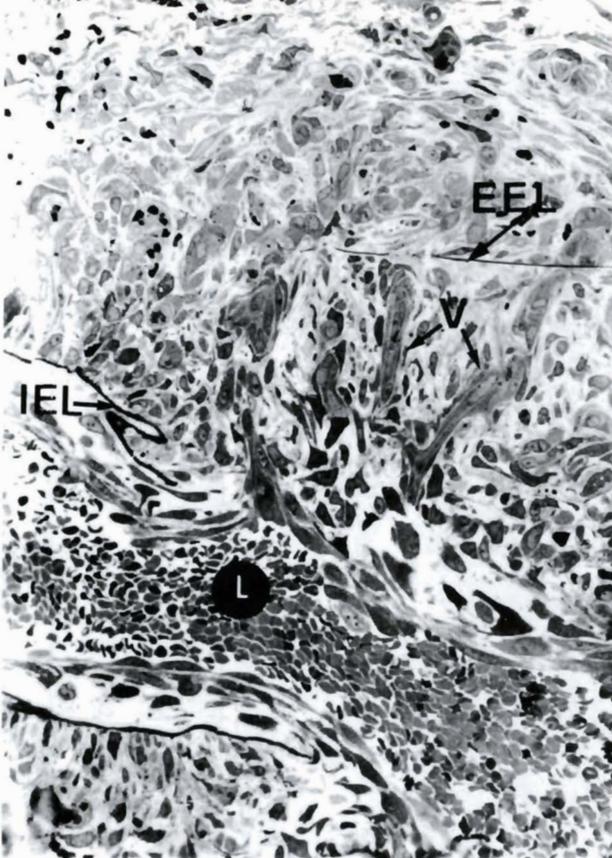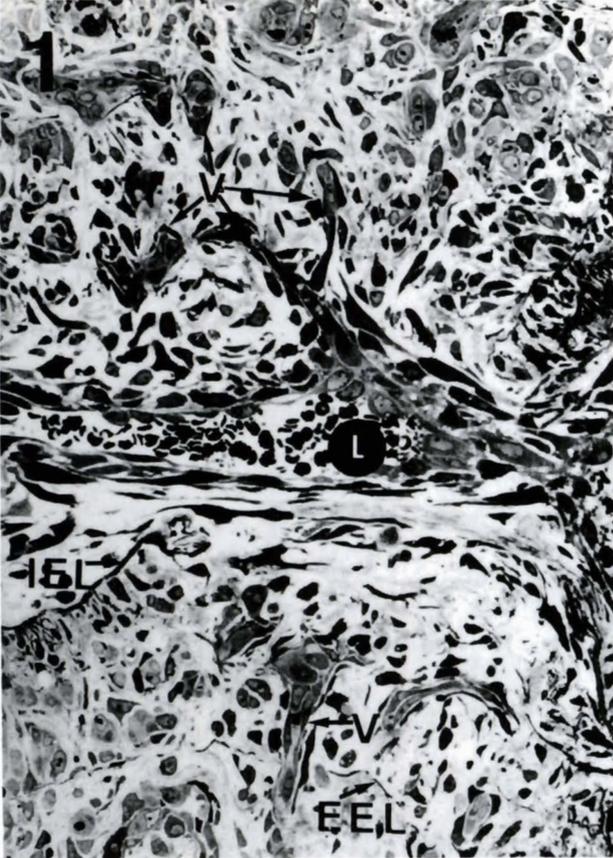
Results

One to three days after the arterial segments were dissected free and ligatures applied, the endothelium disappeared, or was necrotic, and the internal elastic lamina (I.E.L.) showed platelets adhering to the luminal surface. Some of the medial SMC revealed degenerative changes, loss of myofilaments and necrosis. Few polymorphonuclear leukocytes were observed both in the arterial lumen and through the arterial wall. On the third day, capillary sprouts were beginning to form from the surrounding arterial microcirculation. The vascular sprouts extended from the preformed postcapillary venules and capillaries into the adventitia of the artery.

Between days 4 and 7 newly-formed capillaries, together with large numbers of fibroblast-like cells and macrophages were observed growing into the adventitia and tunica media. These findings were more frequent in the proximity of the collateral ostium (Fig. 1), with and without collateral ligature.
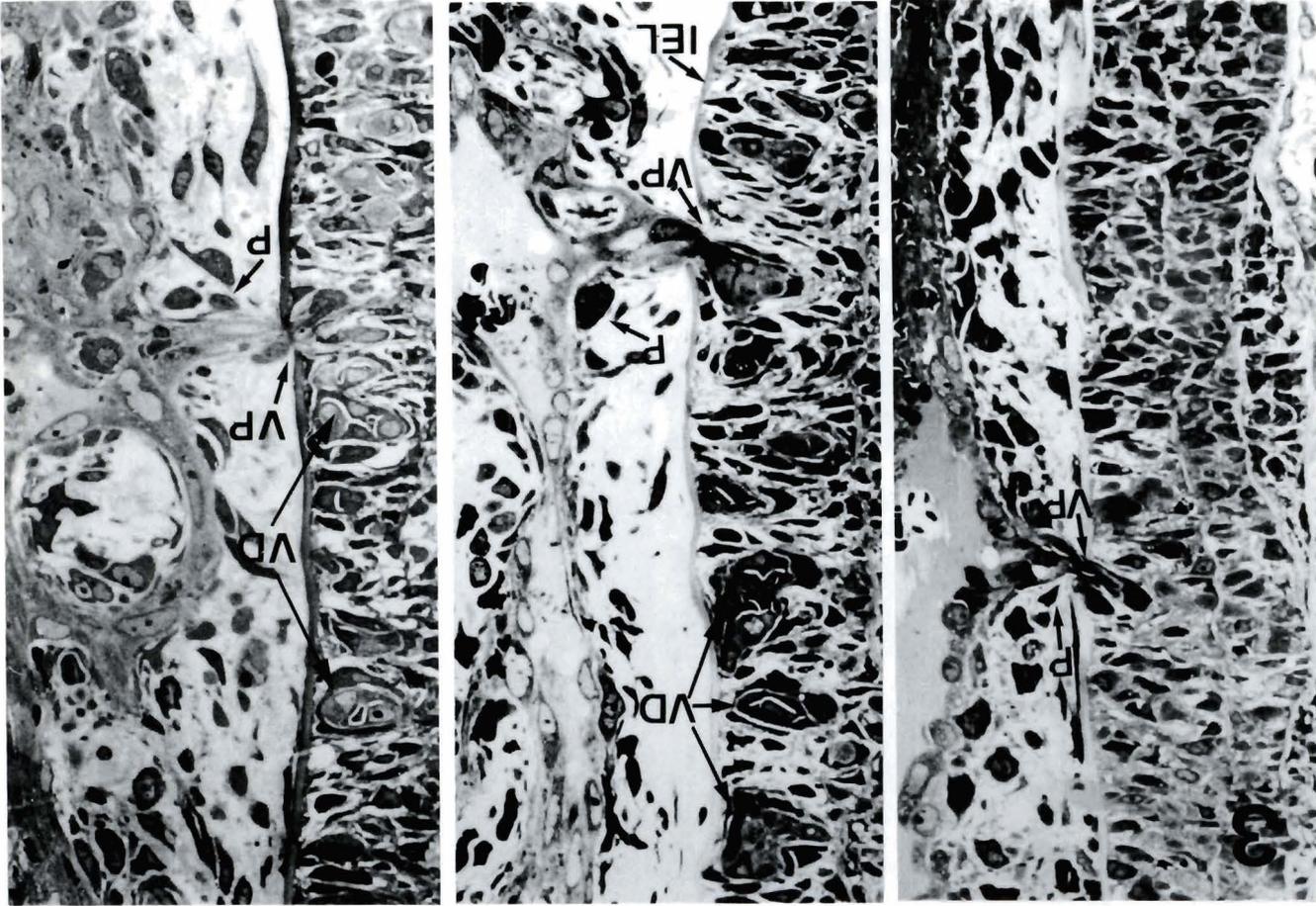
From day 8 onwards many myointimal cells and capillary sprouts were present in a newly-formed intimal thickening lining the I.E.L. The pericytes of these capillaries were hypertrophied and they showed an increased rough endoplasmic reticulum and a wide separation from the EC (Fig. 2A). Mitotic figures were observed in some pericytes (Fig. 2B). Numerous transitional cell forms between hypertrophied pericytes and myointimal cells were also seen.

In those cases where the ligatures were applied without damaging the microcirculation surrounding the artery, similar results were observed, except that capillary and cellular ingrowth into arterial lumen was

granulation tissue were observed penetrating the arterial wall. By means of serial longitudinal sections, a high number of vessel buds lying on the external surface of the I.E.L. (Fig. 3) and penetrating the I.E.L. through narrow channels (Fig. 3) were also demonstrated. Zonal breaking of I.E.L. with an evident continuity between the vessels penetrating the tunica media, and those of the intimal thickening was observed (Fig. 4). When the new capillary buds penetrate through the I.E.L. they provide multiple sources of endothelium at the arterial luminal surface and their pericytes form part of the intimal thickening (Figs. 3, 4). These findings were very prominent in the area of the ostium of the collateral (Fig. 4).

In the cases where vascular perfusion with barium solution was undertaken, the contrast material was observed in the newly-formed vessels present in the adventitia, tunica media and in the intimal thickening (Fig. 5). Connections were seen between these vessels and the arterial lumen (Fig. 5B, C, D). These findings were more evident in the ostium of the collateral (Fig. 5B).

The autoradiographic studies of the arterial segment showed a considerably increased rate of DNA synthesis when compared to other non-affected arterial areas. At first, numerous EC and pericytes were labelled in preformed postcapillary venules and capillaries of

Numerous capillaries derived from surrounding number of capillary sprouts and fibroblast-like cells. external surface of the tunica media, there were a great prominent. On the neoadventitia, specially on the penetration in the arterial tunica media became more described, but the events of cellular and microvascular obtained were not quite as regular as those previously When the adventitia was also removed, the results observed as soon as day 4.

Fig. 1. Arterial segment, 7 days after occlusion, dissected free from surrounding tissue. The collateral was not ligated. Vessel buds are observed (V) penetrating into the adventitia and tunica media in close proximity to the collateral ostium. I.E.L.: internal elastic lamina. E.E.L.: external elastic lamina. L: arterial lumen. Semithin sections, toluidine blue. × 520

Fig. 2. Ultrastructural characteristics of new ingrowing capillary buds through the arterial wall 7 days after ligature. Activated pericytes (P), prominent endothelial cells (E), disruption of the basal lamina and mitotic figure in a pericyte (MP) are observed. L: capillary lumen. × 10,200

Fig. 3. Occluded arterial segment without fibrous layers of the adventitia. 6 days postsurgery. Examples of vessel buds located on the external surface of the I.E.L. (V.D.) and other ones penetrating the I.E.L. through narrow channels (V.P.). The new arterial endothelium is continuous with the endothelial cells of ingrowing capillaries. The pericytes (P) of these ingrowing capillaries become part of the intimal thickening. Semithin sections, toluidine blue. × 480
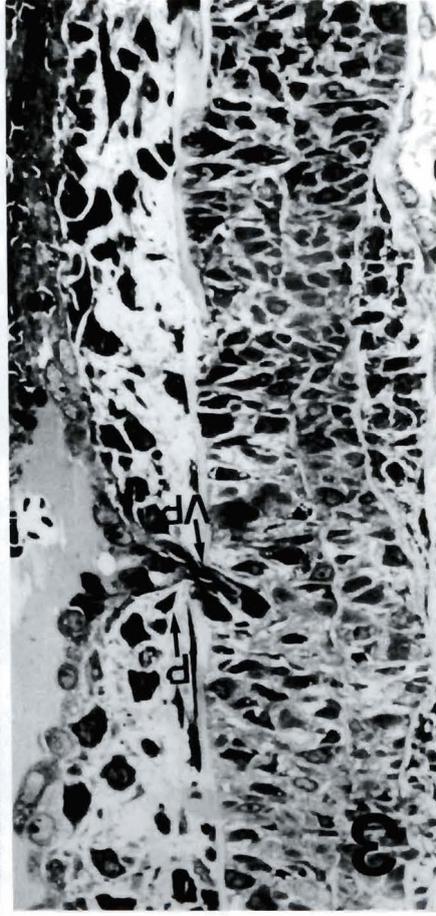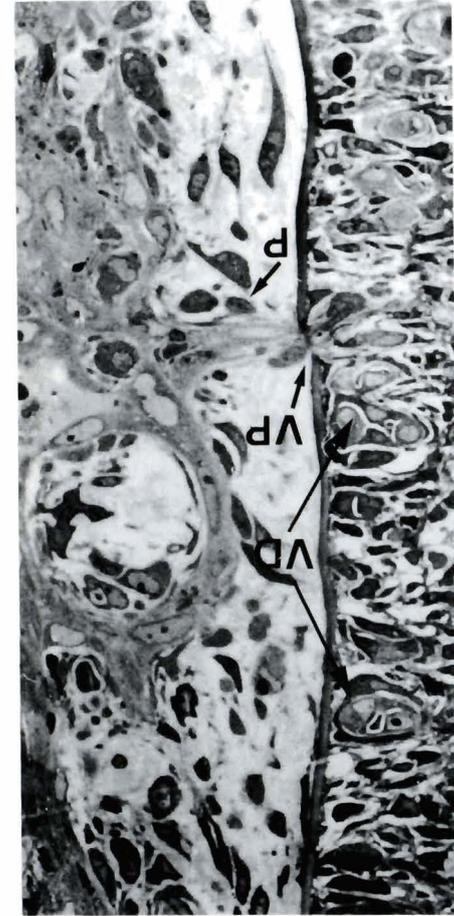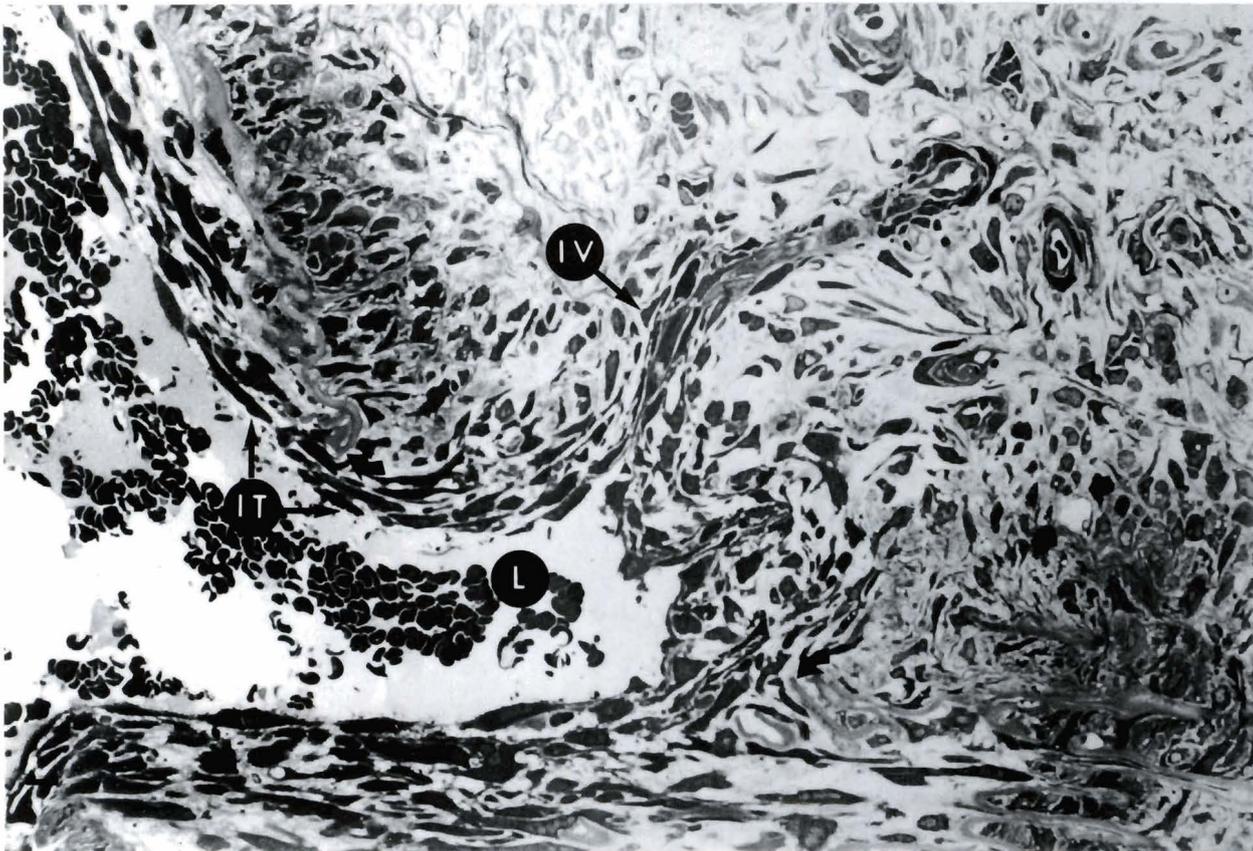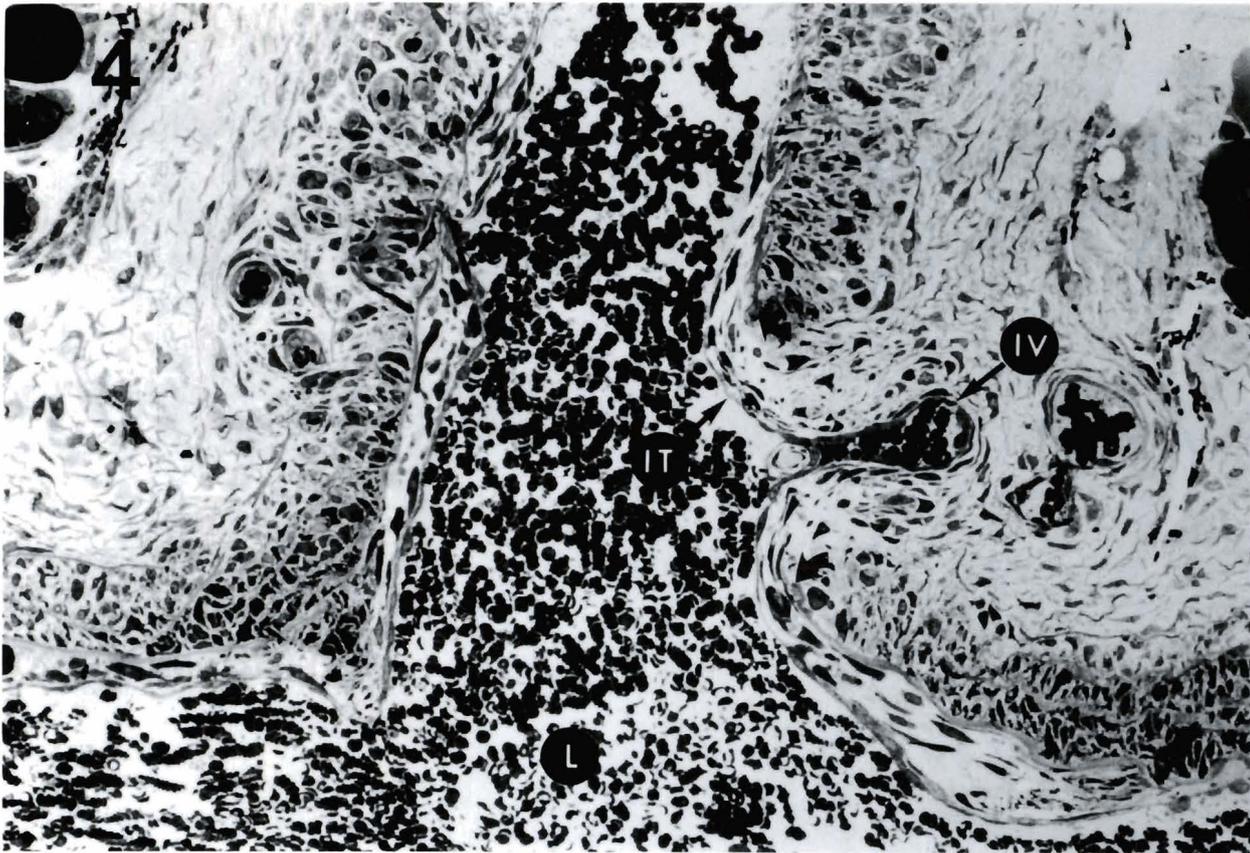
**Fig. 4.** Semithin sections through arterial segment without fibrous layer of the adventitia. 5 and 6 days postsurgery. Zonal breakings of I.E.L. (between incurvated arrows) are observed in collateral ostium with the presence of ingrowing vessels (I.V.) which extend from the periarterial tissues to the lumen (L). The continuity between the ingrowing vessels and the intimal thickening (I.T.) is evident. Toluidine blue × 90 and 260
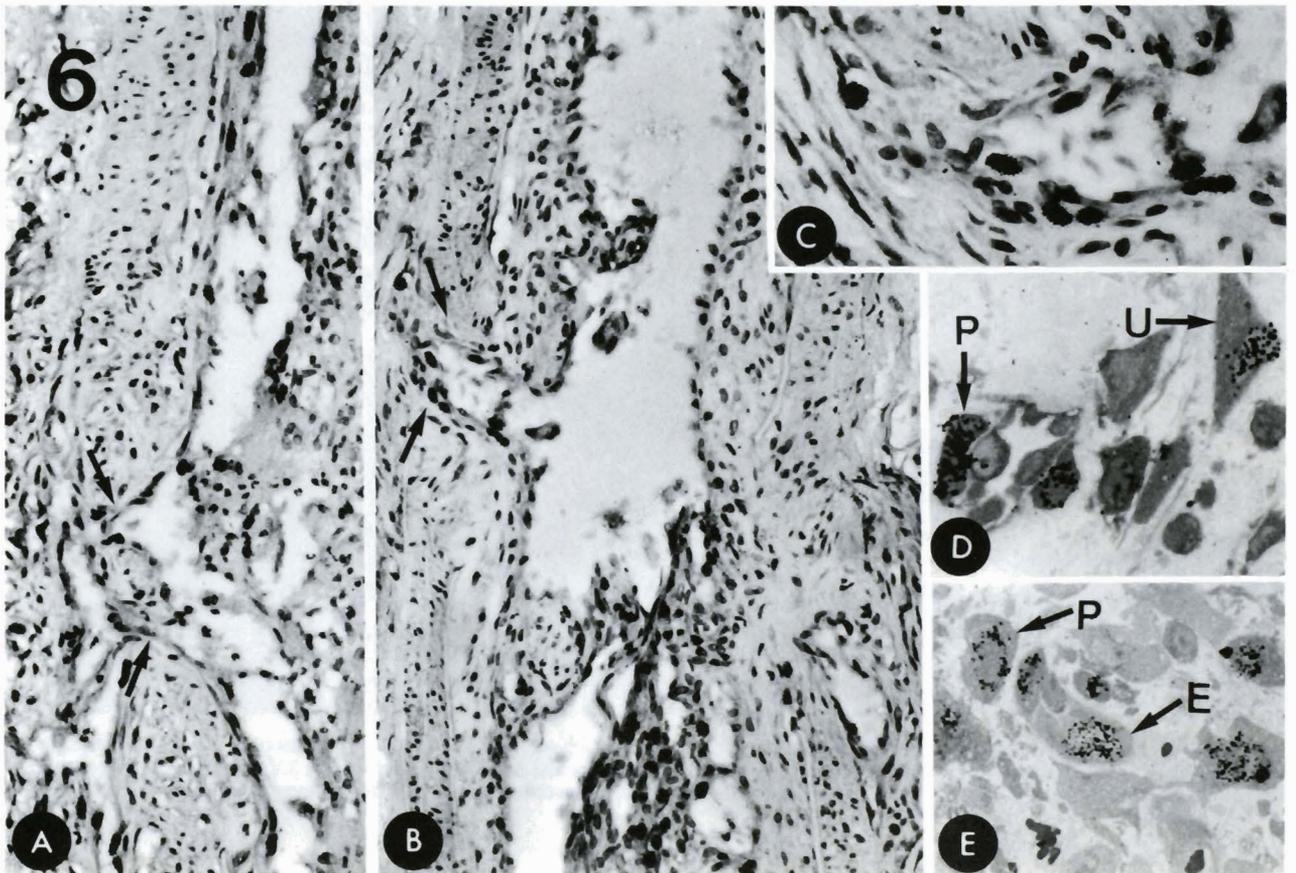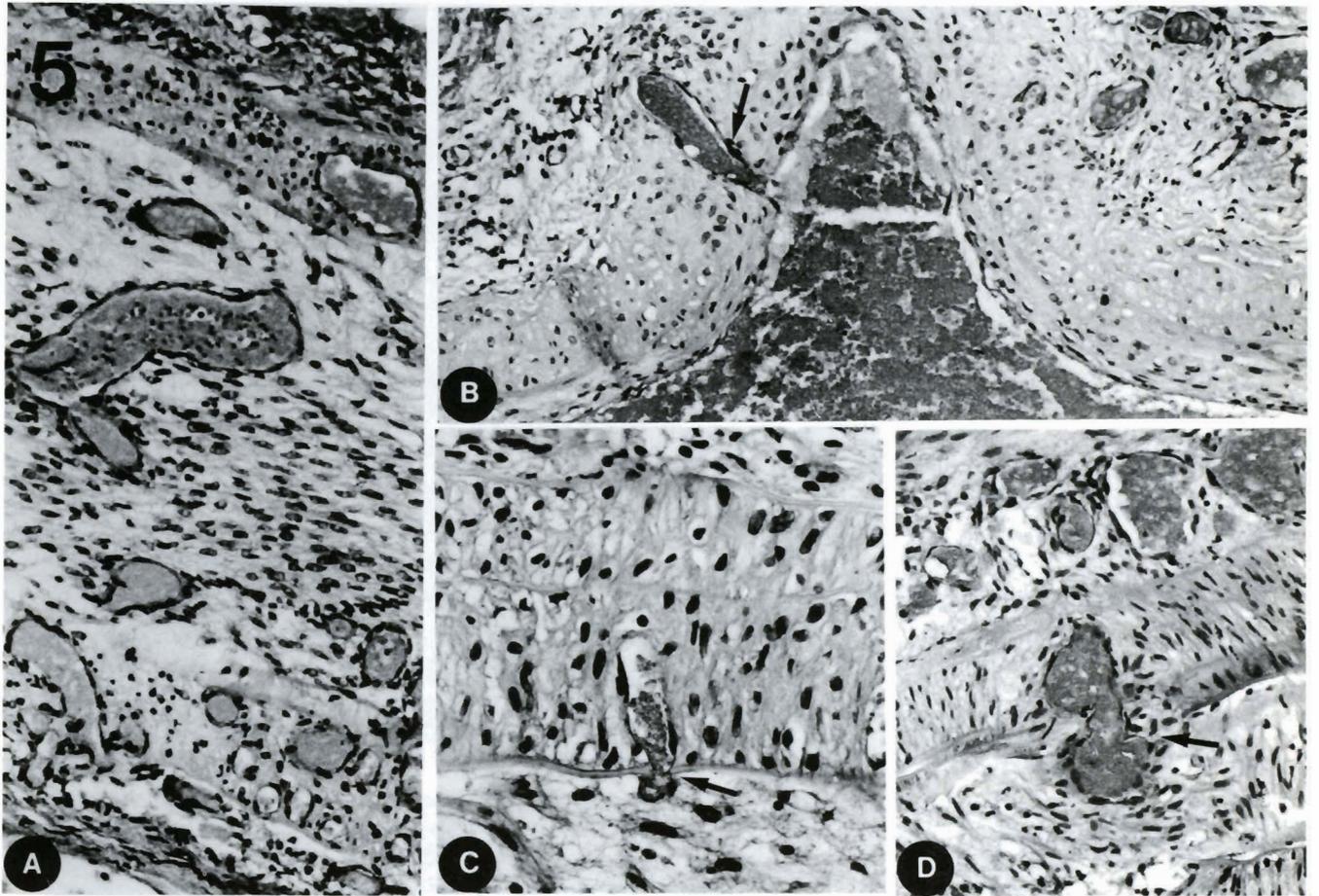
Fig. 5. Different examples of occluded arterial segments with vascular perfusion 9-13 days after ligatures. Contrast technique make evident a dense plexus of microvessels typically extended from the adventitia through the media and into the thickened intima (Fig. 5A). In Fig. 5B the vascular penetration is shown in the ostium of the collateral (arrow), and in Figs. 5C and 5D some vessels are observed penetrating I.E.L. (arrows). H & E. Figs. A, B, C × 70 and Fig. D× 80

Fig. 6. Autoradiograms from occluded arterial segments. (Figs. 6A and 6B) narrow strips of the arterial wall (arrows) are observed with an increasing gradient of labelling percentage. The increased number of ^{3}H-labelled cells is evident in vessel buds penetrating the tunica media and the thickened intima (× 70. Hematoxylin). Fig. 6C, enlarged area between arrows of Fig. 6B (× 180. Hematoxylin). Figs. 6D and 6E, detail of H^{3}labelled pericytes, endothelium and undifferentiated cells from ingrowing vessel buds (× 440. Toluidine blue).
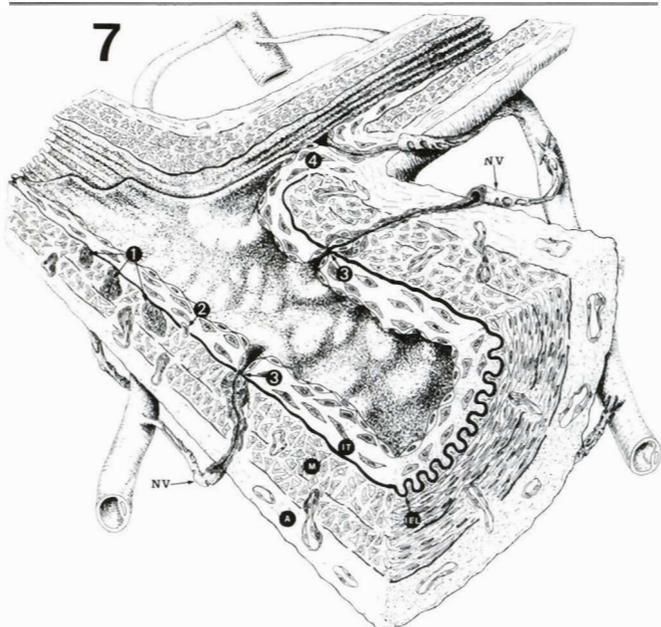
Fig. 7. Schematic drawing showing the different ways in which the newly-formed capillaries cross the arterial wall in a segment between ligatures. 1: Vessel buds delayed on the external surface of the I.E.L.; 2: undifferentiated cell penetrating the I.E.L.; 3: vessel buds penetrating the I.E.L.; 4: zonal breaking of I.E.L. at collateral ostium with ingrowing vessels. The endothelial cells of the vessel buds are continuous with the new arterial endothelium (3 and 4) and the pericytes become part of the intimal thickening. A: adventitia, M: tunica media, IT: intimal thickening, and NV: newly-formed adventitial microcirculation.

surrounding arterial tissue. Later, labelling was observed in newly-formed vessels penetrating the arterial wall and reaching the intimal thickening (Fig. 6A, B). In these vessels, numerous hypertrophied pericytes appeared labelled (Fig. 6D, E).

Discussion

Most results in the occluded arterial segments are in agreement with other studies on the occlusion of large vessels in similar conditions (Buck, 1961; Bhawan et al., 1977; Guyton and Karnovsky, 1979). Moreover, our observations on longitudinal arterial sections showed capillary ingrowth through the adventitia and tunica media, mostly in the proximity of the ostium of the

collaterals. Microvessels were also seen in the intimal thickening. The connection between the capillaries in the arterial wall and those of the intimal thickening was observed in serial longitudinal sections, while this fact was difficult to demonstrate in transversal sections, because the ingrowing capillaries cross the arterial wall spirally at several irregularly arranged points. This form of vascular ingrowing may explain why, by means of indirect immunoperoxidase technique, in the tunica media of the healing rabbit thoracic aorta the fibrinogen/fibrin and degradation products were seen in scattered areas only (Chemnitz and Collatz Christensen, 1984).

Bhawan et al. (1977) observed that in some segments of the carotid arteries between two ligatures the original lumen showed one or more newly-formed «channels» containing what appeared to be circulating «fresh» blood and concluded that this minimal recanalization arises from the point of the ligations. In our observations, when the collateral was not ligated, numerous vessels penetrating the adventitia and tunica media were observed in the proximity of the ostium of the collateral, which rules out the intervention of the ligatures. The more prominent response in the ostium of the collateral might be related to the fact that the newly-formed and ingrowing capillaries follow the paths of least resistance, as do the intramural fluids of the arteries.

When at the moment of applying ligatures the artery was dissected free of surrounding tissue, the normal continuity of the adventitial vessels was interrupted. In these conditions, the formation of new vessels in the adventitia required some time, and the capillary ingrowth through the arterial wall appeared later than when the normal continuity of the adventitial vessels was not interrupted. If the vessels growing into the arterial wall intervened in the formation of the intimal thickening, it can be supposed that this one will appear at different times according to whether the adventitial vessels are interrupted or not. To assess this hypothesis, in some cases the surrounding arterial microcirculation was not damaged, because the arterial segment was not dissected free from surrounding tissue except in the ligature area. In these cases, the results were similar, but the intimal thickening appeared earlier. This further supports the intervention of the adventitial microvasculature as being the genesis of the intimal thickening.

The course of the ingrowing capillaries crossing the adventitia is difficult to follow even in serial longitudinal sections. For this reason, some arterial segments were dissected free from surrounding tissue and the adventitia removed. In these cases, the relationship between the newly-formed adventitial vessels with those present in the tunica media and in the intimal thickening was easily established. Likewise, it was possible to observe a continuity between the endothelial cells of ingrowing vessels and the new endothelium of the arteries, and to demonstrate that their pericytes form part of the intimal thickening. The arterial perfusion of contrast medium confirmed the connections between adventitial and luminal vessels. The different ways in which the capillaries cross the I.E.L. were clearly shown, ranging

from narrow perforations to large breakings (Fig. 7).

The presence of ³H-labelled undifferentiated cells in the internal zone of the medium layer, prior to the myointimal cells appearing in the intimal thickening, has been previously described (Hassler, 1970; Stary, 1974; Webster et al., 1974). This fact seems to support the hypothesis that intimal cells are derived from medial SMC. Our autoradiographic studies demonstrated that the pericytes and endothelial cells of the newly-formed vessels presented the most marked proliferation. The distribution of tritiated thymidine uptake appeared first in the cells of preformed postcapillary venules and capillaries of surrounding arterial microcirculation and later in those of the newly-formed vessels growing into the arterial wall. According to these observations, the presence of the ³H-labelled undifferentiated cells in the internal zone of the medium layer, can have another interpretation than that previously mentioned, since these labelled cells seem to coincide more with the solid vessel buds, which reached as far as the external surface of the I.E.L., than with undifferentiated SMC.

The observations reported here suggest that capillaries can penetrate the arterial wall and serve as a source of intimal thickening cells: luminal EC and myointimal cells. In relation to this, Díaz-Flores and Domínguez (1985) hypothesized that intimal thickening results from intra-arterial penetration of the vasa-vasorum and a differentiation of its pericytes into myointimal cells when the arterial circulation has been interrupted. Alternatively, the thickening could result from the migration and proliferation of the pericytes of the vasa-vasorum when the arterial flow has remained unchanged after arterial injury. With regard to this, it has been suggested that perivascular cells (pericytes) retaining considerable mesenchymal potentiality may have the capacity to differentiate into other cell types, such as fibroblasts (Ross et al., 1970), chondroblasts, osteoblasts (Tahayasi, 1986; Díaz-Flores et al., 1988), preadipocytes (Richardson et al., 1982) and vascular smooth muscle cells (Movat and Fernando, 1964). Likewise, some authors consider the pericytes as the progenitor cells of several pseudosarcomatous soft tissue lesions (Díaz-Flores, et al., 1989), malignant fibrous histiocytoma (Iwasaki et al., 1987) and mixoid liposarcoma (Bolen and Thorning, 1980).

The hypothesis generally accepted that myointimal cells may come from the medial SMC is supported by numerous observations (Hassler, 1970; Stemerman and Ross, 1972; Benditt and Benditt, 1973; Fisham et al., 1975, 1979; Mustard and Packman, 1975; Ross and Glomset, 1976, 1979; Benditt, 1977; Bhawan et al., 1977; Guyton and Karnovsky, 1979; Thomas et al., 1979; Ross, 1981; Karnovsky, 1981; Thomas, 1983) but, in the results obtained by most of the authors that hold to this hypothesis, there are some findings which cannot be easily explained by means of this theory. For example: a) the high degree of differentiation of SMC, with tensile and biosynthetic properties, which, according to this hypothesis, should also be multipotential and migratory elements «in vivo» (Wissler, 1968;

Stemerman and Ross, 1972); b) the presence of some phenotypic difference between myointimal cells and medial SMC (Kocher et al., 1984); and c) the few surviving SMC of tunica media found in occluded arterial segments, coinciding with the formation of the intimal thickening (Bhawan et al., 1977; Guyton and Karnovsky, 1979). The data of the present study of a previous one (Díaz-Flores and Domínguez, 1985) add a new possibility to the origin of the intimal thickening cells when the arterial circulation has been interrupted. It is possible that the intimal thickening is derived from pericytes of the vasa-vasorum or from those which accompany the ingrowth of new vessels from regenerated adventitia. The pericytes, while conserving undifferentiated properties, would intervene not only in the arteriolization of capillaries but also in the replacement of the vascular SMC (Webster et al., 1974) and in the formation of myointimal cells, by migration, proliferation and differentiation.

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