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Participation of angiogenesis from rat femoral veins in the neovascularization of adjacent occluded arteries

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Summary. The neovascularization of the arterial wall in human and experimental pathology has been demonstrated. The occlusion of the of the rat femoral artery is a suitable model for the study of these angiogenesis processes. Newly formed capillaries growing into the arterial wall have been described in this model. The origin of these ingrowing capillaries has been attribute to the preformed surrounding venules and capillaries. The contribution of the adjacent femoral vein with a supplementary population of vascular sprouts could also be possible. To test this hypothesis in half of the occluded arteries, the adventitia was removed from the side facing the femoral vein. Between 1 and 3 days after surgery several alterations were found both in the endothelial cells and the smooth muscle cells of the tunica media. Between 3 and 6 days, solid or canalized endothelial sprouts were observed arising from the femoral vein. By days 4 and 6, newly formed capillaries grew into the adventitia and tunica media of the femoral artery. Some of them, penetrated the internal elastic lamina. This microvascular penetration from the femoral vein was more prominent in the area of the ostium of the collateral and when the adventitia was removed. Some ingrowing capillaries were in continuity with the endothelial cells of the arterial neointima. At days 7 and 8, regressing capillaries were observed in the neomicrovasculature network between artery and vein, with a selective loss of the smaller vessels. From day 9 onwards, fewer and larger vascular channels were present between the femoral vein and the femoral artery. An arterial neolumen contained what appeared to be circulating "fresh" blood. Quantitatively, the venous neocapillary density increased from days 4 to 6 and then declined significantly by day 8. The arterial neocapillary density increased form days 4 to 8 and declined significantly by day 12. Moreover, both densities were significantly greater when the arterial adventitia was removed. The perfusion with barium solution showed the presence of the contrast material in the newly formed vessels, the lumen of the femoral vein, and the neolumen of the occluded arterial segment. The present findings indicate that putative angiogenic molecules released form the occluded arterial segment may reach the adjacent wall of the vein inducing neovascularization from it. The vein vascular sprouts are connected to the ingrowing capillaries in the occluded arterial wall and to the neocapillaries form the preexisting pericytic microvasculature. When the arterial adventitia were removed up to 2 times greater vein neocapillary's density was observed suggesting an easily access of the putative angiogenic factors to the vein.

Key words: Angiogenesis, Microcirculation, Neovascularization, Arterial intimal thickening, Veins, Endothelial cells, Smooth muscle cells, Pericytes

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Introduction

The microvessel invasion of the arterial wall has been demonstrated in human and experimental pathology (Köester, 1876; Winternitz et al., 1938; Paterson, 1938; Le Compte, 1967; Cliff and Schoefl, 1983; Barger et al., 1984; Eisenstein, 1991; Zhang et al., 1993; O'Brien et al., 1994), with particular reference to atherosclerotic lesions, in which neovascularization may play several important roles in their pathogenesis and their sequelae (Zhang et al., 1993; O'Brien et al., 1994). As far as experimental pathology is concerned, the presence of newly-formed capillaries growing into the arterial wall can be well demonstrated in occluded segments of rat femoral arteries, while an intimal thickening is developing (Díaz-Flores et al., 1990; Díaz-Flores and Domínguez, 1985). The origin of these ingrowing capillaries in the segments of rat femoral arteries occluded between ligatures has been attributed to the surrounding arterial pericytic microvasculature, in other words, the preformed venules and capillaries (Díaz-Flores and Domínguez, 1985; Díaz-Flores et al., 1990). However, it is possible that the adjacent femoral vein augments the process of neovascularization from venules and capillaries by contributing a supplementary population of vascular sprouts. We base this hypothesis

on the fact that an intense vascular sprouting from the rat femoral vein has been induced in our laboratory by different substances, such as glycerol, some acylglycerols, and prostaglandins E1 and E2 (Díaz-Flores et al., 1994, 1996).

Given these considerations, the purpose of this study was to test the hypothesis that rat femoral veins participate in the neovascularization of occluded adjacent femoral arteries in which an intimal thickening is developing. To facilitate its demonstration, in half of the cases the adventitia of the femoral artery was removed from the side facing the femoral vein. The results, obtained by light and electron microscopy, and by perfusion of intravascular contrast medium, provide evidence that the femoral vein is in part responsible for the origin of ingrowing capillaries in the wall of the occluded femoral arteries.

Materials and methods

Experimental animals

Adult Sprague-Dawley rats (average weight 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 ad libitum and were maintained under pathogen-free conditions. During surgical procedures and tissue removal, the rats were anesthetized with Ketamine (150 mg/Kg i.p.).

Microsurgical performance in the femoral vessels

Using a surgical microscope, the femoral vessels of both legs in each animal were exposed. Ligatures with a 10/0 thread were applied to the ends of a segment of the femoral artery 1.5 cm in length. A collateral present in the occluded femoral segment was also ligated at a distance of 0.3 cm from the ostium. In the left legs, the adventitia of the occluded segment of the femoral artery was removed from the side facing the femoral vein, leaving the arterial media layer in direct contact with the vein.

Vascular perfusion

In the rats in which vascular perfusion was to be undertaken, an aqueous barium solution (Micropaque, Pan Quimica-Farmaceutica, S.A., Madrid) was administered as a contrast medium, before removing the femoral vessels. The animals were divided in two groups for the following two procedures: 1) without damaging the microcirculation, a segment of the femoral vein adjacent to the occluded femoral artery was isolated from circulation to avoid any possible escape of the perfused solution other than through the newly-formed microvessels from the femoral vein. Then, through an opening in the distal end of the occluded venous segment, the contrast medium was administered. 2) The micropaque solution was introduced by arterial perfusion from the aorta.

Tissue processing

Specimens for conventional light and electron microscopic histology (LM and EM), 5 mm long, were fixed in a glutaraldehyde solution, diluted to 2% with sodium cacodylate buffer, pH 7.4, for 6 h at 4 °C, washed in the same buffer, postfixed in 1% buffered osmium tetroxide for 2 hours, dehydrated through a graded alcohol series and embedded in Epon 812 resin. The specimens were orientated in such a way that the femoral vein and the femoral artery were sectioned longitudinally, and 1.5 μm-thick sections were cut, mounted on acid-cleaned slides, and stained with 1% toluidine blue. Thin sections were obtained from selected areas, double stained with uranyl acetate and lead citrate, and examined under a JEOL electron microscope. Vascular perfused specimens were fixed in formalin, embedded in paraffin and cut into serial longitudinal sections for LM. Sections were stained with Haematoxylin and Eosin, and Orcein.

Quantitative studies

From each specimen of the femoral vessels, at the level of the collateral, five 1.5 μm-thick sections were cut out at approximate intervals of 20 μm. A 4 mm-length of femoral vessels in each section, with a total length of 20 mm, was considered for the quantitative study. Both the numbers of newly formed capillaries arising from the femoral vein (vein neocapillary density - VNCD -) and of those present in the media and intima of the femoral artery (artery neocapillary density - ANCD -) were independently counted in the 20 mm length of these vessels. Following that, the mean value and standard deviation of the mean were calculated for days 4, 6, 8 and 12. Statistical analysis was made with analysis of variance (ANOVA), followed by t-test comparisons. Analysis was carried out using the Statistic Software program (NH Analytical Software). Statistical significance was defined as $p< 0.05$.

Experimental design

To study whether the rat femoral vein may participate in the neovascularization of occluded segments of femoral arteries, microsurgical procedures, with and without removal of the arterial adventitia, were undertaken in 52 animals, 36 for conventional LM and EM histology and 16 for vascular perfusion. Specimens from femoral vessels were removed at days 1-12, 28 and 42 after surgery; 2 rats per time point, except for days 4, 6, 8 and 12, in which 4 were used for quantitative studies. To confirm that the newly formed vessels in the occluded segments of the femoral artery connected with the lumen of the femoral vein, an aqueous barium solution was perfused, either from the femoral vein

(n: 8), or from the aorta (n: 8) at days 6, 8, 10 and 12 after surgery. Control rats (n: 20) were sham-operated, being submitted to the microsurgical procedure described above, except for the application of ligatures and the removal of the arterial adventitia. Of these sham-operated rats, 16 were for conventional LM and EM histology and 4 for vascular perfusion.

Results

Control cases

The morphology of the femoral vein and femoral artery remained unmodified, except for the presence of a few neutrophils and mononuclear cells in the surrounding tissues. Using conventional LM and EM histology or LM studies after vascular perfusion, neocapillaries were neither observed arising from the femoral vein, nor were they present in the arterial wall.

Test cases

Between 1 and 3 days after surgery, the femoral vein showed hypertrophy of the endothelial cells (EC), some of which were in mitosis. The EC of the femoral artery were either necrotic or had disappeared, while some of the medial SMC revealed degenerative changes, loss of myofilaments and necrosis.

Between 3 and 6 days, solid or canalized endothelial sprouts were observed arising from the femoral vein (Figs. 1, 2), while some SMC of the vein media layer adopted a periendothelial location (Fig. 2). Mitoses were observed in EC and periendothelial cells of the vascular buds. Capillary sprouts emerging from the preformed microvasculature were also present. Some newly formed vessels appeared to be connected to each other, and their lumens were continuous with the interior of the vein (Figs. 1, 2). By days 4 and 6, newly-formed capillaries were observed growing into the adventitia and tunica media of the femoral artery (Fig. 3). Several ingrowing capillaries were also located on the external surface of the arterial internal elastic lamina (IEL) and occasionally were seen penetrating it through narrow fenestrations (Fig. 4). At this stage, an arterial intimal thickening was present and some of the ingrowing capillary EC penetrating the IEL were observed in continuity with the EC of the arterial neointima (Fig. 4). The phenomena of angiogenesis from the femoral vein and the microvascular penetration in the arterial adventitia or neoadventitia, tunica media and neointima were present in all test cases, but they were more prominent in the area of the ostium of the collateral and when the adventitia was removed.

At days 7 and 8, regressing capillaries were observed, showing degenerated EC, as well as lumens occluded by platelets, erythrocytes or dead cells. Likewise, the neomicrovasculature network between artery and vein appeared to be simplified with a selective loss of the smaller vessels, leaving fewer, larger channels, some of them in connection with the femoral vein lumen (Fig. 5). In the femoral artery, capillaries were still present in the media and intima, while the intimal thickening was increased by numerous myointimal cells (Fig. 5).

From day 9 onwards, fewer and larger vascular channels were present between the femoral vein and the femoral artery. An occasional continuity of these persistent vessels, either with the lumens of the vein or the artery, was demonstrated. The femoral arteries showed a well developed intimal thickening, lined by EC and made up of cells with varying degrees of differentiation toward SMC. An arterial neolumen contained what appeared to be circulating «fresh» blood. Channels, also lined by EC, were occasionally observed crossing the arterial wall.

In the quantitative studies, the venous neocapillary density (VNCD) was defined as the number of neocapillaries arising from the intimal endothelial cells of the femoral vein, while the number of neocapillaries in the media and intima of the femoral artery was the arterial neocapillary density (ANCD). The VNCD increased from days 4 to 6 and then declined significantly by day 8. The VNCD, before its decrease, was significantly greater when the arterial adventitia was removed than when it was not. The ANCD increased from days 4 to 8 and declined significantly by day 12. It was also significantly grater in cases without arterial adventitia than in those with it.

In specimens where vascular perfusion with barium

Table 1. VNCD or vein neocapillary density (number of neocapillaries that appear arising from the femoral vein wall) and ANCD or artery neocapillary density (number of capillaries growing into the media and intima of the femoral artery wall) per 20mm length of the longitudinally sectioned femoral vessels.

	DAY 4		DAY 6		DAY 8		DAY 12	
	VNCD	ANCD	VNCD	ANCD	VNCD	ANCD	VNCD	ANCD
Test cases (occluded arteries)								
Arterial adventitia unremoved	95±33.66*	6.25±2.5^{+}	120.5±49.9**	30.25±12.89^{++}	48.5±23.58	33.25±22.09^{+++}	21±7.48	12.75±4.03
Arterial adventitia removed	159.75±40.50	26.25±14.54	252.5±54.35°	181.25±48.21	84.75±28.12	207.75±40.17°°	27.5±11.67	14.5±9.29
Control								
Sham operated rats	0	0	0	0	0	0	0	0

VNCD and ANCD in test groups vs. control group: significant (VNCD and ANCD of control group: 0). *: p<0..55; **: p<0.02 vs arterial adventitia removed; $^{+}$: p<0.04; $^{++}$: p<0.001; $^{+++}$: p<0.0003 vs arterial adventitia removed; °: p<0.002 vs VNCD of 8 days; °°: p<0.0009 vs ANCD of 12 days.

solution was performed through the femoral vein isolated from the circulation, the contrast material was observed in the newly formed vessels originating from the femoral vein and in most of those crossing the arterial wall, as well as in the neolumen of the occluded arterial segment (Fig. 6). Similar findings were seen when the contrast medium was perfused from the aorta.

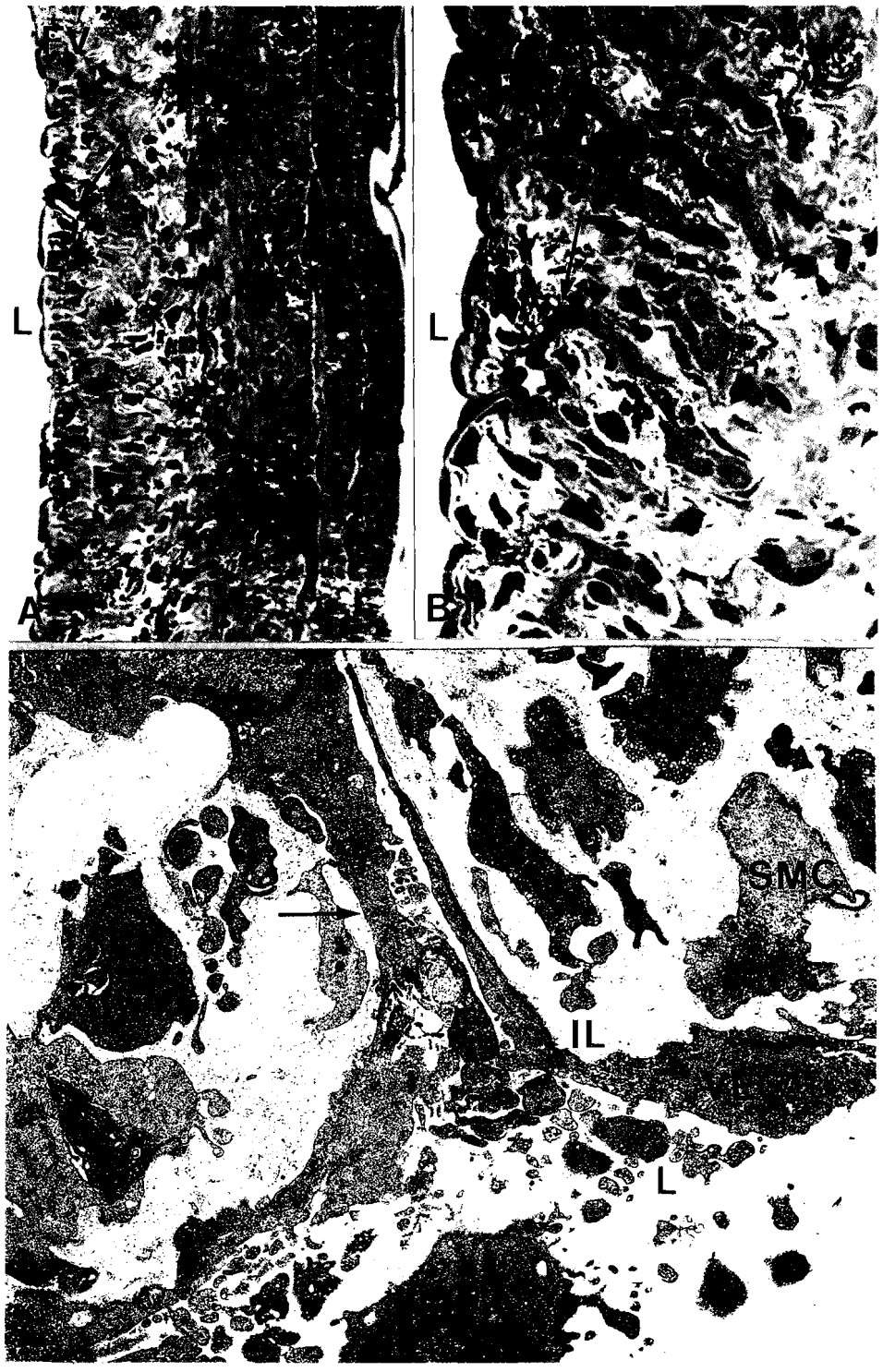

Fig. 1. Femoral vessels 4 days after occlusion of the femoral artery. The adjacent walls of both the femoral vein (FV) and femoral artery (FA), and the connective tissue between them are shown (A). Vascular buds arising from the femoral vein are observed in semithin **(A and B)** and ultrathin **(C)** sections (arrows). L: Femoral vein lumen; IL: vein discontinuous internal elastic lamina; VEC: vein endothelial cell; SMC: Smooth muscle cell. A and B: Toluidine blue, x 86 and 320. C: Uranyl acetate and lead citrate, x 7,200

Discussion

In the current work we confirm the development of an intimal thickening in occluded segments of arterial vessels (Bahwan et al., 1977; Guyton and Karnovsky, 1979; Díaz-Flores and Domínguez, 1985; Díaz-Flores et al., 1990), as well as previous observations showing an extensive adventitial capillary ingrowth through the rat femoral arterial wall, while an intimal thickening is developing; a pehnomenon that is more easily

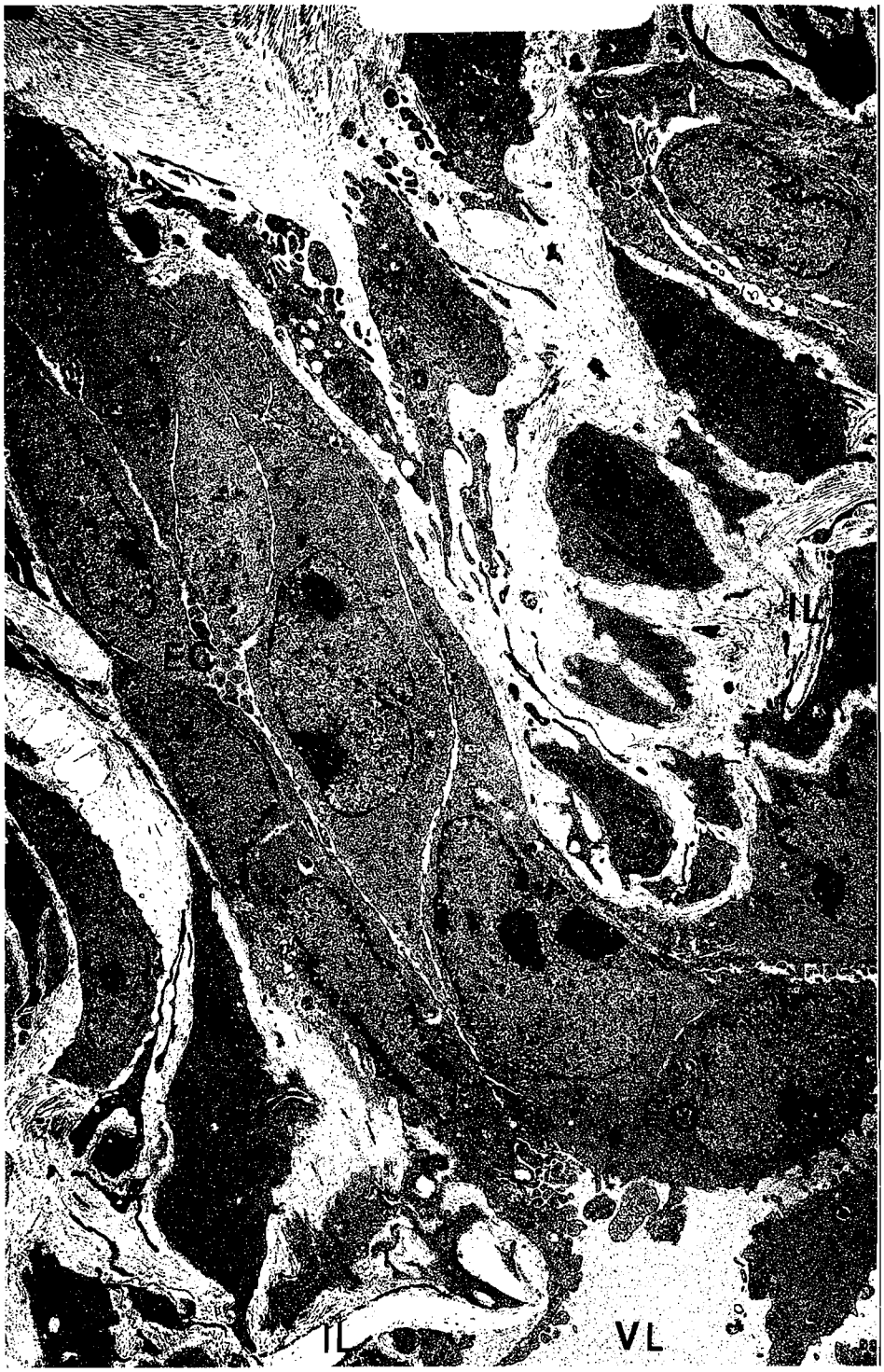

Fig. 2. A capillary sprout extending into the medial layer of the femoral vein, through a gap in the discontinuous internal elastic lamina (IL), is shown 4 days after occlusion and adventitia removal of the femoral artery. Between sprouting EC, a slitlike lumen is present. Smooth muscle cells (SMC) of the vein media layer are observed, some of them adopting a periendothelial location on the vascular bud. VL: vein Lumen. Ultrathin section; Uranyl acetate and lead citrate, x 8,000

demonstrated on longitudinal sections of the artery, mainly in the ostium of collaterals (Díaz-Flores and Domínguez, 1985; Díaz-Flores et al., 1990).

The neovascularization of the occluded arteries may represent a response to vessel wall ischemia. This mechanism is also proposed to explain the angiogenesis of many tumors (Shweiki et al., 1992), the presence of intraplaque vessels in human atherosclerotic lesions

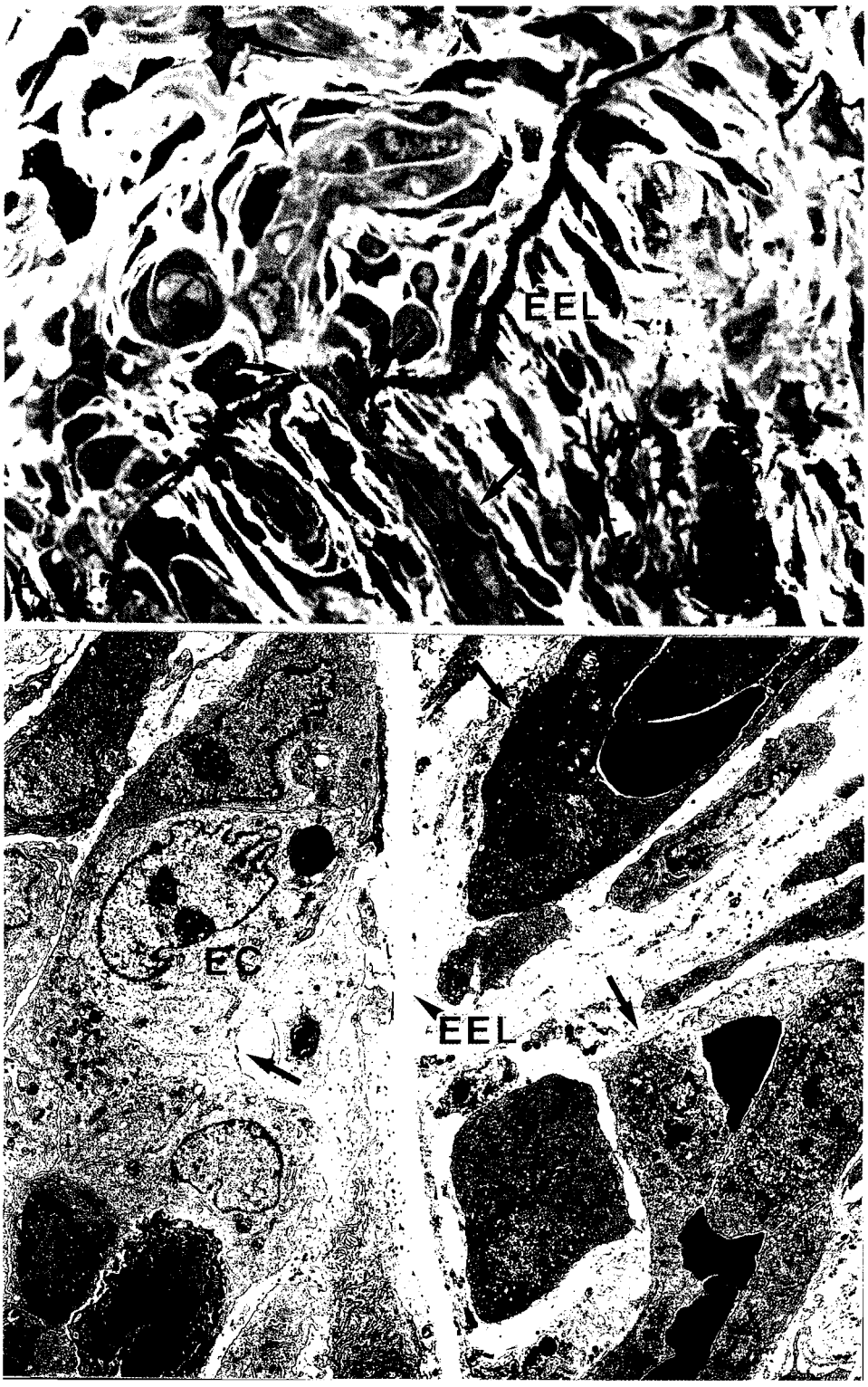

Fig. 3. Media layer and neoadventitia of femoral arterial walls 6 days after femoral artery occlusion and adventitial removal. Vascular buds (arrows) are observed in the neoadventitia and tunica media. **A.** A capillary penetrating the external elastic lamina is shown in a semithin section. Toluidine blue. x 750. **B.** Neocapillaries at both sides of the external elastic lamina are identified in an ultrathin section. EEL: External elastic lamina; EC: Endothelial cells. Uranyl acetate and lead citrate. x 7,200

(Martin et al., 1991; O'Brien et al., 1994), and the development of neovascular collateral vessels in tissues at risk of irreversible ischemic injury (D'Amore and Thompson, 1987). This hypothesis is supported by several studies which demonstrate that hypoxia controls the expression of angiogenic molecules (Knighton et al., 1983; Kourembanas et al., 1990, 1991), including those released by macrophages (Knighton et al., 1983; Koch et

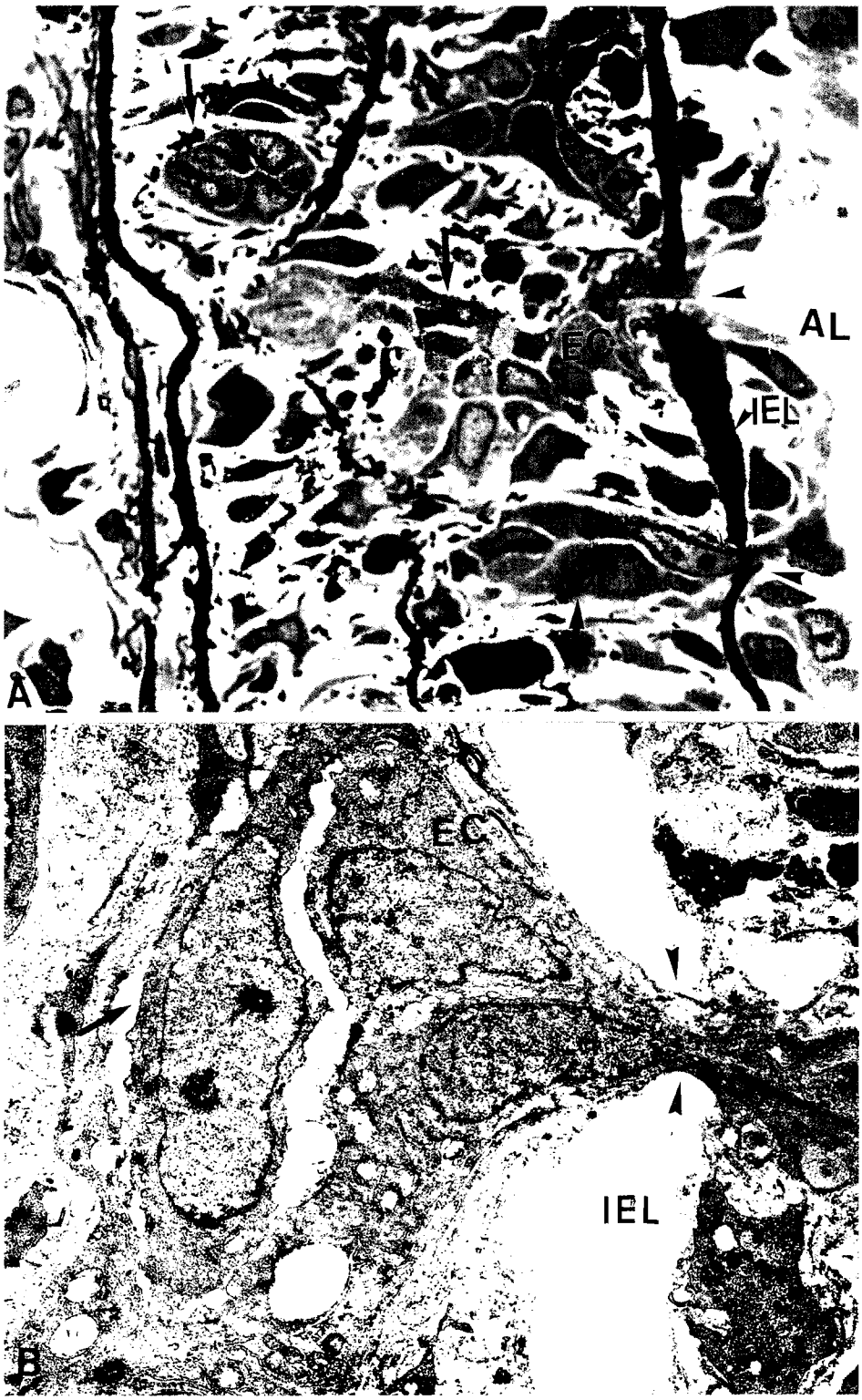

Fig. 4. Semithin and ultrathin sections of femoral arteries 6 days after their occlusion and adventitial removal. Ingrowing capillary buds (arrows) in the tunica media are observed. Some ingrowing capillary endothelial cells (EC) penetrating the Internal Elastic Lamina (IEL) through narrow fenestrations (arrowheads), are shown in continuity with the EC of the arterial neointima. AL: arterial Lumen. A, Toluidine blue, x 750. B, Uranyl acetate and lead citrate, x 7,200

al., 1992).

The main objective of the current work was to investigate the behaviour of the rat femoral vein when it was adjacent to an occluded segment of the femoral artery, in particular to assess the vein participation in neovascularization. The morphological and quantitative studies in test cases, when compared with sham controls, demonstrated a significant capacity of an occluded

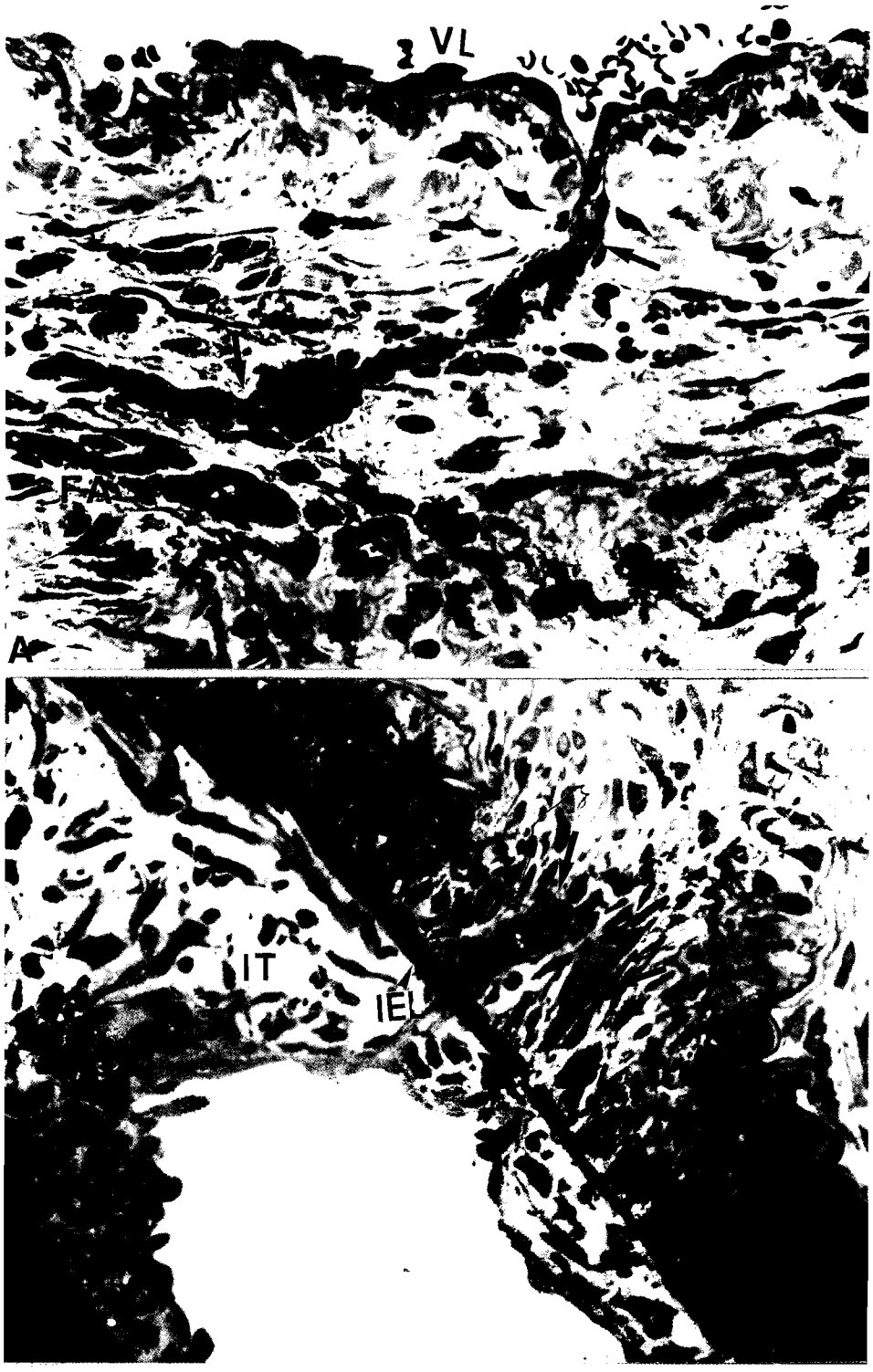

Fig. 5. Semithin sections of both the femoral vein and artery 8 days after occlusion and adventitial removal. **A.** A persistent newly formed vessel from the femoral vein (arrow) is shown extending towards the femoral artery (FA). VL: vein lumen. **B.** some persistent vascular channels (arrows), penetrating through arterial media layer zonal breakings and reaching a well developed intimal thickening, are still observed. IEL: Internal elastic lamina. Toluidine blue. A, x 320; B, x 280

segment of the rat femoral artery to induce neovascularization, not only from preformed venules and capillaries, but also from the femoral vein. Therefore, the putative angiogenic molecules released from the occluded arterial segments may reach the adjacent wall of the vein. The neovascularization from the femoral

Fig. 6. Femoral vessels after vascular perfusion with contrast material through the femoral vein at day 6 **(A, B)** and day 10 **(C, D and E)** postsurgery. Contrast material in newly formed microvessels from the femoral vein (A and B) (arrows), in the microvessels penetrating the arterial wall (C, D and E) (arrowheads) and in the arterial neolumen (AL), is observed. VL: femoral vein lumen; I: interstitium between vein and artery; ML: media layer of the femoral artery; IEL: Arterial internal elastic lamina; IT: intimal thickening of the femoral artery. H-E. A, B, x 280; C, x 2130; D, x 290; E, x 86

vein agrees with previous studies in our laboratory demonstrating that vein with smooth muscle cells in their media layer and a discontinuous internal elastic lamina, can augment the process of neovascularization from pericytic microvasculature by contributing a supplementary population of vascular sprouts (Díaz-Flores et al., 1994, 1996).

The increased neovascularization occurring around days 3 to 6, and the decrease in the following days, agree with previous studies on the chronology of angiogenesis (Burger et al., 1983). Likewise, the morphological findings observed in the vascular sproutings and in the regressing capillaries were consistent with those previously reported (Ausprunk and Folkman, 1977; Ausprunk et al., 1978; Azmi and O'Shea, 1984).

The results of the present study also reveal that the vein vascular sprouts are connected to the ingrowing capillaries in the occluded arterial wall and to the neocapillaries from the preexisting pericytic microvasculature. Indeed, the perfusion of contrast medium, either through the vein after isolating it from the circulation, or through the aorta, demonstrated the continuity of these newly-formed vessels and the establishment of a neocirculation between the preformed microvasculature, the occluded artery and the vein. The afore-mentioned may be of interest to understand how a functional circulation is established during postnatal angiogenesis, the mechanisms of which are difficult to explain (Burger et al., 1983; D'Amore and Thompson, 1987; Phillips et al., 1991; Díaz-Flores et al., 1994). This might occur in two different ways. First, by the remodeling of the newly formed capillaries and their parent vessels; and second, by anastomoses of the new capillaries with preexisting vessels of greater caliber in the venous and arterial sides of the circulation. Our observations support these two possibilities, both of which are probably associated. In other words, a similar neovascularization to that described herein, with some capillaries outgrowing from veins and ingrowing into arterial walls, along with vascular remodeling, may facilitate the establishment of a functional circulation through the neomicrovasculature networks.

The increase of the neovascularization from the vein when the adventitia was removed, with a VNCD of up to 2 times greater than when the adventitia had not been removed, suggests that the putative growth factors released in the media and in the intima of the occluded artery reach the most crucial areas of the vein wall more easily when the adventitia of the artery is removed. Indeed, it is known that the angiogenic response can be modified by the topographic relationship and the proximity of the target tissue to the source of the factors that control angiogenesis (Edelman et al., 1990; Folkman and Shing, 1992). This could also be argued to explain the dramatic intensification of the number of ingrowing capillaries in the occluded femoral artery when the adventitia was removed, with an ANCD of up to 6 times greater than when it was not. However, we should also consider the possibility that the absence of the fibrous layers of the adventitia facilitates the capillary ingrowth into the arterial media.

On the other hand, our observations also suggest that, in some conditions, migration of endothelial and pericytic cells from the adventitial side can be an important route of cell entry into the arterial wall. Recently, Nicosia and Villaschi (1995), demonstrated that pericytes of microvessels formed during rat aortic angiogenesis in vitro derive from a subpopulation of intimal/subintimal SMC. These authors hypothesized that capillary and venule pericytes may be the microvascular component of a widespread system of multipotential SMC. In our observations, the periendothelial cells of the newly formed capillaries from the femoral vein appear to be modified SMC. Likewise, the pericytes of the ingrowing capillaries in the occluded artery seem to be incorporated into the arterial wall after microvascular involution. Therefore, it is intriguing to speculate that vein SMC may participate in the origin of the new pericytes during the neovascularization from the vein, while the pericytes of the capillaries penetrating the arterial wall may contribute SMC to the medial layer and intimal thickening of the artery. However, these hypotheses have not been clearly demonstrated by the technique used here and are topics worthy of future consideration.

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