Histol Histopathol (2007) 22: 1379-1386

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Cell contribution of vasa-vasorum to early arterial intimal thickening formation

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Summary. In occluded femoral artery segments, intimal thickening occurred and abundant neovascularization from the surrounding microcirculation developed. Under these conditions, the contribution of vasa-vasorum as a source of supplementary population of cells during the early intimal thickening formation was studied. Using a technique that specifically labels venules, predominantly postcapillary venules, a marker-Monastral Blue B-was used as a tracer to follow the pericyte, endothelial cell and monocyte/macrophage lineages. In the first two days of the experiment, the marker was restricted to the wall of the periarterial microcirculation, being incorporated by endothelial cells, pericytes and some monocytes/macrophages crossing the venule walls. Later, the marker continues to be observed in some of the following cells: endothelial cells and pericytes of the newly-formed vessels, fibroblast-like cells, transitional cells between pericytes and fibroblast-like cells, macrophages migrating into the interstitium, myointimal cells and neoendothelial cells of the arterial lumen. These findings provide evidence that, during arterial intimal thickening formation in occluded arterial segments, the periarterial microvascularization contributes, in addition to recruited macrophages, newly-formed endothelial cells and a supplementary population of fibroblast-like cells and myointimal cells.

Key words: Vasa-vasorum, Arterial adventitia, Arterial intimal thickening, Arterial intimal hyperplasia, Neovascularization, Stem and progenitor cells, Pericytes

Introduction

An emerging concept is that the vascular adventitia control vascular remodeling and that adventitial fibroblasts undergo the earliest and most significant increase in proliferation of all vascular cell types, including endothelial and smooth muscle cells (for review, see Stenmark et al., 2006). Indeed, the possibility that the adventitia contains progenitor cells capable of differentiating into smooth muscle cells has recently been raised (Hu et al., 2004; Torsney et al., 2005). Prior to the above, during early intimal thickening formation in occluded arterial segments, some of us demonstrated transitional forms between pericytes and myointimal cells and that DNA synthesis was first seen in the adventitia, fundamentally in the periarterial microvessel pericytes, suggesting the participation of these cells in the intimal thickening development (Díaz-Flores and Domínguez, 1985; Díaz-Flores et al., 1990). Therefore, the adventitia could contribute progenitor cells from the interstitium or from the vasa-vasorum. In this order, limitations in available methodology have precluded confirmation of the early participation of vasa-vasorum in the cell contribution to the arterial intimal thickening. The main difficulty is that cells from vasa-vasorum, either circulating or in the vessel wall, should migrate and be induced to differentiate far away from the preformed (parent) vessels.

To confirm this hypothesis, it is necessary to obtain a tracer for labelling cells in the wall or crossing the wall of the periarterial preformed vessels and for following up their lineage. In this order, venules and capillaries (predominantly postcapillary venules) of a defined tissue area can be exclusively labelled with a local injection of histamine followed by Monastral Blue B intravenously (Joris et al., 1982; Díaz-Flores et al., 1991a,b, 1992a, b). This copper phtalocyanine pigment is visible in paraffin and plastic embedded sections and has a distinctive appearance in electron micrographs. Furthermore, it is non-toxic, remains trapped by the venule basal membrane without leaking out into the interstitium and has the capacity to be incorporated by endothelial cells, pericytes and monocytes/macrophages. Likewise, we have observed that this tracer can be followed in daughter cells (Díaz-Flores et al., 1991a,b, 1992a,b).

Given these considerations, the object of this study was to assess the role of vasa-vasorum as a source of supplementary population of cells during early intimal

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thickening formation. For this, the rat femoral periarterial venules, predominantly the postcapillary venules, were selectively labelled and their cell lineage was followed during intimal thickening formation in occluded femoral artery segments.

Materials and methods

Adult Sprague-Dawley rats (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 ad libitum and maintained under pathogen-free conditions.

The rats were anesthetized with Ketamine (150 mg/Kg i.p.). An inguinal incision was made and the femoral vessels with their connective tissue were exposed. To label the cells in the wall or crossing the wall of postcapillary venules, 1 ml of histamine diphosphate (Sigma Chemical Co., St. Louis, MO; H-7375) at 1 mg/ml in saline was administered on the connective tissue surrounding the femoral vessels. Following, Monastral Blue B (3% suspension) (Sigma Chemical Co., M-3764) was administered intravenously (contralateral saphenous vein) at 0.1 ml/100 g of body weight. Immediately after MB labelling was identified within the periarterial microcirculation, ligatures were applied to the proximal and distal parts of a femoral artery segment 1.5 cm long. In the arterial segment between ligatures, the surrounding microcirculation was not damaged.

To follow the cell lineage, histamine-induced MB labelling was undertaken in 32 rats. The animals were sacrificed under general anesthesia at days 1, 2, 4, 6, 8, 10 and 12, 4 rats per time point. To study the number of MB-labeled cells semiquantitative studies were carried out.

Two specimens of each time point were processed by means of standard protocol and 5 μm thick sections stained with hematoxylin and eosin were obtained from paraffin-embedded tissue. The other two specimens of each time point were fixed in a glutaraldehyde solution, diluted to 2% with sodium cacodylate buffer, pH 7.4, for 6 hours at 4ºC, 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 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 an electron microscope.

Results

At day 1, Monastral Blue B, trapped by microvessel basal membrane, was detected in the space between pericytes and endothelium, and in the cytoplasm of both endothelial cells and pericytes of the periarterial venules (predominantly in postcapillary venules) (Fig. 1, Table 1). Edema and infiltration of polymorphonuclear leukocytes were observed in the interstitium but no tracer was present in this area. The endothelium of the arterial occluded segment disappeared or was necrotic and some of the medial SMC revealed degenerative changes. Few polymorphonuclear leukocytes were present both in the arterial lumen and the arterial wall.

Between days 2 to 6, vascular buds, arising from small venules and capillaries of the surrounding arterial microcirculation were also observed. The vascular sprouts extended from the preformed postcapillary venules and capillaries into the adventitia toward the media layer of the artery. Monocyte/macrophages were also observed crossing the activated vessel walls. Bulging from small venules, the pericytes had shortened their processes and increased their somatic volume. Their nuclei contained prominent nucleoli and, in the extensive cytoplasm, a large number of ribosomes were present. Many of the endothelial cells and pericytes were in mitosis. Fibroblast-like cells, some of them transitional with pericytes, were seen in interstitial areas next to small venules. Some of these cells (endothelial cells of the vascular buds, pericytes, fibroblast-like and transitional cell forms with pericytes) showing MB in their cytoplasms (Table 1) were seen in interstitial areas of the adventitia and crossing the external elastic lamina

Table 1. Semiquantitative study of MB-labeled cells.

CELL TYPE	DAYS						
	1	2	4	6	8	10	12
Adventitial microcirculation							
Endothelial cells	++/-	+++	++/-	+	+/-	+/-	+/-
Pericytes	++/-	+++	++/-	+	+/-	+/-	+/-
Monocyte/macrophages crossing microvascular wall	-	+/-	+/-	+/-	-	-	-
Adventitia interstitium							
Fibroblast-like and transitional cells	-	+/-	+	+	+	+/-	+/-
Macrophages	-	++/-	++/-	+	+	+/-	+/-
Endothelial cells and pericytes of vascular buds	-	+/-	+	+	+	+/-	+/-
Arterial media layer							
Endothelial cells of vascular buds	-	-	+/-	+	+	+/-	+/-
Fibroblst-like/SMC-like cells	-	-	+/-	+	+	+/-	+/-
Macrophages	-	-	+/-	+/-	+/-	+/-	+/-
Intimal thickening							
Myointimal cells	-	-	-	+/-	+	+	+
Endothelial cells	-	-	-	+/-	+	+	+
Macrophages	-	-	-	++/-	+	+/-	+/-

SemiQuantitative study of MB-labeled cells obtained from 4 cases for each time point and from 5 sections in each case. (+++: Marker in most of the cells; +: marker in moderate number of cells; +/-: Marker in scarce cells).

of the artery (Fig. 2). Monocytes/macrophages present in the venule walls and in connective tissue outside the vessels also showed the tracer (Table 1).

By day 6 onwards, the following findings occurred: vascular buds, fibroblast-like cells and macrophages penetrate and spirally dissect the different layers of the arterial wall, reaching the lumen. The endothelial cells migrate in arterial lumen and the points of vascular growth in the different areas of penetration coalesce with each other originating a luminal neoendothelium. Macrophages and myointimal cells, with some characteristics of pericytes, myofibroblasts or smooth muscle cells, appear between the neoendothelium of the arterial lumen and the internal elastic lamina. Interstitial

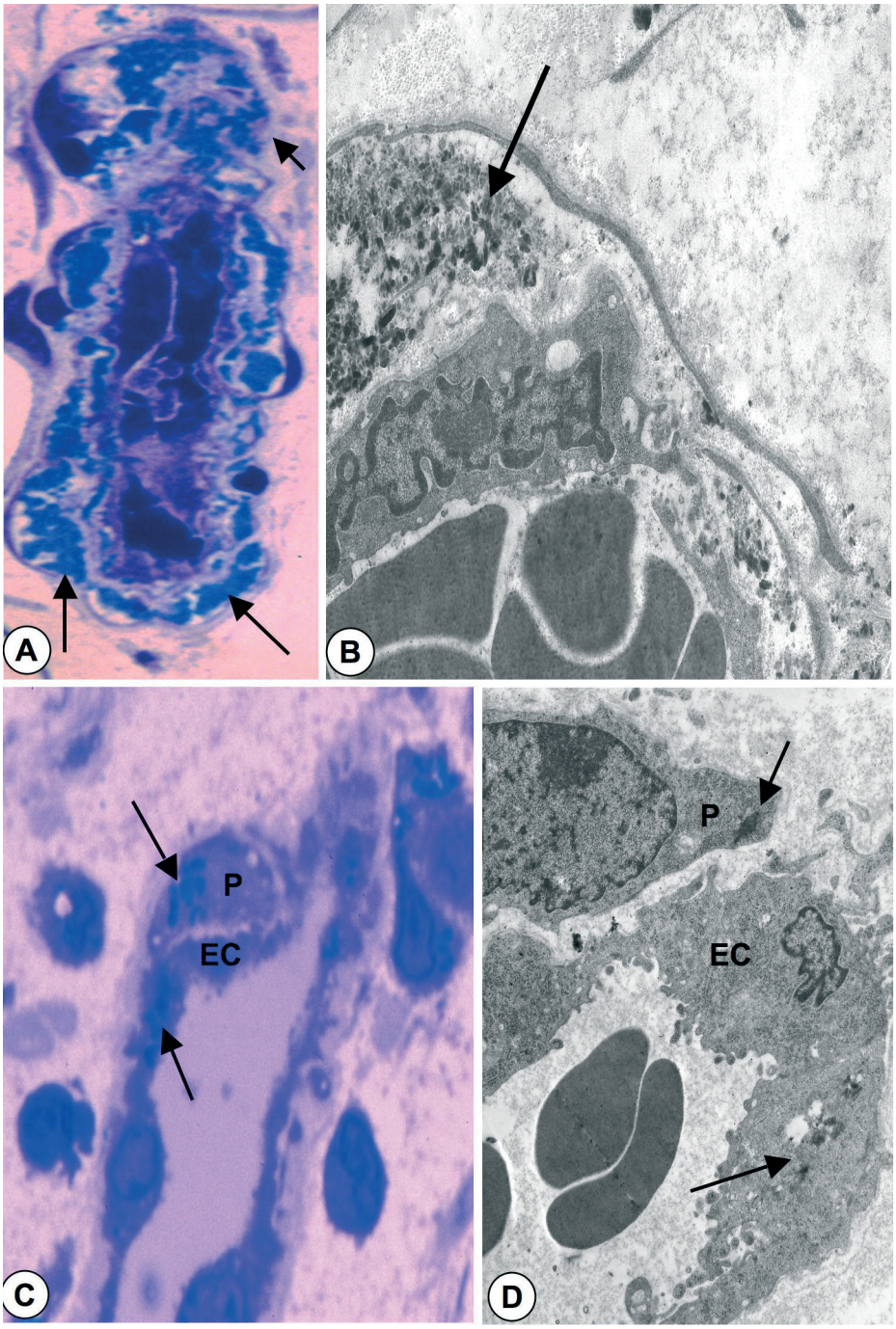

Fig. 1. Postcapillary venules labelled with Monastral Blue B (MB), in occluded arterial segments. Day 1 (**A and B**) and 2 (**C and D**) after ligature. In Fig. **1A** and **1B**, the marker (arrows) is trapped by the basal membrane. In Fig. **1C** and **1D**, MB (arrows) is observed in the cytoplasm of both endothelial cells (EC) and pericytes (P). Fig. 1A, C: Semithin sections. Toluidine Blue, x 600 and x 1,100, respectively. Fig. 1B, D: Uranyl acetate and lead citrate, x 12,500

material similar to the basal membrane, collagen and young elastic fibers are produced concurrently. Some of the aforementioned cells (endothelial cells, macrophages and myointimal cells) were labelled with Monastral Blue B (Fig. 3, Table 1).

Discussion

More than a century ago, late neointimal vascularization was associated with progressive atherosclerotic disease and the development of lesion intensity (for review, see Doyle and Caplice, 2007). In previous studies, increased neovascularization from the vasa-vasorum during early stages of intimal thickening formation and the possible participation of cells from the vasa-vasorum in the origin of myointimal cells was pointed out (Díaz-Flores and Domínguez, 1985; Díaz-Flores et al., 1990). Subsequently, numerous authors have described early increased neovascularization in the vessel wall at sites of intimal hyperplasia originated in

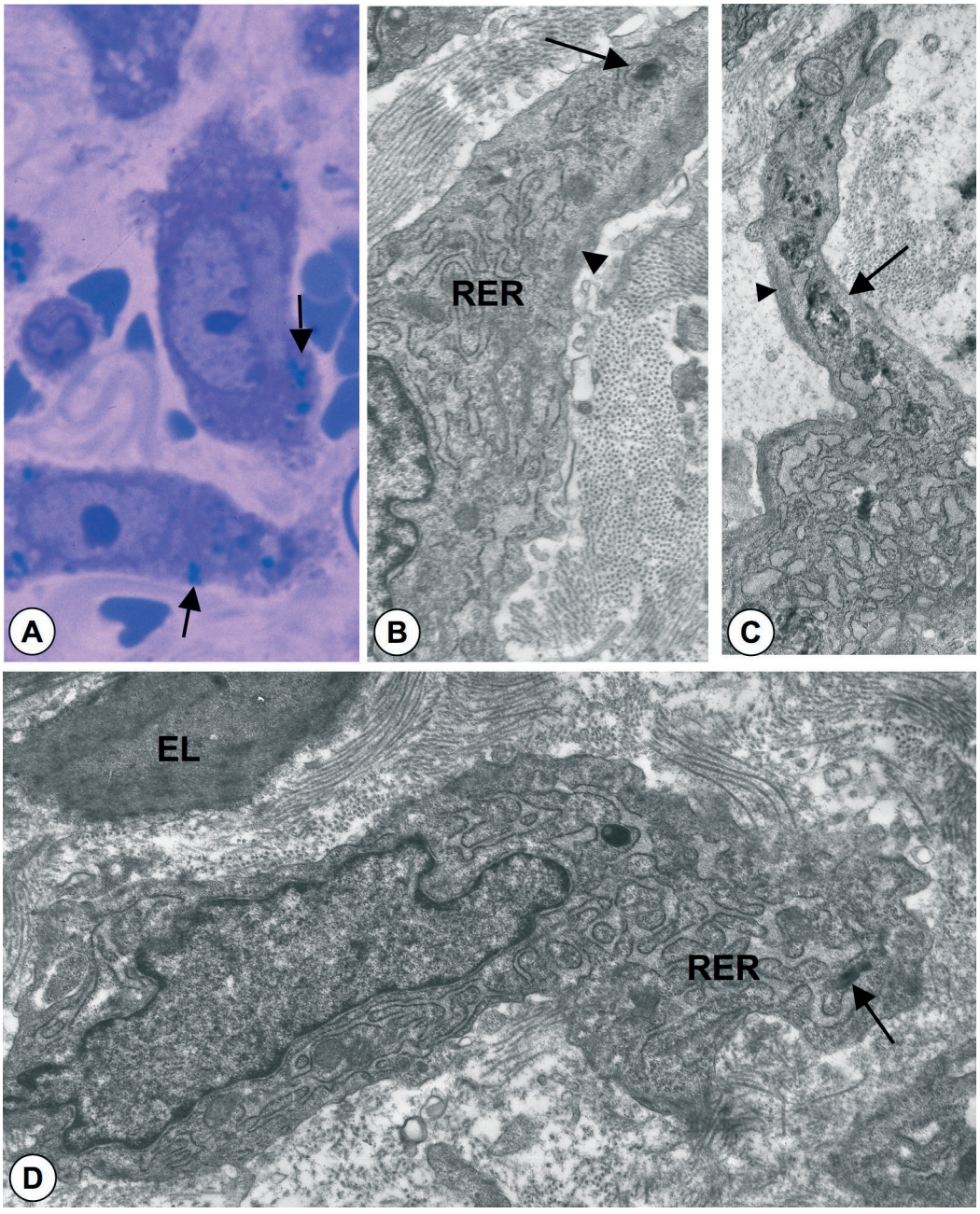

Fig. 2. Fibroblastic-like-myofibroblast cells presents in the adventitia of occluded arterial segments, 4 days after ligature. Monastral Blue B (arrows) is observed in the cytoplasm of these cells. RER: rough endoplasmic reticulum. Arrowheads: intracytoplasmic filaments. In Fig. **2D** a cell is crossing the external elastic lamina (EL). Fig. 2A: Semithin section. Toluidine Blue. x 1,100. Fig. 2B, C and D: Uranyl acetate and lead citrate, x 15,000

different conditions (Edelman et al., 1992; Kwon et al., 1998; Westerband et al., 2000; Shibata et al., 2001; Shigematsu et al., 2001; Khurana et al., 2004). In the present work, with the objective to study the role of the arterial vascularization as a source of cells during intimal thickening formation, in occluded femoral artery segments, using a technique that specifically labels venules (Joris et al., 1982; Díaz-Flores et al., 1991a, 1992a,b), Monastral Blue B was used as a tracer for labelling cells in their walls (pericytes and endothelial

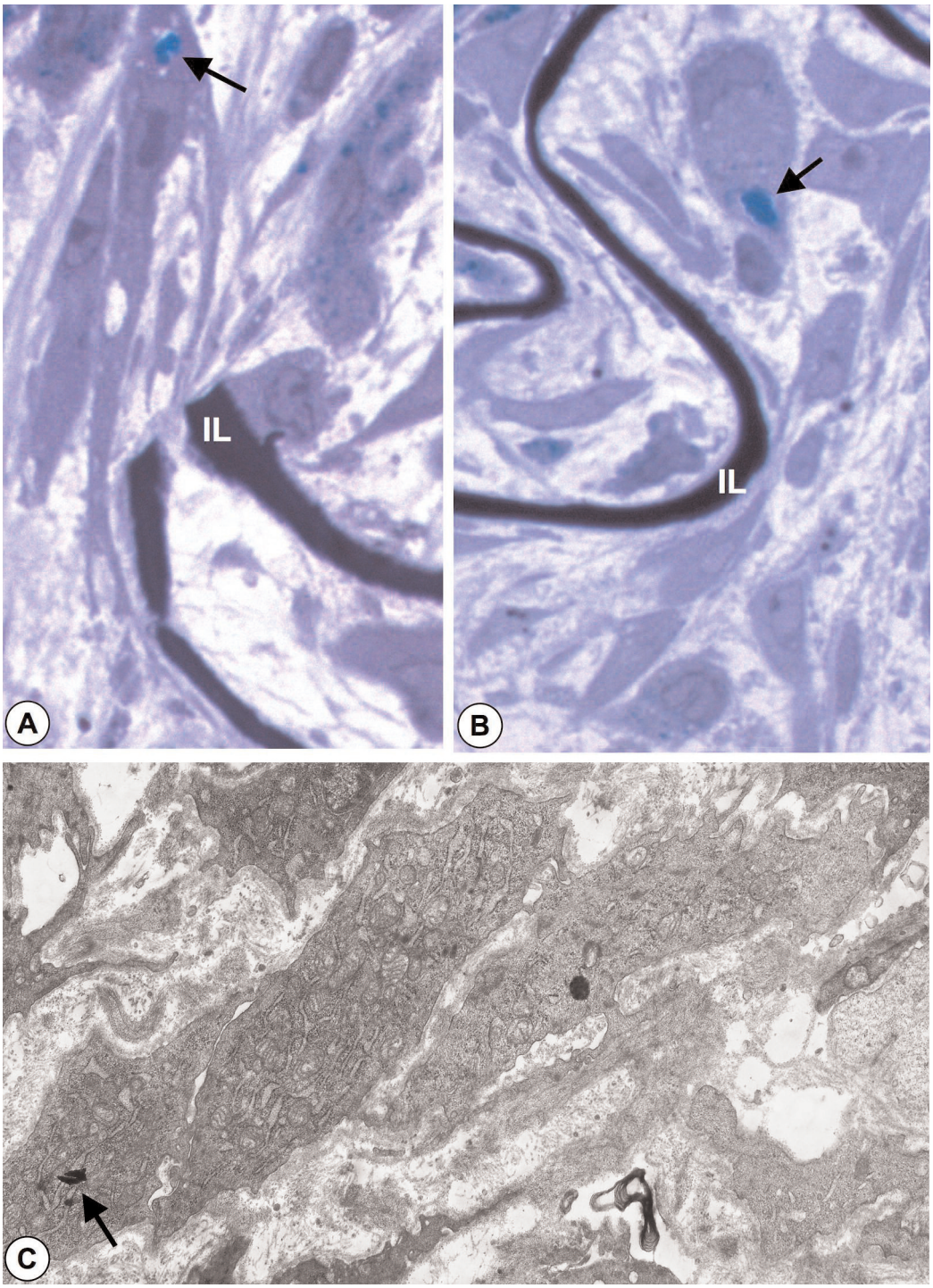

Fig. 3. Myointimal cells in the intimal thickening, 8 days after ligature. MB is present in their cytoplasms (arrows). Interstitial material similar to basal membrane is observed. Internal elastic lamina: IL. Fig. 3A and 3B: Semithin sections. Toluidine Blue. x 1,100. Figs 3C: Uranyl acetate and lead citrate, x 15,000

cells) or crossing their walls (monocyte/macrophages). Indeed, the technique is based on the principle of vascular labelling, whereby an insoluble tracer can be deposited in the wall of those vessels of the microcirculation that have become abnormally permeable. For this labelling method, vascular leakage was induced locally by histamine, which has a high degree of specificity for the postcapillary venules where histamine receptors are largely located. In these conditions, the basal lamina remains intact and the labelling is restricted to the walls of the graft bed venules without leaking into the interstitial space, as it was trapped against the vascular basal membrane. For the objective of this study, it was important that MB be incorporated by ECs, pericytes and crossing monocytes/macrophages in the postcapillary venules but that it did not compromise cell viability (Díaz-Flores et al., 1991a, 1992a,b). Although the quantity of Monastral Blue B diminishes in each cellular division, this tracer persists in some of the daughter cells and, as a foreign element, has the advantage of not being present in the unexposed cells. Furthermore, this tracer is visible in paraffin and plastic-embedded sections and has a distinctive appearance in electron micrographs.

The results reported have clearly shown that, in the first days of the experiment, the Monastral Blue B was restricted to the wall of the periarterial microcirculation. Subsequently, the marker appeared in their endothelial cells, pericytes and in some monocytes/macrophages crossing the wall of venules and capillaries. Later, some endothelial cells and pericytes of newly formed vessels, fibroblast-like cells and transitional cell forms with pericytes showed Monastral Blue B in their cytoplasms. Finally, the marker was observed in some of the myointimal cells, macrophages and neoendothelial cells of arterial lumen. Since the preformed capillaries were scarcely labelled and the quantity of Monastral Blue B disminished with each cellular division, it is probable that the cell contribution of vasa-vasorum is greater than that reflected by this study. The above provides evidence that, during intimal thickening formation, the periarterial microvascularization contributes some neoformed endothelial cells, fibroblast-like cells and myointimal cells, as well as recruited macrophages. Therefore, the findings support the hypothesis that vasa-vasorum preexisting endothelial cells originate vascular buds, which penetrate the arterial wall and form a neoendothelium in the lumen of the occluded arterial segments (Díaz-Flores and Domínguez, 1985). On the other hand, some of the myointimal cells originate from preformed postcapillary venules, that is, from pericytes and/or circulating progenitor cells. Indeed, during the earlier stages, excluding the endothelial cells, Monastral Blue labelling was restricted to the pericytes and blood monocytes, which may include macrophages precursors and circulating progenitor cells, such as the denominated fibrocytes (Abe et al., 2001; Kuwana et al., 2003; Varcoe et al., 2006).

Resident progenitor cells in the artery wall have not been conclusively identified (Liu et al., 2007). Although the traditional view is that the myointimal cells are derived from medial smooth muscle cells through the¡r luminal migration and proliferation (Ross et al., 1977), our group hypothesized the possibility that myointimal cells originate from progenitor cells from periarterial vascularization, in particular from vasa-vasorum pericytes (Díaz-Flores and Domínguez, 1985; Madrid et al., 1998). This agrees with the results given here. In this order, pericytes retaining considerable mesenchymal potentiality (Díaz-Flores et al., 1991b) may have the capacity to differentiate into other cell types, such as fibroblasts (Ross et al., 1970; Farrington-Rock et al., 2004), chondroblasts (Díaz-Flores et al., 1988), osteoblasts (Takahashi and Urist, 1986; Schor et al., 1990; Díaz-Flores et al 1992b; Collet and Canfield, 2005), skeletal muscle cells (Dellavalle et al., 2007), preadipocytes (Richardson et al., 1982; Farrington-Rock et al., 2004), vascular smooth muscle cells and myointimal cells (Movat and Fernando, 1964; Díaz-Flores and Domínguez, 1985; Madrid et al., 1998). Furthermore, cells with pericyte characteristics have been localized within the artery wall (Andreeva et al., 1998; Böstrom et al., 1993; Iwanov et al., 2001; Lamagna and Bergers, 2006) and the pericytes may form part of the stem or progenitor cells in the adventitia, which is currently considered as an important source of stem or progenitor cells (Siow et al., 2003; Hu et al., 2004). On the other hand, circulating progenitor cells could also contribute to pericyte, fibroblast-like, smooth muscle and myointimal cells (Campbell et al., 2001; Hu et al., 2002; Sata et al., 2002; Simper et al., 2002). In our experiments, the circulating progenitor cells only could reach the arterial wall and the intimal thickening through the periarterial vessels, since the arterial lumen was occluded between ligatures. Bearing in mind the above, the periarterial microcirculation, predominantly the postcapillary venules, may form part of a general inflammatory-reparative system in which these vessels seem not only to be a source of inflammatory cells and endothelial cells, but also of contractile and matrix-forming cells in different tissues.

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Accepted July 19, 2007