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Peg-and-socket junctions between smooth muscle cells and endothelial cells in femoral veins are stimulated to angiogenesis by prostaglandin E_2 and glycerols

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Summary. The administration of prostaglandin (PG) E_2, triacetylglycerol and glycerol induce the formation of numerous vascular buds arising from the femoral vein, as previously demonstrated by our group. In the present study, a great number of peg-and-socket junctions (PSJs) between smooth muscle cells (SMCs) (providing the pegs) and ECs (forming the sockets) were demonstrated. At the first stage, days 1 to 3, PSJs connect subendothelial penetrating processes from activated SMCs with activated ECs of the intima. Subsequently, during angiogenesis (days 4 to 6), SMCs, showing transitional aspects with pericytes, also form PSJs with intimal ECs, but also new PSJs between SMCs and sprouting ECs in the media layer were now observed. Immunohistochemically, α-smooth muscle actin (α-SMA) and H-caldesmon are positive in the cytoplasm of the SMCs, showing a higher expression in pegs. Desmin, however, although it is also positive in the cytoplasm of the SMCs, is negative in the pegs. The expression of CD34 in ECs reveals abundant positive folding that appears to correspond to the sockets. The peculiar expression of caldesmon, whose isoforms may contribute to the regulation of cell motility, and to vasculogenesis and angiogenesis, may have a role in the different mechanisms by which PSJs act in the vein wall.

Key words: Immunohistochemistry, Electron microscopy

Introduction

The peg-and-socket junction (PSJ) is a homocellular or heterocellular contact, which involves a bulb- or finger-shaped (peg) protrusion from a cell that penetrates an invagination (socket) in another cell (Díaz-Flores et al., 1991, 2009a; Huizinga et al., 2008; Aranishi et al., 2009; Rumessen et al., 2009). The pericytic microvasculature shows heterocellular PSJs between pericytes and endothelial cells (ECs) (Wakui et al., 1989; Díaz-Flores et al., 1991). Their number increase in maturing angiogenic vessels (Díaz-Flores et al., 1991; Wakui, 1992, Wakui et al., 2006; Caruso et al., 2001).

Capillary-like structures arising from the intimal ECs which break through the vein media layer have been described (Díaz-Flores et al., 1994, 1996). A single application of prostaglandin (PGE_2) diluted in triacetylglycerol or glycerol solution into the rat femoral vein surrounding soft tissue induces vascular sprouts from the vein ECs, and a sudden, brief and intense neovascularization in their media layer. The immature, newly formed capillaries in the media layer showed a number of PSJs between ECs and neighbouring periendothelial cells (Díaz-Flores et al., 1994, 1996). Our aim is to determine the distribution and the ultrastructural and histochemical characteristics of the PSJs between vein media layer smooth muscle cells (SMCs) and vein intimal ECs, as well as between medial SMCs and ECs of the newly formed capillaries at the media layer.

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. For surgical procedures and tissue removal the rats were anesthetized with ketamine (150

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mg/b.w. Kg i.p.).

Procedures

0.1 mg of PGE_2 (Sigma Chemical Co., P-5640) was diluted in 1 ml of triacetylglycerol (Sigma Chemical Co., T-5376) and 1 ml of glycerol (Sigma Chemical Co., G-9012) and injected in the connective tissue surrounding the femoral vein with the help of a surgical microscope. Both the femoral vein and surrounding connective tissue were removed at days 1 to 9, 3 rats every day. As a control a saline solution was administered in a similar way (n=9).

For light microscopy the femoral veins were orientated to get longitudinal sections, fixed in a buffered neutral 4% formaldehyde solution and embedded in paraffin. 4 μm-thick sections were stained with Haematoxylin and Eosin (H&E). For immunohistochemistry, 3 μm-thick sections were attached to silanized slides. Sections were pre-treated for labelling enhancement ("antigen retrieval" process), and with 3% hydrogen peroxide for endogenous peroxidase blocking. Then, they were incubated with the following antibodies (10-40 mins); alpha smooth muscle actin (Dako, Glostrup, Denmark; dilution 1:50), desmin (Dako, Glostrup, Denmark; dilution 1:50), H-caldesmon (AbCam, Cambridge, UK; dilution 1:250) and CD-34 (Abbiotec, San Diego, USA; dilution 1:500). The immunoreaction was developed in a solution of diaminobenzidine and the sections cleared in xylene, and mounted in Eukitt®.

For electron microscopy, small pieces were fixed in 2% glutaraldehyde diluted in sodium cacodylate buffer, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in a graded ethanol series and embedded in epoxy resin. Semithin sections (1.5 μm thick) were mounted on acid-cleaned slides, and stained with 1% Toluidine blue. Ultrathin sections (60-90 nm thick) were counterstained with Uranyl acetate and lead citrate.

Results

Control group

The femoral vein showed the characteristic structural organization of the collecting veins. The media layer,

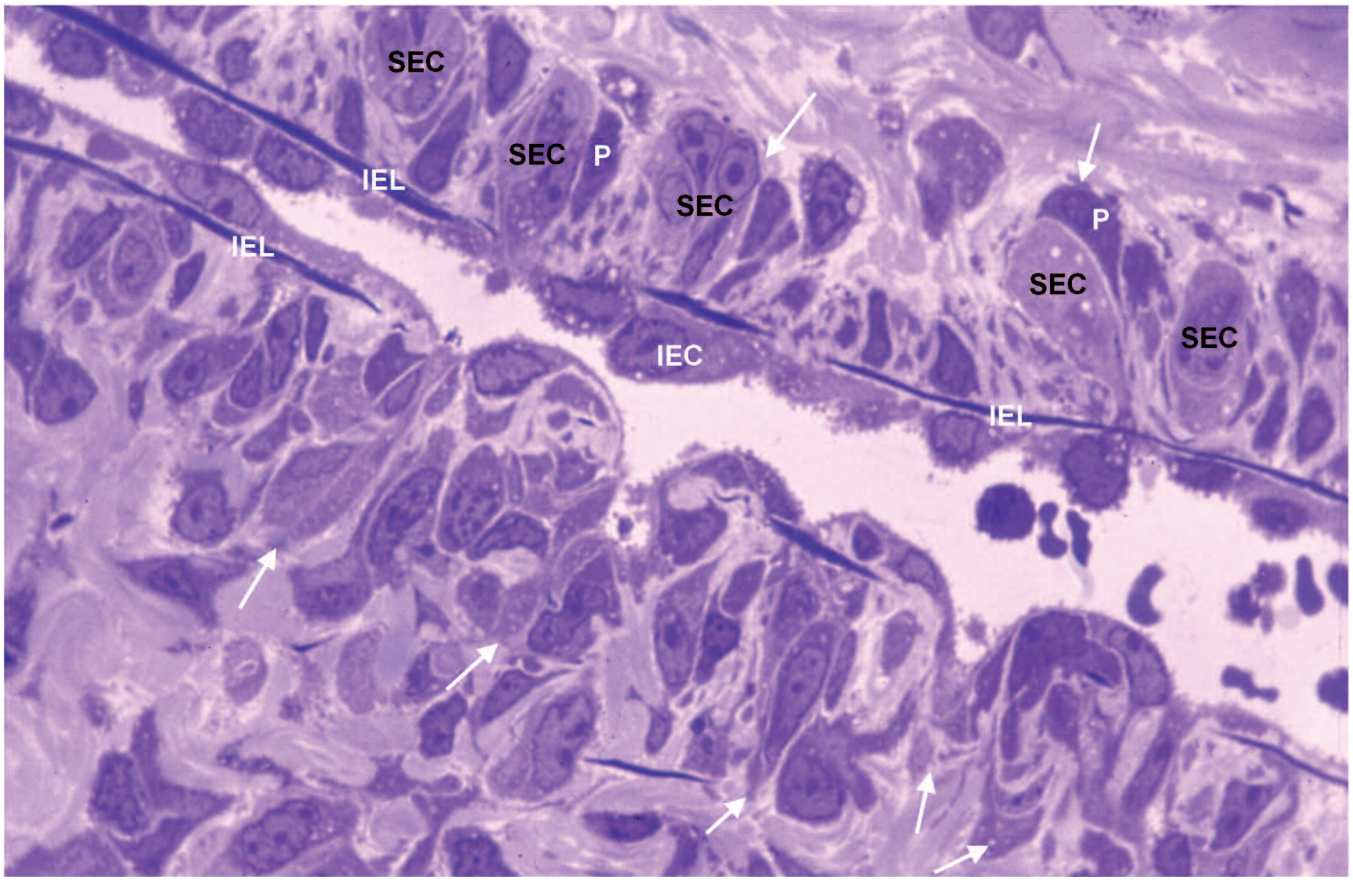

Fig. 1. Femoral vein after stimulation. A great number of vascular buds (arrows) are observed in the media layer of the vein by day 5. Periendothelial cells with pericytic (P) appearance are observed around the sprouting endothelial cells (SEC). IEL: Internal elastic lamina. LEC: Luminal endothelial cells. Semithin section, Toluidine blue, x 1,100

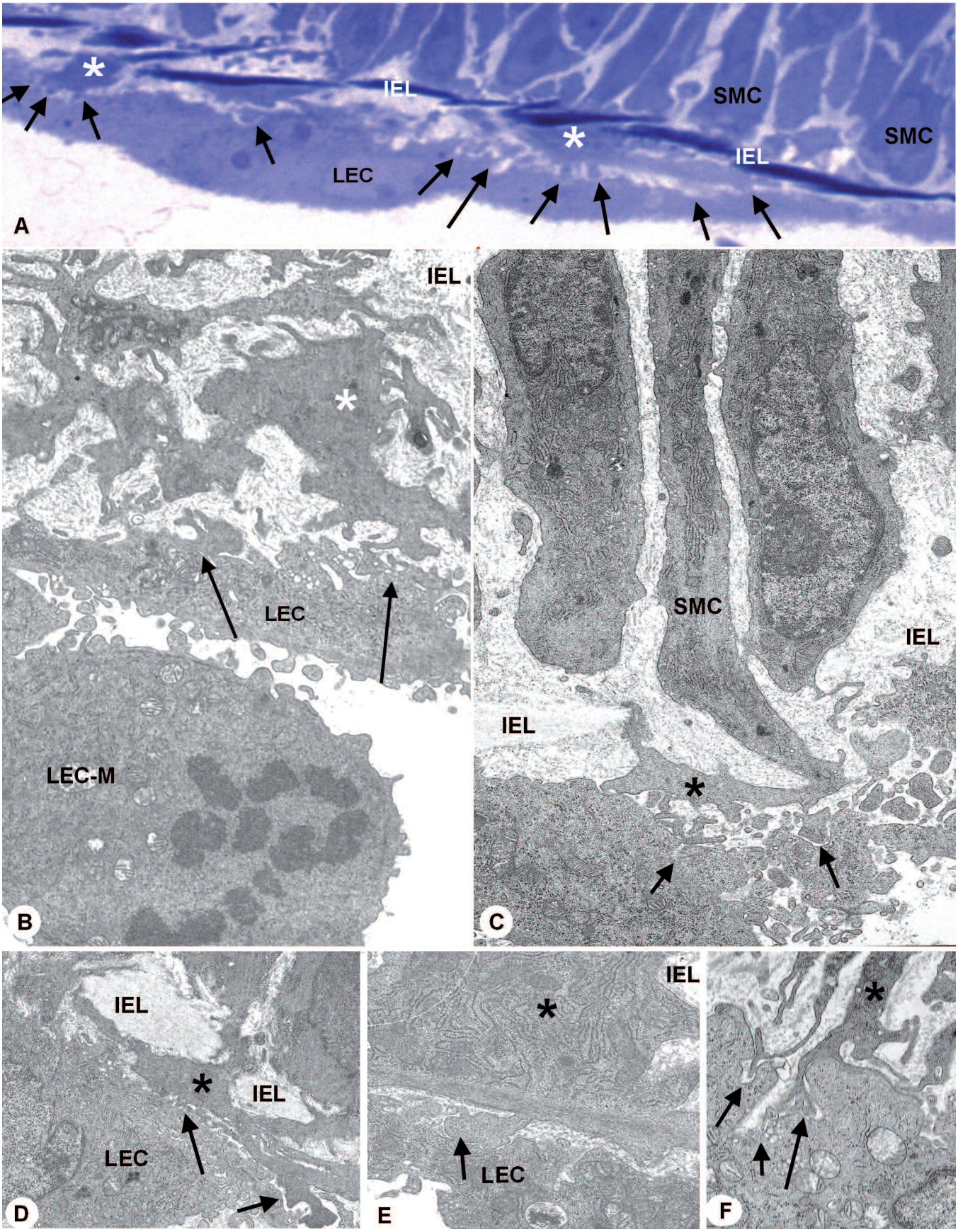

Fig. 2. Femoral vein at 3 days after stimulation. The luminal endothelial cells (LEC) are hypertrophied; one of them protrudes into the lumen of the vein showing mitosis (**B**, LEC-M). The subendothelial space is enlarged. Some SMCs in the media layer show a different aspect with an increased number of organelles involved in protein synthesis and a lower expression of contractile proteins (**C**). These cells form cytoplasmic processes (asterisk) that penetrate the discontinuous internal elastic lamina (IEL), run across the subendothelial space and originate numerous pegs (arrows), while the ECs form the socket. The pegs appear as bulb- or finger-shaped protrusions. A, Semithin section, Toluidine blue; B-F, Ultrathin sections, Uranyl acetate and Lead citrate. EC: endothelial cell; SMC: smooth muscle cell. A, x 1,100; B, C, x 15,000; D, F, x 18,000; E, x 20,000.

composed by a continuous layer of SMCs, was separated from the intima layer by an usual discontinuous inner elastic lamina.

Test Group

General characteristics of the femoral vein

A considerable number of vascular buds arising from the ECs of the intima layer penetrating the media layer were observed (Fig. 1). The capillary sprouts were more evident at days 4 to 6. They consisted of parallel ECs surrounded by a variable number of periendothelial cells. Occasionally, two or more neighbouring neo vessels appeared interconnected in the most external region of the media layer, generating a microvascular network. At days 7 to 9 most of the newly formed microvasculature regressed.

SMC/EC modification and heterocellular contacts: PSJs

Early stage (1-3 days). In the early stages (days 1-3) (Fig. 2), the femoral vein ECs appeared hypertrophied, with an increased cell volume. Their slightly indented nuclei showed prominent nucleoli. Some mitosis was observed. In the large cytoplasm, the cell organelles were preserved and there were numerous ribosomes, either isolated or aggregated. The EC surface presented abundant folds and the subendothelial space was often enlarged. Some medial SMCs showed a modified phenotype with a high number of intracytoplasmic organelles involved in protein synthesis, and with a lower expression of SMC contractile proteins. These SMCs showed cytoplasmic processes closely associated to the internal elastic lamina (IEL). The cytoplasmic processes penetrated into the intima layer of the vein, through the gaps of the discontinuous IEL, and formed several PSJs with ECs. PSJs appeared as bulb- or finger-shaped protrusions, which established a close association between SMCs and ECs, originating a disruption in the basement membrane of both cells. The basement membrane was absent in extensive zones. Because the pegs are formed by cytoplasmic processes, it was difficult to know which cells originated them. However, by means of serial sections, it was determined that the SMCs originated the pegs in most of the cases, while the ECs formed the sockets. The SMC pegs protrude from the intimal surface of the SMC processes to the abluminal surface of the ECs. The SMC pegs showed scarce or no organelles, and an abundant granular/filamentous material. Although usually there was a close relation between SMCs and ECs in the PSJs, only occasional specialized junctions were found. Additional rudimentary cytoplasmic processes without peg and socket junctions were observed protruding from the other surfaces of the cells.

Immunohistochemically, the contractile proteins were differentially expressed in the cell cytoplasm of SMCs and in the pegs. The cell cytoplasm expressed desmin, α-SMA and H-caldesmon (Fig. 3). However, in the pegs, desmin was negative, while α-SMA and H-caldesmon showed a higher staining intensity (Fig. 3). CD34 immunostaining showed abundant abluminal folding in the ECs, which corresponds to the electron microscopic images of the socket. Indeed, when the expression of contractile proteins on pegs and of CD34 in ECs are compared, CD34-positve EC abluminal folding appeared to correspond to the socket, which houses the α-SMA- and H-caldesmon-positive (Fig. 3).

Later stage (4-6 days). At days 4 to 6, a great number of PSJs are observed between activated SMCs and ECs of the intima, and between the SMCs and the ECs of the numerous capillaries emerging from them toward the media layer of the vein (Fig. 4). The modified SMCs, with increased organelles for cell synthesis, frequently showed transitional characteristics between activated SMCs and activated pericytes, mainly around the neocapillaries in the media layer (Fig. 4). Likewise, the same SMCs form PSJs with the intimal ECs, as well as with those in the media layer (Fig. 4). The immunohistochemical expression of desmin, α-SMA, H-caldesmon and CD34 was similar to that described in the previous stage.

Discussion

The general characteristics of the test group agree with the previous studies on the vascular sprouting from rat femoral vein induced by PGE_2 (Díaz-Flores et al., 1994). In this way, it has been demonstrated that mechanical wounding of the EC monolayer stimulates rapid cyclooxigenase-2 expression and secretion of enhanced levels of PGE_2 (Jiang et al., 2004). Cyclooxigenase-2 was expressed within neovascular structures, while the inhibition of PGE_2 production was associated with a cellular increase in apoptosis and a decrease in proliferation (Leahy et al., 2002). Furthermore, present and previous results show that glycerol, and some acylglycerols, may constitute a large family within the wide variety of non-peptidic molecules, which have a direct or indirect angiogenic activity "in vivo" (Díaz-Flores et al., 1996). Neovascularization stimuli are necessary for both the formation and the maintenance of blood vessels. Thus, the regression of most of the newly formed capillaries in the vein wall may be due to failure in the activity of the administered substances or the factors activated by them.

In this work, we have considered the heterocellular contacts between SMCs and ECs during experimental vascular sprouting in the femoral vein wall after PGE_2 and glycerol administration. Firstly, the numerous processes from the modified SMCs penetrating the IEL originate a great number of PSJs with the activated ECs of the vein intima layer. Subsequently, when ECs sprout from the intima layer toward the media layer, the activated SMCs showed a similar characteristic to

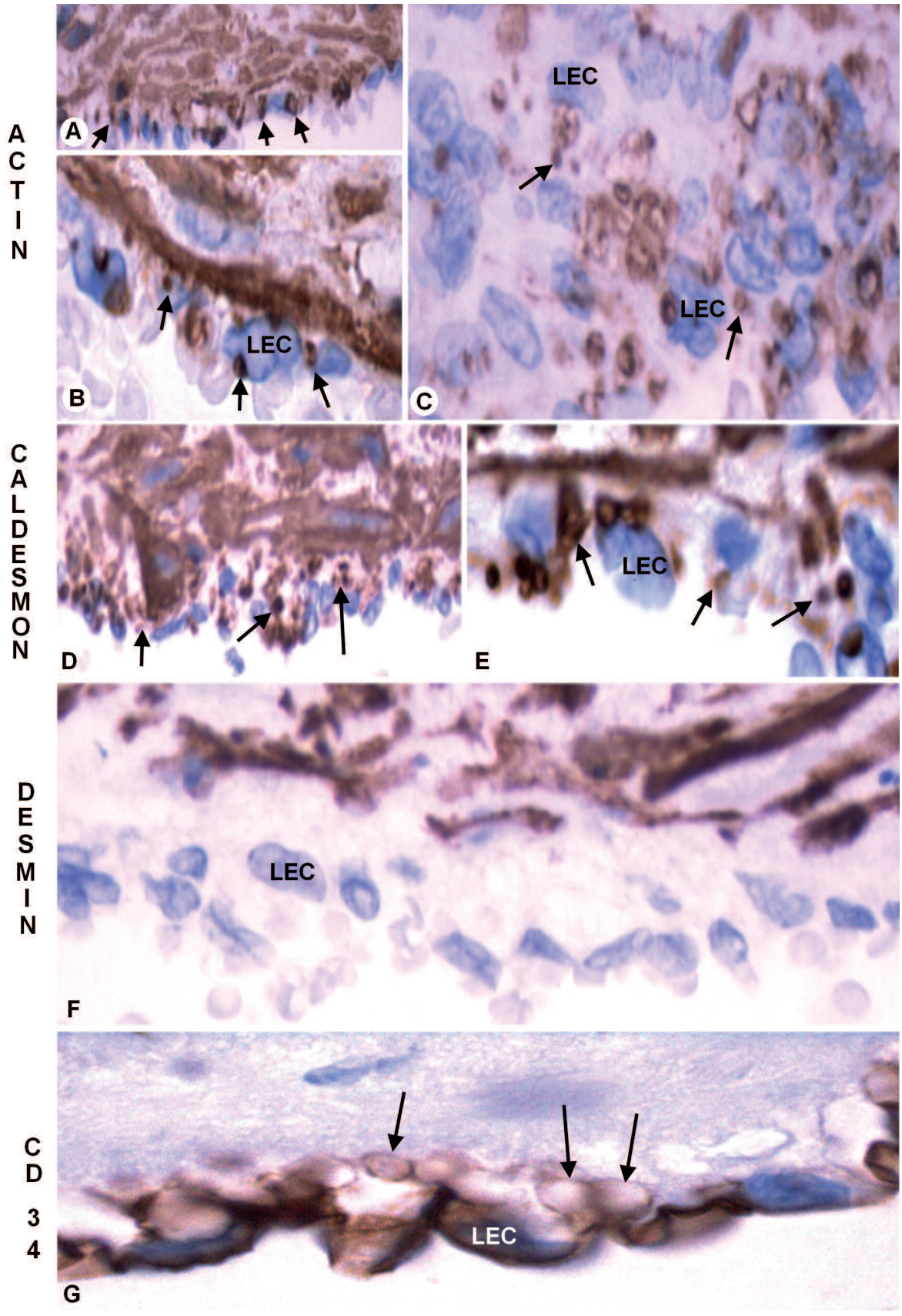

Fig. 3. Immunohistochemical demonstration of α-SMA, H-caldesmon, desmin and CD34. α-SMA **(A-C)** and H-caldesmon **(D-E)** are expressed in the cytoplasm of SMCs showing a higher intensity at their pegs (arrows). **C** is a tangential section of intimal ECs with α-SMA positive pegs. Desmin expression **(F)** is observed in the cytoplasm of SMCs but it is negative in pegs. CD34 **(G)**, reveals abundant folding (arrows) on the abluminal surface of the ECs, which appears to correspond to the socket where the peg is housed. EC: endothelial cells. α-SMA, H-caldesmon, desmin and CD34 immunohistochemistry. A, x 250; B, C: x 600; D: x 250; E-G: x 600

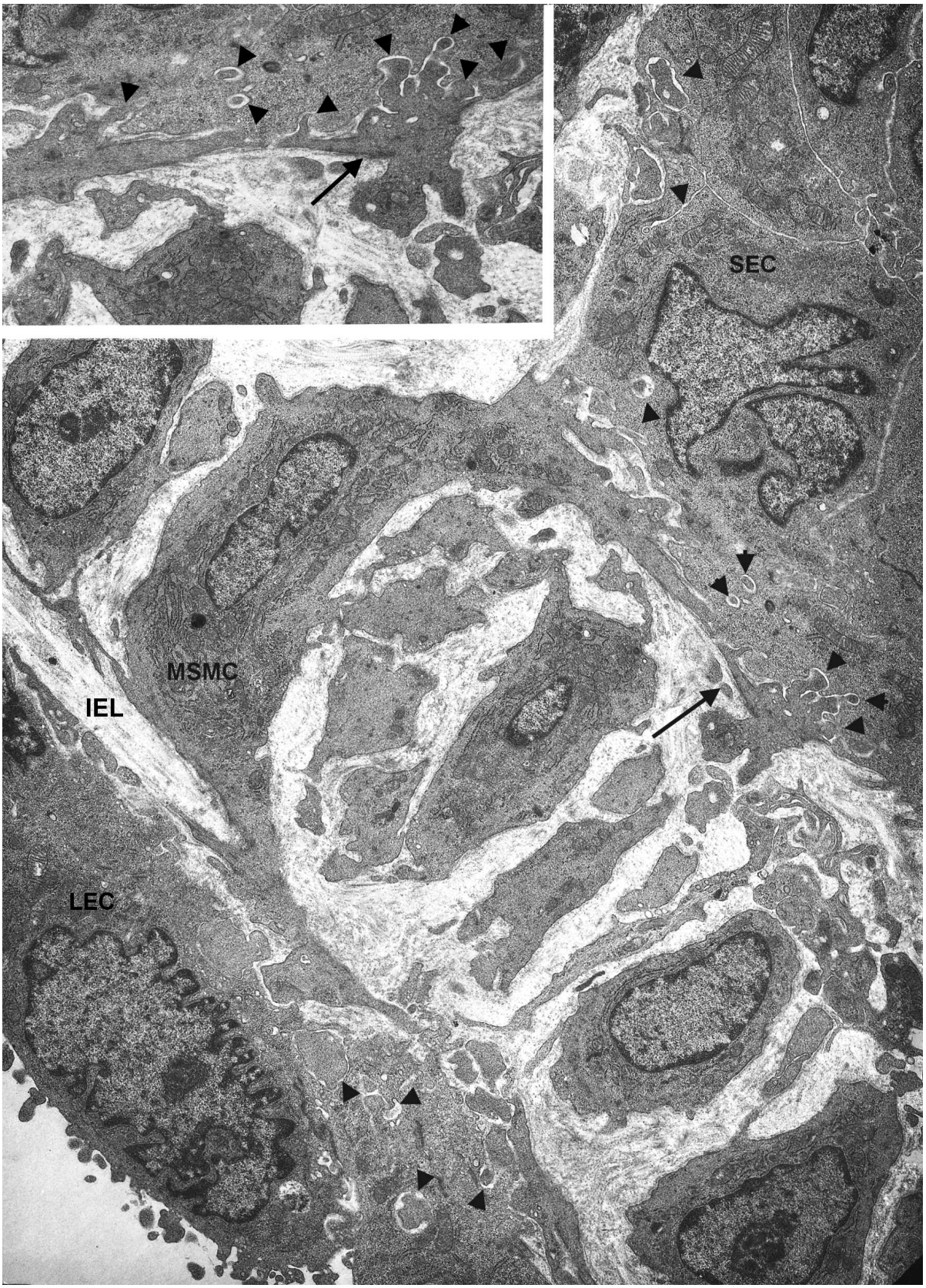

Fig. 4. Femoral vein 5 days after stimulation. One modified smooth muscle cell (MSMC), showing an increased number of organelles involved in the protein synthesis and, with transitional characteristics to activated pericyte, forms PSJs with luminal endothelial cells (LEC) and sprouting endothelial cells (SEC) in the media layer. The insert shows magnification of several pegs between one SMC projection and SEC (arrows). Other pegs are indicated by arrowheads. Ultrathin sections. Uranyl acetate and Lead citrate, x 16,000; Insert, x 20,000

activated pericytes, and continue forming pegs that penetrate the EC socket of both the intimal ECs and those of the sprouting ECs in the media layer. Therefore, in our current observations, we present a peculiar and predominant type of contact between SMCs and ECs during neovascularization of the vein media layer, while SMCs adopt pericytic characteristics and participate in the origin of new pericytes during vein angiogenesis. In this way, a type of contact between pericytes and ECs includes the PSJs (Díaz-Flores et al., 1991), also called EC-pericyte interdigitations (Wakui, 1992; Ishibashi et al., 1995; Wakui et al., 2006), which increase during neovascularization, as happens in granulation tissue and stroma tumour formation (Díaz-Flores et al., 1991, 2009b; Wakui 1992; Ishibashi et al., 1995; Wakui et al., 2006). In the vein, similar structures have been described in grafts that undergo severe contraction, with striking extensions of SMCs in the immediate subendothelial region of the wall; although they have only been considered as herniation in the endothelial layer and as a possible cause of EC disruption (Baumann et al., 1981).

The PSJ action mechanisms in the vein wall may be similar to those proposed in other vessels and other systems. EC-pericyte interdigitations described in pericytic microvasculature during angiogenesis might serve as a pathway for the Ang-1/Tie-2 system, with vascular endothelial growth factor (VEGF) promoting pericyte recruitment for microvasculature integrity (Wakui et al., 2006). Likewise, the PSJs have been considered as an alternative to the gap junctions in coupling SMCs (Thuneberg and Peters, 1998), interstitial cells of Cajal or interstitial cells of Cajal and SMCs (Rumessen et al., 2009). Some authors consider that these structures facilitate field coupling and propagation of a depolarization pulse through accumulation of K^+ in the extensive narrow gap (Vigmond et al., 2000). Our immunohistochemical observations demonstrating a higher expression of α-SMA and H-caldesmon in pegs, while desmin disappears, may be of interest in PSJ function. Caldesmon is a major calmodulin- and actin-binding protein found in SMCs and nonmuscle cells, and the expression of its isoforms plays a crucial role in the regulation of cell motility (by modulating the actin cytoskeleton - Jiang et al., 2010), vasculogenesis and angiogenesis "in vivo" (Zheng et al., 2009). However, more studies are required to elucidate the mechanism by which PSJs act in the activated vein wall to complement our morphogical observations.

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