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Arterial wall neovascularization induced by glycerol

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Summary. An intense and significant neovascularization, with numerous capillaries growing into the media layer of the rat femoral artery, was demonstrated when glycerol was administered into the interstitium between the femoral vein and the femoral artery. The maximum microvascularization was observed at days 7 and 9 after glycerol administration. Afterwards, involution of the majority of the newly-formed microvessels in the arterial wall occurred. Other substances containing glycerol in their molecules, such as triacetyl-glycerol and tributyril-glycerol, failed to produce significant neovascularization in the media layer of the femoral artery. Neovascularization of the arterial wall was preceded by a considerable decrease in the number of the smooth muscle cells, which experienced apoptosis and necrobiosis, disappearing in extense areas of the arterial segment affected by glycerol. Coinciding with neovascularization and microvascular involution, repopulation of the media layer by smooth muscle cells was observed.

Key words: Arterial wall, Angiogenesis, Glycerol, Smooth muscle cells, Pericytes, Neovascularization

Introduction

The microvessel invasion of the artery wall has been demonstrated in human pathology (Köester, 1876; Paterson, 1938; Winternitz et al., 1938; Le Compte, 1967; Cliff and Schoefl, 1983; Barger et al., 1984; Eisenstein, 1991; Zhang et al., 1993; Jeziorska and Woolley, 1999; Moulton, 2001), and in experimental conditions (Díaz-Flores and Domínguez, 1985, Díaz-Flores et al., 1990) with particular reference to the atherosclerotic lesions. The formation of these intraplaque microvessels is part of the complex process of neovascularization, which depends on several angiogenic factors, capable of stimulating the EC migration and/or proliferation (Folkman and Sing, 1992; Díaz-Flores et al., 1994a; Jeziorska and Woolley, 1999; Feige and Bailly, 2000; Moulton, 2001). However, the mechanism responsible for the ingrowth of new blood vessels into the arterial wall is not understood. Recently, an angiogenic action has been demonstrated for glycerol and mono and triacylglycerols (Dobson et al., 1990; Díaz-Flores et al., 1996). Thus, in our laboratory, glycerol and triacetylglycerol, with and without the combination of PGE_2, injected into a site between the femoral vessels of the rat, were found to have a neovasculogenic ability "in vivo" from small venules and capillaries, and from muscular vasculature in the venous side of the circulation. Since components of glycerol are present in the atherosclerotic plaques, it is of interest to test the hypothesis that glycerol and/or acylglycerols may induce microvessel invasion of the arterial wall.

Given these considerations, the object of this study was to assess the possible action "in vivo" of glycerol and some acylglyrerols on the neovascularization of the rat femoral artery wall. For this, the substances were administered in the adventitial side of the arterial wall using an "in vivo" rat model previously developed in our laboratory, which facilitates identification and quantitation of the newly-formed capillaries present in easily measurable sections of the media layer of the femoral arteries. The capacity of glycerol to produce intense microvessel invasion of the artery wall is described in this report.

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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 Commitee 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.)

Surgical procedure

To study the angiogenic action, the test substances were administered by means of a modification of a

procedure which has been previously described (Díaz-Flores et al., 1994b). Briefly, the femoral vessels and the branches with their surrounding connective tissue were exposed. Using a surgical microscope, a syringe connected to a polyethylene tube was used to pierce through the connective tissue between the superficial femoral artery and vein, next to their origin, following the interstitium longitudinally between the femoral artery and the femoral vein, and 0.1 ml of each test substance was introduced into this interstitium. At the moment of removing the tube, the perforation was sutured to prevent the administered substance from escaping.

Tissue processing

Specimens 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 for 2 h in 1% osmium tetroxide, dehydrated through a graded acetone series, and embedded in epoxy resin. For light microscopic histology, the specimens were orientated in such a way that the femoral vein and the femoral artery were longitudinally sectioned, 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 an electron microscope.

Experimental design

To assess the possible action of glycerol, triacetylglycerol and tributyril-glycerol on the neovascularization of the rat femoral artery wall, three test groups of 24 animals each were established, according to the substance administered: Group A: Glycerol (Sigma Chemical Co., Ref. G-9012); Group B: Triacetylglycerol (Sigma Chemical Co., Ref. T-5376), and Group C: Tributyrilglycerol (Sigma Chemical Co., Ref. T-8626). In a control group (n = 24) saline solution (0.1 ml) was administered, following the same procedure. The animals were sacrificed under general anesthesia with removal of the femoral vessels and surrounding tissues at days 3, 5, 7, 9, 12 and 15 after surgery; 4 rats per time point for each group.

To calculate to what extent the test substances intervened in the neovascularization of the rat femoral artery wall, we chose a simple and reproducible procedure. In each specimen, three 1.5 μm-thick sections were cut through the longitudinally-orientated femoral vessels, and a 4 mm-length of femoral artery in each section, with a total length of 12 mm, was considered for the quantitative study. The number of newly formed capillaries present in the media layer of the femoral arteries was counted. Following that, the mean value and standard deviation of the mean were calculated for days 3, 5, 7, 9, 12 and 15 for each group. Statistical analysis was made with analysis of variance (ANOVA), followed by t-test comparisons. Analysis was carried out using the Statistix software programme (NH Analytical Software). Statistical significance was defined as a $p<0.05$.

Results

Sham femoral arteries

During the experiment, the morphology of the femoral artery wall remained unmodified. At no time were capillary sprouts observed in the media layer of the artery.

Test femoral arteries

At days 3 and 5 after glycerol administration, degenerative and necrotic smooth muscle cells (SMC) were observed in the arterial wall and extensive areas of the media layer appeared without SMC (Fig. 1A). In the innermost part of this layer, some macrophages and spindle-shaped cells were observed (Fig. 1A,B). The endothelial cells (EC) of the artery showed hypertrophy and some of them were in mitosis (Fig. 1B). Some mononuclear cells were also seen adhering to the EC and within the subendothelium of the femoral artery wall (Fig. 1B). By day 5, some capillary sprouts appeared in the media layer contacting with the internal elastic lamina.

At days 7 and 9, numerous solid or canalized capillary sprouts were present in the innermost part of the femoral artery media layer (Fig. 2). The new

Table 1. Quantitative studies of the vascular sprouts in the media of the femoral artery.

SUBSTANCES	DAY 3*	DAY 5*	DAY 7*	DAY 9*	DAY 12*	DAY 15*
Glycerol	0	30.0±10.32	238.5±46.88	259±46.88	30.5±12.12	5.25±4.42
Triacetylglycerol	0	3±2.58	3.25±3.30	1±1.41	0	0
Tributirylglycerol	0	0.75±0.95	7.25±6.80	3.5±3.51	0	0
Control saline solution	0	0	0	0	0	0

*: NCD or neocapillary density: Number of capillaries present in the media layer of the longitudinally sectioned femoral artery per 12 mm length of the artery.

NCD in glycerol group vs control group: significant (NCD of control group: 0); increase by days 7 and 9 and decline by days 12 and 15; significant $p< 0.0001$). Triacetylglycerol vs control group: not significant. Tributyrilglycerol vs control group: not significant. Glycerol vs triacetylglycerol: significant ($p<0.0001$). Glycerol vs tributyrilglycerol: significant ($p<0.0001$).

capillary consisted of EC arranged in parallel fashion, as well as a variable number of periendothelial cells (Fig. 3). The nucleus of the EC displayed some foldings and contained prominent nucleoli. In the extensive cytoplasm, numerous ribosomes, some profiles of rough endoplasmic reticulum, and vacuolated mitochondria were observed. The periendothelial cells showed pericytic characteristics, with electrondense cytoplasm and some cisternae of the rough endoplasmic reticulum. Relatively frequent mitosis appeared in EC and periendothelial cells.

The newly-formed vessels in the tunica media appeared to be connected to each other and an evident continuity between their EC and the EC in the intima of the femoral artery was observed, with EC processes extending through the gaps of the internal elastic lamina (Fig. 4). By day 9, numerous transitional cell forms between periendothelial cells and SMC in the media layer were also seen.

Between days 12 and 15, most of the new capillaries in the media layer of the femoral artery had regressed. Some degenerated EC and capillaries occluded with platelets, erythrocytes or dead cells were still observed.

At 12 days, the arterial media layer showed cellular

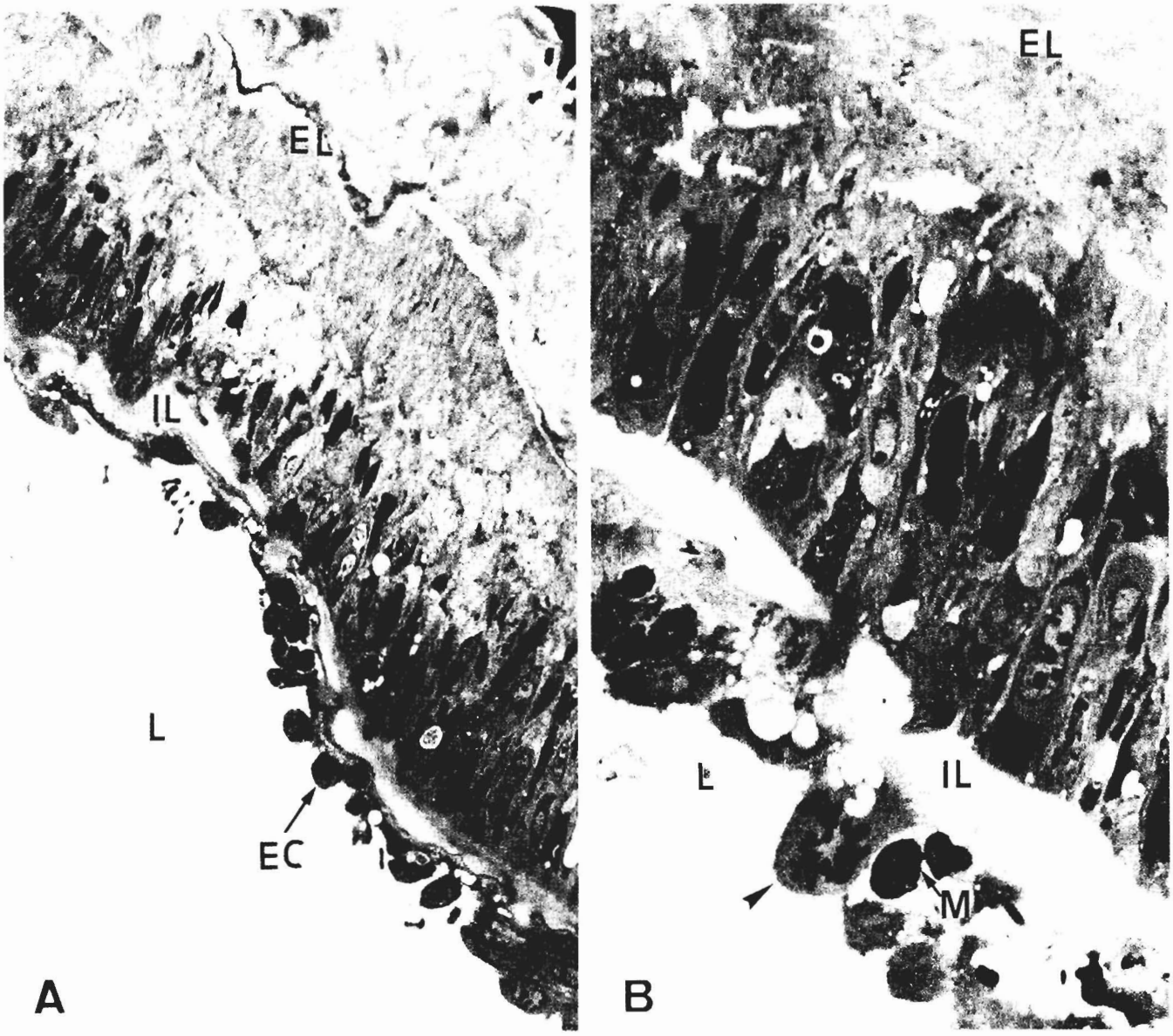

Fig. 1. Longitudinal sections of the femoral artery 5 days after glycerol administration in the interstitium between the femoral artery and the femoral vein. The endothelial cells (EC) are hypertrophied and one of them appears in mitosis (arrowhead). Some mononuclear cells (M) are observed within the subendothelium. In the innermost part of the media layer, spindle-shaped cells and macrophages are present, while the rest of the media layer appears without SMC. Cells extending through the gaps of the internal elastic lamina (IL) into the media layer are also shown (arrow). L: artery lumen; EL: external elastic lamina. Semithin sections; toluidine blue, x 360 and 900, respectively.

repopulation and many of the transitional cell forms had acquired a synthetic state of SMC. Their nuclei showed prominent nucleoli, and some of these cells were in the process of cell division. The cytoplasm appeared filled with numerous ribosomes, either simply or in aggregates, some profiles of rough endoplasmic reticulum and few bundles of myofilaments. At 15 days, some of these SMC cells increased their bundles of myofilaments, acquiring a contractile state. Collagen fibrils and elastin were present among the SMC.

The quantitative studies of the vascular sprouts in the media of the femoral artery are shown in the table. The neocapillary density (NCD) was defined as the number of neocapillaries present per 12 mm length of the artery (obtained in three longitudinal sections of the artery of 1.5 μm thickness). The glycerol showed a significant capacity to induce neovascularization. The NCD was moderate by the fifth day, increased sharply on days 7 and 9 and then declined significantly on days 12 and 15. The capacity to induce neovascularization in the arterial wall by triacetylglycerol and tributyrilglycerol was scarce and insignificant.

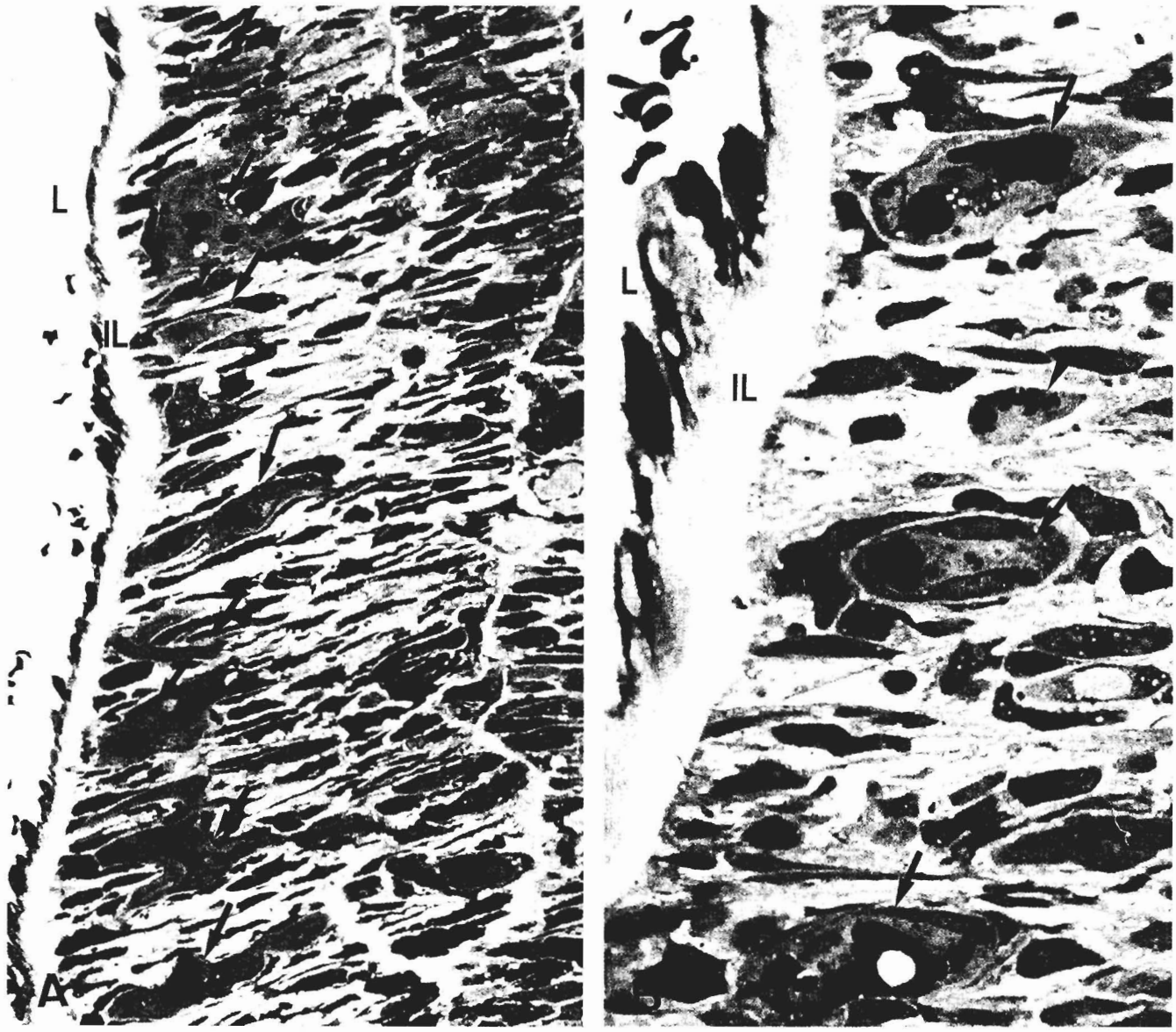

Fig. 2. Numerous newly-formed capillaries (arrows) are present in the innermost part of the femoral artery media layer 9 days after glycerol administration. The capillary lumens contain erythrocytes and platelets. The arterial media layer is repopulated by cells with transitional features between pericytes and SMC and one of them appears in mitosis (arrowhead). L: artery lumen; IL: artery internal elastic lamina. Semithin sections; toluidine blue, x 360 and 900, respectively.

Discussion

The current model has supplied us with a precise, reproducible, and comprehensive analysis of the processes triggered by glycerol, triacetylglycerol and tributyrilglycerol in the femoral artery, when the substances are administered into the interstitium between the femoral vein and the femoral artery. This model combines the following conditions: little damage to the tissues for testing, and an adequate procedure to prevent the administered substance from escaping from the extravascular position at the moment in which it is injected (Díaz-Flores et al., 1994b).

The findings of the present work, when compared with sham controls, have demonstrated that a single application (0.1 ml) of glycerol into the soft tissue surrounding the rat femoral artery induces a sudden, brief, and intense neovascularization, with numerous capillaries growing into the media layer of the artery. Triacetylglycerol and tributyrilglycerol failed to produce a significant neovascularization of the arterial wall. Previous studies (Dobson et al., 1990; Díaz-Flores et al., 1996) have demonstrated that glycerol, and some acylglycerols, may constitute a large family among the wide variety of non-peptide molecules which have been shown to have angiogenic activity "in vivo". The results described here show that glycerol may also intervene in the neovascularization of the arterial wall. Its biological activity is probably due to the capacity to behave as an angiogenic trigger, previously shown in the pre-existing microcirculation (Díaz-Flores et al., 1996). The events and sequence of capillary formation in our experiments agree with the previous studies on the morphology and chronology of angiogenesis (Ausprunk and Folkman, 1977; D'Amore and Thompson, 1987; Díaz-Flores et al., 1992, 1994a), including EC proliferation, solid sprout formation with posterior canalization, development of the new basal lamina, and the appearance of pericytes around the new capillary. The most obvious feature was the large number of vessel sprouts forming a conspicuous microvascular network in the media layer of the artery.

Since neovascularization stimuli are required for both the formation and maintenance of blood vessels (Ausprunk et al., 1977; Ziche et al., 1982, 1992), it is possible that the cessation in the perfomance of the

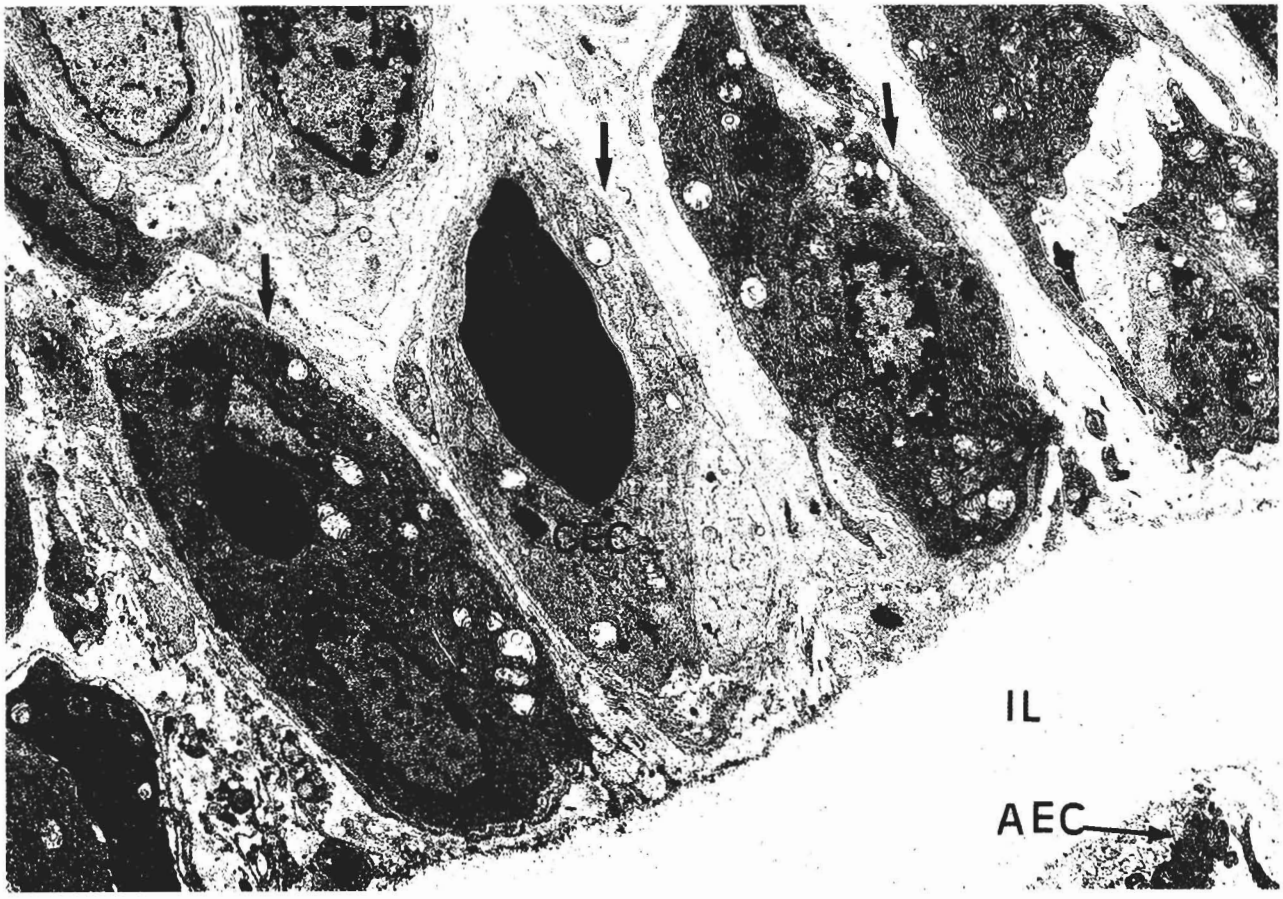

Fig. 3. Characteristics in ultrathin section of the newly-formed capillaries (arrows) in the innermost part of the media layer of the femoral artery 9 days after glycerol administration. AEC: artery endothelial cells; CEC: capillary endothelial cell; IL: Internal elastica lamina. Uranyl acetate and lead citrate, x 12,500

glycerol or the factors activated by them are the mechanism by which many of the newly-formed capillaries regress. This regression showed some of the structural changes described in regressing capillaries in different locations and conditions (Ausprunk et al., 1978; Azmi and O'Shea, 1984; Latker et al., 1986; Díaz-Flores et al., 1994a, 1996).

Neovascularization is a prominent feature of the early and late stages of atherosclerosis (Jeziorska and Wooley, 1999; Moulton, 2001), and there is a positive correlation between the density of new vessels with the incidence of luminal stenosis (Kumamoto et al., 1995). Our current findings suggest that glycerol may intervene in the complex molecular and cellular mechanisms responsible for the expression of angiogenic factors in arteries (Eisenstein, 1991, Battegay, 1995)

The observations reported here also suggest that the capillary sprouts in the innermost part of the arterial media layer originate from migrating and proliferating EC arising from the intima of the femoral artery. Although, in atherosclerotic lesions and other types of vascular injury, the network of capillaries in the lesion arise from adventitial vasa-vasorum and extend into the medial and intimal layers (Díaz-Flores and Domínguez, 1985; Díaz-Flores et al., 1990; Moulton, 2001), it is also possible that arterial endothelium may contribute to the new vessel formation in some conditions. In this way, it is generally thought that vascular sprouts originate during angiogenesis from nonmuscular or pericytic microvasculature (Ausprunk and Folkman, 1977; Burger et al., 1983; Phillips et al., 1991; Díaz-Flores et al., 1992), but the results described here and those of the previous studies (Díaz-Flores et al., 1994b; Nicosia and Vilashi, 1995) show that vessels of greater calibre, with smooth muscle cells in the medial layer, can participate in the process of neovascularization.

In our observations, the origin of pericytes of the newly formed vessels may be controversial, although in the early stages the periendothelial cells appear to be modified SMC. With regard to this, it has been demonstrated that rat SMC of the femoral vein (Díaz-Flores et al., 1994b) and of aortic intimal/subintimal origin (Nicosia and Vilashi, 1995) become pericytes during angiogenesis. Likewise, the presence of

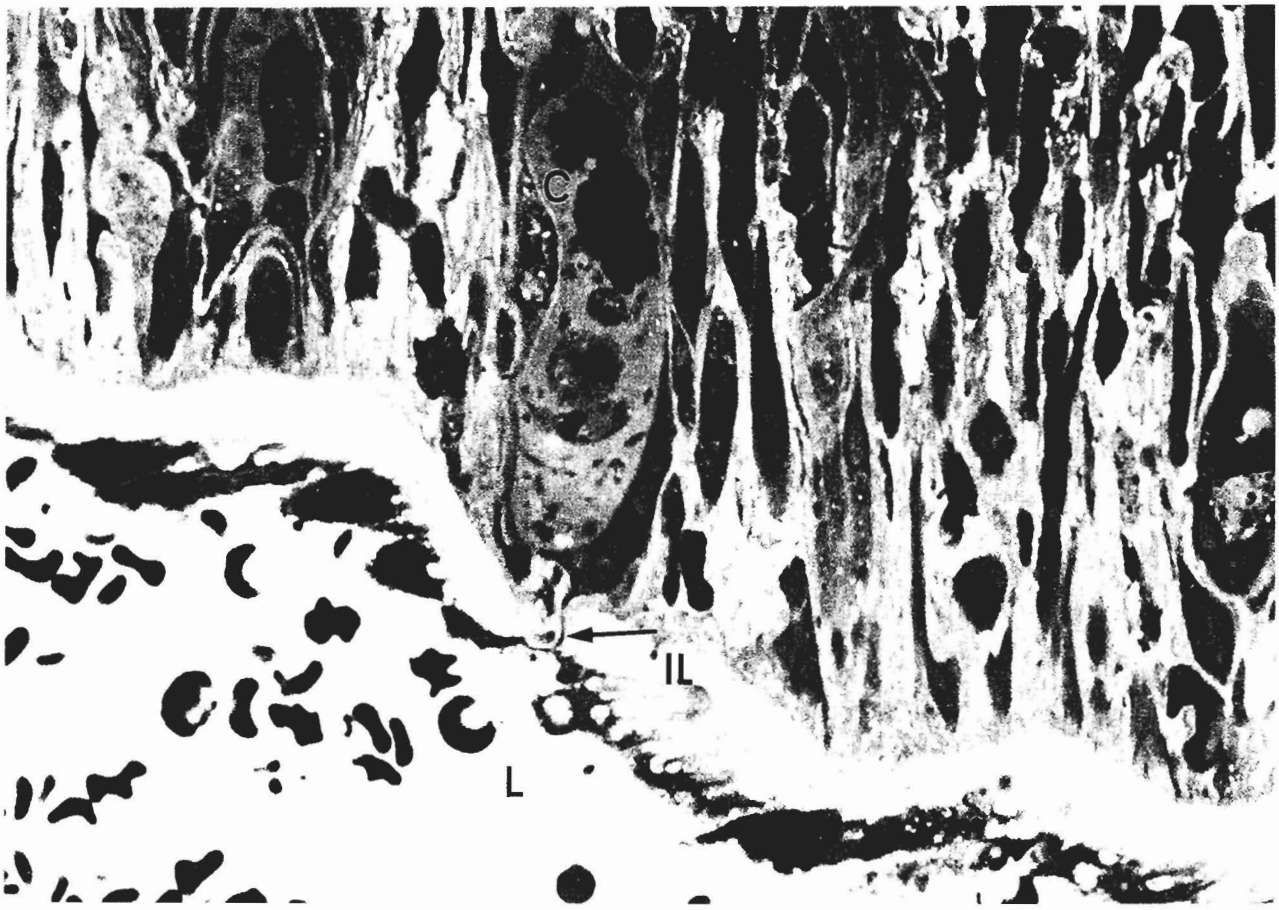

Fig. 4. Semithin section of the femoral artery 9 days after glycerol administration. An endothelial cell of a newly-formed capillary (C), present in the innermost part of the media layer, appears connecting (arrow) with an EC of the arterial endothelium trough a gap of the internal elastic lamina (IL). L: artery lumen. Toluidine blue, x 900

transitional cell forms between periendothelial cells and SMC also suggests that the former may contribute to the repopulation of SMC in the media layer of the artery. In other words, our findings also highlight a possible mechanism in arterial SMC repopulation by means of endothelial-SMC interaction similar to that which occurs in the formation of the arterial muscle wall in developing vasculature (Gale et al., 2001). This is a topic worthy of future exploration.

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