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Research Articles

Induction of neovascularization in vivo by glycerol

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Abstract. Glycerol, injected into a site between the femoral vessels of the rat, induced neovascularization, both from the preexisting microcirculation and from the side of the femoral vein facing the artery-vein interstitium where the glycerol was administered. The use of glycerol together with a known angiogenic substance (PGE_2) did not modify the neocapillary density (NCD) obtained with glycerol alone. In contrast, the lower level of NCD achieved with an acylglycerol (triacetylglycerol) was increased when the latter was associated with PGE_2. Values reached were similar to, but never higher than, those for glycerol alone, or combined with PGE_2. The results suggest that glycerol and some substances containing glycerol, amongst which 1-butyrylglycerol has been previously considered[1], may stimulate angiogenesis by a direct or indirect mechanism of action.
Key words. Angiogenesis; glycerol; acylglycerols; endothelial cells; smooth muscle cells; veins; microcirculation; neovascularization.

Angiogenesis, characterized by the sprouting of capillaries from pre-existing vessels, is particularly important in a great number of processes during prenatal and postnatal life. A greater knowledge of the control of this mechanism may lead to the development of a potential therapy in angiogenesis-related pathological processes, which include neoplasia, inflammation, wound repair and collaterization in response to ischemic stimuli[2,3,4,5]. Recently, a novel angiogenic substance, 1-butyrylglycerol, secreted by differentiating adipocytes, was discovered[1]. In our laboratory triacetylglycerol was found to have a slight neovasculogenic ability in vivo, which increased when it was associated with PGE_1 and PGE_2 [6]. In our experiments, the new capillaries originated not only from non-muscular or pericytic microvasculature (small venules and capillaries), but also from vessels of greater caliber in the venous side of the circulation, such as the rat femoral vein. This angiogenic capacity of 1-butyrylglycerol and triacetylglycerol suggested that as well as molecules containing glycerol, glycerol alone may have angiogenic capacity.

The object of this study was to assess the possible angiogenic action of glycerol in vivo, comparing the results with those of triacetylglycerol, or mixtures of these substances with a known angiogenic factor (PGE_2)[6,7,8]. A modified in vivo rat model[6], which enables easy identification and quantitation of the newly formed capillaries arising from easily measurable sections of the femoral vein wall, was used. The capacity of glycerol, and to a lesser extent triacetylglycerol, to produce intense vascular sprouting from the rat femoral vein and surrounding microvasculature is described in this report.

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Materials and methods

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.). To study the angiogenic action, the test substances were administered by means of a modification of a procedure which has been previously described[6]. 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.

Four 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); Group C: Solution of PGE_2 (0.1 mg/ml) (Sigma Chem-

ical Co., Ref. P-5640) in glycerol, and Group D: Solution of PGE_2 (0.1 mg/ml) in triacetylglycerol. In a control group (n = 24) saline solution (0.1 ml) was administered, following the same procedure. The animals were killed, under general anesthesia, with removal of the femoral vessels and surrounding tissues at days 3, 5, 8, and 12 after surgery; 6 rats per time point for each group.

5 mm long pieces 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.

To calculate the angiogenic capacity of the different substances and solutions we chose a simple and reproducible procedure. In each specimen, five 1.5 µm-thick sections were cut, through the longitudinally orientated femoral vessels, at approximate interval of 20 µm , and a 4 mm length of femoral vein in each section, with a total length of 20 mm, was considered for the quantitative study. The number of newly formed capillaries arising from the intimal endothelial cells of the sectioned femoral vein, and present in its wall, were counted in the 20 mm length of the vein. Two independent counts were made, one on the side of the femoral vein nearest to the femoral artery, facing the interstitium where the test substance was administered, and one on the opposite side. Following that, the mean value and standard deviation of the mean were calculated for days 3, 5, 8 and 12 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

Control group. In the interstitium between the femoral artery and the femoral vein, three days after surgery, a few neutrophils and mononuclear cells were present. Throughout the remaining days of the experiment, the morphology of the femoral vein wall or the surrounding tissues remained unmodified. No capillary sprouts arising from the femoral vein were ever observed.

Test groups. In all the test groups, new capillaries were observed arising from the femoral vein, as well as from the preexisting venules and capillaries of the soft tissues surrounding it.

At day 3 (fig. 1), the femoral vein endothelial cells (EC) nearest to where the test substance was administered

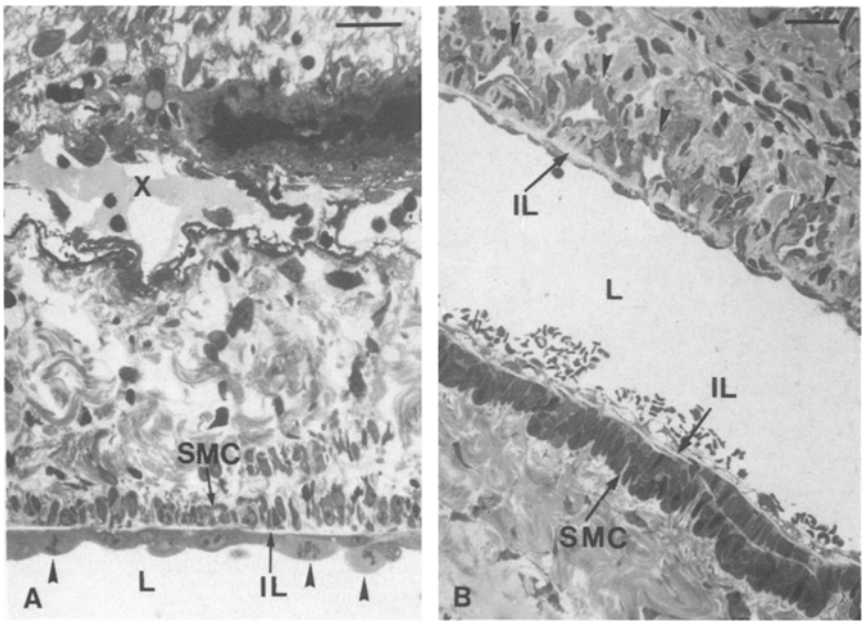

Figure 1. Semithin sections of femoral veins 3 days after glycerol administration. *A)* Wall of the femoral vein and connective tissue between the femoral vein and femoral artery (the latter not shown in the microphotography). X: site of the femoral vein-artery interstitium where glycerol was administered. Hypertrophied endothelial cells, some of them in mitosis (arrowheads), are present in the intima of the vein. *B)* Vascular buds (arrowheads) arising from the side of the femoral vein next to the test substance are observed. Note the spatial orientation of the angiogenic phenomena, since the opposite side of the femoral vein remains unmodified. L: Femoral vein lumen. IL: Internal elastic lamina. SMC: Smooth muscle cells. Semithin section. Toluidine blue. Bar: 15 µm.

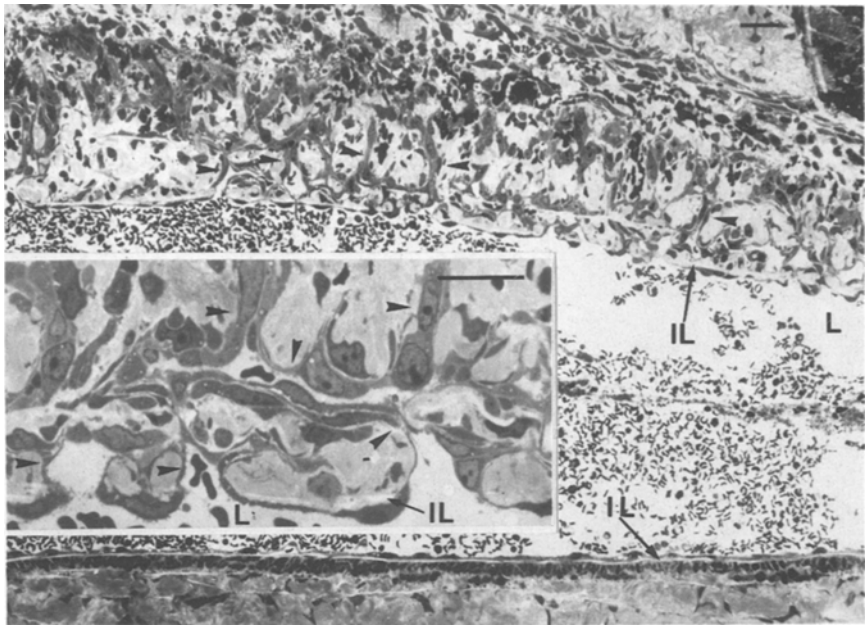

Figure 2. Longitudinal section of the femoral vein 5 days after glycerol administration in the interstitium between the femoral artery and the femoral vein. A great number of vascular buds (some of them indicated by arrowheads) arising from the side of the femoral vein facing the femoral artery (the latter not shown in the microphotography), near where the glycerol was introduced, is observed. Note how the vascular sprouts form an interconnected plexus. The opposite side of the femoral vein appears unmodified. In the area of the femoral vein wall where the neocapillaries originate, the number of smooth muscle cells decreases. In the insert, an area of the numerous vascular buds arising from the femoral vein is shown. IL: Vein discontinuous intimal elastic lamina. L: Vein lumen. Semithin sections. Toluidine blue. Bar: 15 µm.

showed hypertrophy and some of them were in mitosis (fig. 1A). In most cases at this stage, numerous endothelial cells were also observed protruding through the wall of the femoral vein, as well as arising from the venules and capillaries of the perivenous microvasculature. Frequently, several pairs of EC processes extended outwards in unison to form endothelial sprouts with a narrow slit-like lumen. Some neocapillaries with prominent lumens were also observed (fig. 1B). EC mitoses were present in both the parent vessels and the newly formed vessels. A one-sided spatial distribution of the capillary sprouts originating from the femoral vein was

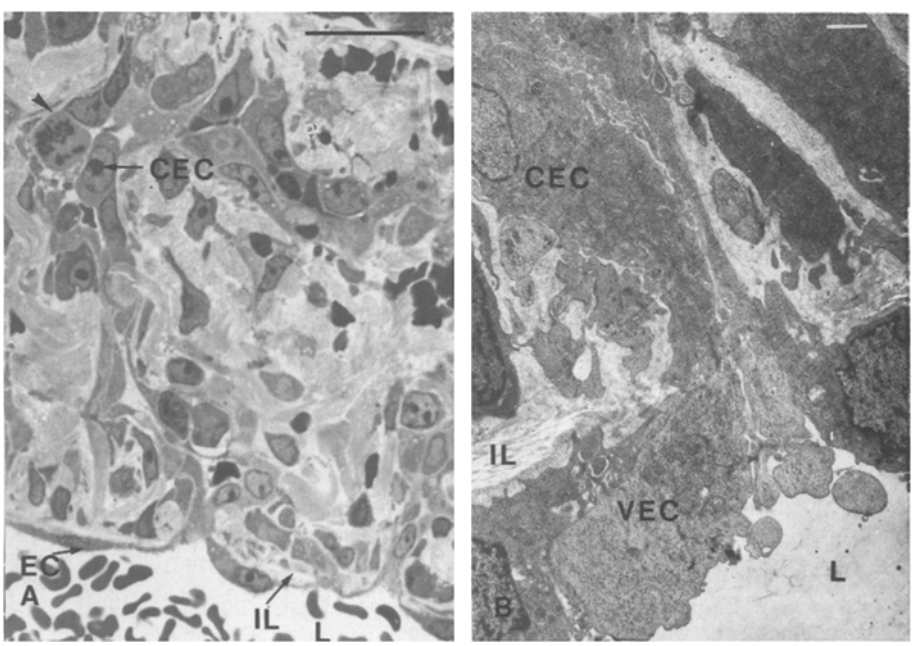

Figure 3. Characteristics of the newly formed capillaries originating from the femoral vein 5 days after glycerol administration in semithin (*A*) and ultrathin (*B*) sections. The capillary sprouts show prominent endothelial cells (CEC) and periendothelial cells. The latter appear to be modified smooth muscle cells. A capillary endothelial cell in mitosis is observed (arrowhead). L: Femoral vein lumen. IL: Vein discontinuous intimal elastic lamina. VEC: Vein endothelial cell, *A*: toluidine blue. Bar: 15 µm. *B*: Uranyl acetate and lead citrate. Bar: 2 µm.

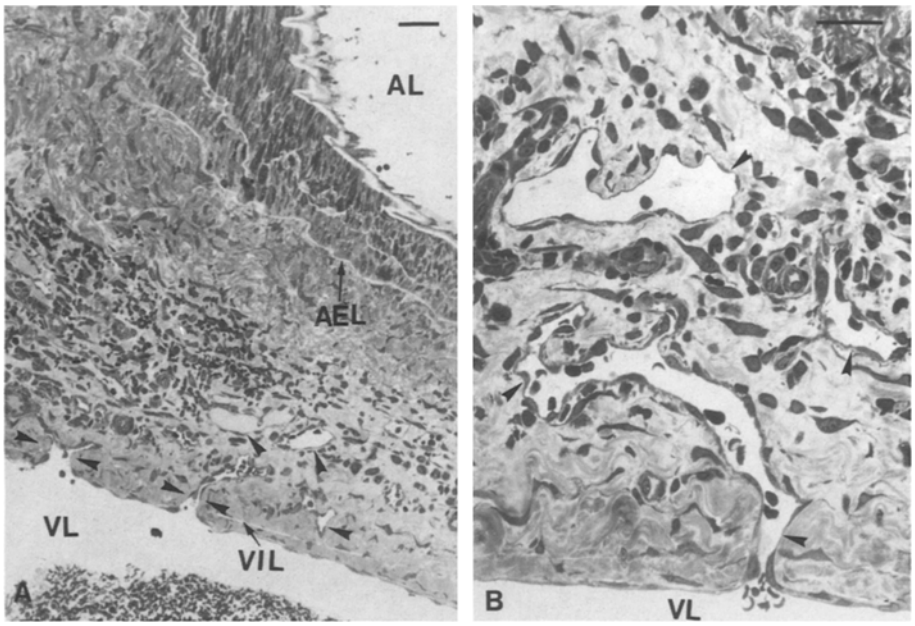

Figure 4. The persistent newly formed microvasculature is observed in the space between the femoral vein and the femoral artery, 12 days after glycerol administration. A detail of the persistent vessels is shown in *B*. Note that only some large channels (arrowheads) are present, most of the neocapillaries having regressed at this stage. The region of the femoral vein wall, where the large channels persist, appears devoid of or with a reduced number of SMC. VL: Femoral vein lumen. AL: Femoral artery lumen. VIL: Femoral vein internal elastic lamina. AEL: Femoral artery external elastic lamina. Semithin sections. Toluidine blue. Bar: 15 μm.

noted. The vascular sprouts that appeared mainly arose from the side of the femoral vein facing the femoral artery, exactly where the test substances were introduced, while the opposite side of the femoral vein remained scarcely modified (fig. 1B). In the area of the femoral vein where the neocapillaries originated, modified smooth muscle cells were observed around the sprouting EC.

By day 5, a considerable number of vascular buds had arisen from both the femoral vein (figs 2 and 3), and the perivenous microvasculature. The new capillaries emerging from the EC in the intima of the femoral vein, present in the media layer and in the most internal part of the adventitia of the vein, continued to show the same spatial distribution in all the test cases (fig. 2). These solid or canalized capillary sprouts consisted of EC, arranged in parallel fashion, as well as a variable number of periendothelial cells. The latter showed pericytic characteristics, while the number of smooth muscle cells considerably decreased in these vein wall areas with neovascularization. Some mitose were still observed in endothelial cells and periendothelial cells (fig. 3). The newly formed vessels appeared to be connected to each other, making up a conspicuous microvascular network (fig. 2), and their lumens were always in communication with the interior of the vein. The newly formed vessels emerging from the perivenous microvasculature also connected to each other, making another dense network of capillaries. Some connections were observed between the two microvascular plexi.

At days 8 and 12, the newly formed microvasculature network 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. 4). The pattern of vessel disappearance was also similar in all test groups, the regressing capillaries showing degenerated EC and lumens occluded by platelets, erythrocytes or dead cells. At this stage, extensive parietal regions of the femoral vein where the new capillaries had originated were devoid of smooth muscle cells.

The quantitative studies of the vascular sprouts from the femoral vein are shown in the table. The neocapillary density (NCD) was defined as the number of neocapillaries arising from the intimal endothelial cells of the femoral vein, and present in its wall, per 20 mm length of the vein (obtained in five longitudinal sections of the vein of 1.5 μm thickness). All the tested substances showed a significant capacity to induce neovascularization. In all the test groups, the NCD was moderate on day 3, increased sharply by the fifth day, and then declined significantly on days 8 and 12. The NCD was always significantly greater on the side of the femoral vein facing the vein-artery interstitium, where the test substances were administered, than on the opposite side of the femoral vein. Glycerol always showed a significantly greater NCD than triacetylglycerol, its maximum value being 2.78 times that of triacetylglycerol. The NCD for glycerol together with PGE_2 was not significantly different from that of glycerol alone.

Table. NCD or neocapillary density (number of capillaries that appear arising from one longitudinally sectioned femoral vein wall per 20 mm length of the vein) of the side of the femoral vein next to the test substance (side next) and the side away from the test substance (side away).

Test substances	Neocapillary density (NCD)							
	Day 3		Day 5		Day 8		Day 12	
	side next	side away	side next	side away	side next	side away	side next	side away
Glycerol	147.83 ± 70.80	5.33 ± 4.32	684.5 ± 97.67	10.00 ± 11.59	117.66 ± 47.84	1.66 ± 2.33	53.00 ± 29.40	4.00 ± 2.34
Glycerol + PGE_2	159.00 ± 80.92	4.66 ± 5.46	663.16 ± 886.21	10.33 ± 7.73	130.83 ± 58.06	3.33 ± 2.42	59.90 ± 38.39	2.33 ± 2.94
Triacetylglycerol	74.33 ± 50.56	4.00 ± 2.52	246.5 ± 110.05	7.33 ± 8.45	54.00 ± 29.78	2.33 ± 2.65	33.00 ± 25.25	0.66 ± 1.03
Triacetylglycerol + PEG_2	132.50 ± 72.43	3.33 ± 3.72	612.0 ± 103.60	7.66 ± 6.62	138.16 ± 69.33	2.66 ± 3.50	71.00 ± 42.80	2.00 ± 2.52
Control saline solution	0	0	0	0	0	0	0	0

NCD in all the test groups vs control group: significant (NCD of control group: 0); increase by day 5 and decline by days 8 and 12: significant ($p < 0.0001$); side next to substance vs side away from substance: significant ($p < 0.0001$); glycerol vs triacetylglycerol: significant ($p < 0.0001$); glycerol vs glycerol + PGE_2 and vs triacetylglycerol + PEG_2: not significant.

However, when triacetylglycerol was associated with PGE_2, the NCD increased significantly with respect to triacetylglycerol alone, reaching figures not significantly different from those for glycerol alone or combined with PGE_2.

Discussion

Although there are studies pointing out the angiogenic capacity of some molecules containing glycerol[1,6], to our knowledge, glycerol alone has not been previously investigated in this way. The findings of the present work, when compared with sham controls, demonstrate that glycerol has a potent action in vivo in the induction of neovascularization. The results were not significantly modified when glycerol was associated with a known angiogenic substance (PGE_2), therefore in our experimental model there seems to be an upper limit of neovascularization from a specific area of the femoral vein, which may be reached with glycerol alone.

Under the same conditions, the triacetylglycerol alone had a lesser angiogenic capacity than glycerol, although it was more intense than that found in a previous study[6]. The difference was probably due to the modification of the method used; in the present study the substance was selectively located in the interstitium between the femoral artery and the femoral vein, whereas in the previous study it was applied to the soft connective tissue surrounding the femoral vessels. The increase of vascular sprouting from the femoral vein resulting from triacetylglycerol associated with PGE_2 reached levels similar to, but never higher than, those due to glycerol alone or combined with PGE_2. This observation reinforces the hypothesis that there is an upper limit of neovascularization from the femoral vein.

The results described here and those of the previous studies[1,6] show 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. Although it has been hypothesized that 1-butyrylglycerol acts via an intracellular or cell surface receptor[1], and that diacylglycerol appears to be the major intracellular messenger for angiogenin[9], an indirect route, involving glycerol and triacylglycerol mobilizing other factor(s) or cell(s), could also be possible. Therefore the role of other acylglycerides, and the systems that mediate their actions in endothelial cells, are topics worthy of future consideration.

The events and sequence of capillary formation in our experiments agree with the previous studies on the morphology and chronology of angiogenesis[3,5,10,11]. It has been pointed out that in the process of neovascularization, the EC migrate from the side of the vessel closest to the angiogenic stimulus, while subtle alterations seem to appear around the whole circumference of the parent vessel[10]. This spatial orientation of the capillary sprouts has been significantly demonstrated in our morphological and quantitative studies, since in all the test groups there was a restriction of the vein area from which the neocapillaries originated; specifically, to the side of the femoral vein wall facing the vein-artery interstitium, where the test substances were located.

The regression of most of the newly formed capillaries, with persistence of a few large channels by day 12, showed a similar pattern to that described in regressing corneal capillaries[12]. Since neovascularization stimuli are required for both the formation and maintenance of blood vessels[12,13,14], it is possible that there was a cessation of the activity of the tested substances or the factors activated by them, which resulted in the regression of many of the newly formed capillaries.

On the other hand, the current work confirms previous studies in our laboratory which demonstrated that veins with smooth muscle cells in their media layer, and a discontinuous internal elastic lamina, can augment the process of neovascularization from venules and capil-

laries by contributing a supplementary population of vascular sprouts[6].

Since in the early stages, the periendothelial cells of the newly formed capillaries appear to be modified smooth muscle cells when their parent vessel is the femoral vein, the experimental model may also be of interest for future investigations on the origin of pericytes during angiogenesis, which is controversial[15,16,17]. Indeed, although there is evidence indicating that they may evolve from fibroblasts[17,18,19], it is possible that pericytes of the preformed microcirculation and smooth muscle cells of some veins may participate in the origin of new pericytes[6,11,19].

1 Dobson, D. E., Kambe, A., Block, E., Dion, T., Lu, H., Castellot, J. J. Jr., and Speigelman, B. M., Cell *61* (1990) 223.
2 Folkman, J., and Klagsbrun, M., Science *235* (1987) 442.
3 D'Amore, P. A., and Thompson, R. W., A. Rev. Physiol. *49* (1987) 453.
4 Folkman, J., and Shing, Y., J. biol. Chem. *267* (1992) 10931.
5 Diaz-Flores, L., Gutierrez, R., and Varela, H., Histol. Histopathol. *9* (1994) 807.
6 Diaz-Flores, L., Gutierrez, R., Valladares, F., Varela, H., and Perez, M., Anat. Rec. *238* (1994) 68.
7 Ben-Ezra, D., Am. J. Ophthalmol. *86* (1978) 455.
8 Form, D. M. and Auerbach, R., Proc. Soc. expl Biol. Med. *172* (1983) 214.
9 Bicknell, R., and Vallee, B. L., Proc. natl Acad. Sci. USA. *85* (1988) 5961.
10 Ausprunk, D. H., and Folkman, J., Microvasc. Res. *14* (1977) 53.
11 Diaz-Flores, L., Gutierrez, R., and Varela, H., J. Morphol. *213* (1992) 33.
12 Ausprunk, D. H., Falterman, K., and Folkman, J., Lab. Invest. 38 (1978) 284.
13 Ziche, M., Jones, J., and Gullino, P. M., J. natl Cancer Inst. *69* (1982) 475.
14 Ziche, M., Morbidelli, L., Alessandri, G., and Gullino, P. M., Lab. Invest. *67* (1992) 711.
15 Sims, D. E., Tissue Cell 18 (1986) 153.
16 Diaz-Flores, L., Gutierrez, R., Varela, H., Rancel, N., and Valladares, F., Histol. Histopathol. *6* (1991) 269.
17 Nehls, V., Denzer, K., and Drenckhahn, D., Cell Tissue Res. *270* (1992) 469.
18 Rhodin, J. A. G., and Fujita, H., J. submicrosc. Cytol. Pathol. *21* (1989) 1.
19 Sappino, A. P., Schürch, W., and Gabbiani, G., Lab. Invest. *63* (1990) 144.