

Canine capillary formation *in vitro*

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Summary. Microvascular endothelial cells derived from canine subcutaneous adipose tissue formed knob-like and tube-like structures *in vitro* without tumor-conditioned medium or special substrate. The knob-like structures consisted of acidic and basic glycosaminoglycans arranged in order.

Knob-like structures were built from cell extrudates and were responsible for capillary lumen formation *in vitro*.

Transmission electron microscopy confirmed the endothelial nature of the cells which expressed extensive phagolysosomal activity.

Key words: Endothelial cell cultures, Canine, Glycosaminoglycans, Capillary

Introduction

Cell culture systems have been used and have played an important role in the investigation of endothelial cell biochemistry and metabolism, as well as their involvement in more complex processes such as cell to cell and cell to matrix interactions, and uptake and discharge of macromolecules (Wagner et al., 1980; Gerritsen and Cheli, 1983; Madri and Williams, 1983; Alby and Auerbach, 1984). In addition, endothelial cell cultures from human cloned microvascular (capillary) and umbilical vein as well as from bovine aortic and adrenal cortex endothelium organize into tubular networks *in vitro*, similar to capillaries *in vivo*, providing a valuable system to study angiogenesis *in vitro* (Folkman and Haudenschild, 1980; Maciag et al., 1982; Feder et al., 1983; Milici et al., 1985).

Recently, endothelial cell populations have become clinically useful in seeding of synthetic vascular prostheses in order to develop antithrombogenic endothelial lining

(Stanley et al., 1985; Jarrell et al., 1986).

In the present study, we describe a microvascular canine endothelial cell culture procedure which yielded endothelial cells forming capillary-like tubes *in vitro*, revealing more information on the *in vitro* angiogenesis.

Materials and methods

Isolation and culture of capillary endothelial cells.

Canine neck subcutaneous adipose tissue was removed under sterile conditions and placed in an Erlenmeyer flask containing 30 ml medium RPMI - 1640 (Seromed) supplemented with glutamine, Heppes, penicillin (100 U/ml), streptomycin (100 µg/ml) and heparin (90 µg/ml). The flask was incubated at 37° C being shaken gently for 30 min to remove blood cells. The medium was discarded and the tissue was sliced and squashed gently with a syringe plunger to break the fat cells. The tissue pieces were rinsed and then immersed in a mixture of trypsin (0.25% in Ca⁺⁺ and Mg⁺⁺ free PBS, Seromed) - 0.1% collagenase (Type A1, Sigma C-9891) and incubated at 37° C being shaken gently for 15 min. The enzyme-cell mixture was then replaced by a fresh one and the tissue fragments were incubated at 37° C being shaken gently for another 30 min. The released cells in the enzyme mixture were washed twice with complete medium (CM) RPMI-1640 (Seromed) supplemented with glutamine, Heppes, 15% foetal bovine serum (FBS), streptomycin and penicillin, by centrifugation at 200 g for 10 min. The above enzyme treatment of the tissue was repeated four times, resulting in four subsequent cell harvests, plated in 4 separate Costar flasks (25 cm²), designated as number 1, 2, 3, 4, each containing 10 ml CM and incubated in a humidified 95% air/5% CO₂ incubator at 37° C. Twenty-four hours later, cell plating was completed and the medium was replaced with RPMI-1640 (Seromed), supplemented with glutamine, 15% FBS and antibiotics.

The cell cultures were allowed to grow for 48 hours

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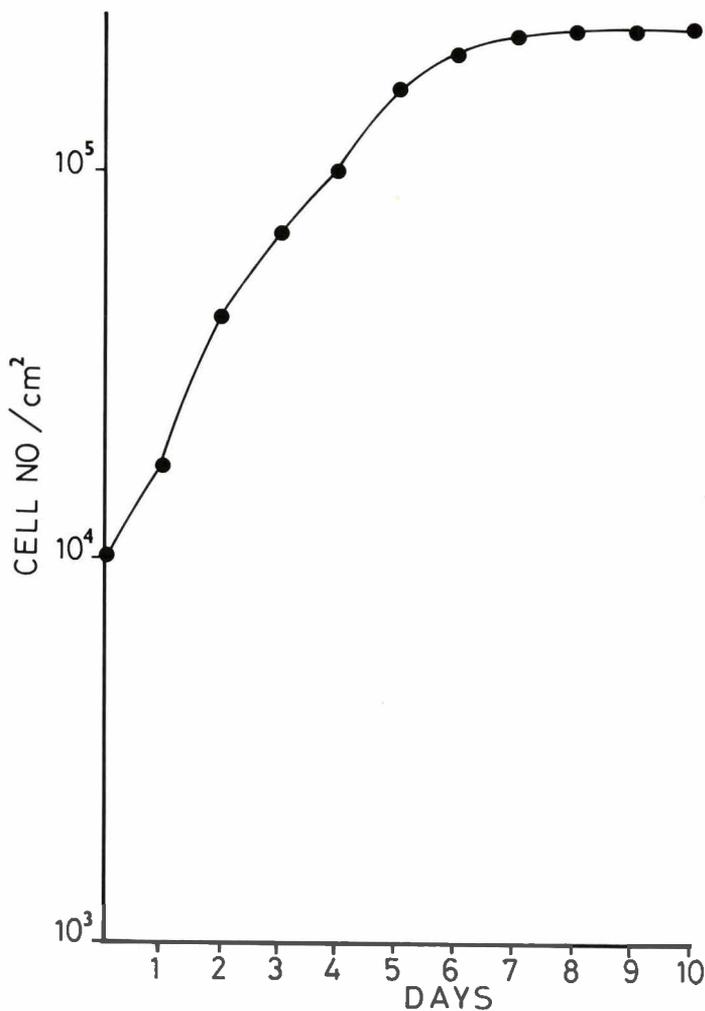


Fig. 1. Growth curve of canine endothelial cells in monolayer culture. SEM were too small to register. $n=3$

and the cells of flasks number 2, 3, 4 were then subcultivated by trypsinization, pooled, counted (2.0×10^6 total cells) and seeded into one stock 75 cm².

Growth Curve

The cell culture of flask number 1, first cell harvest, was used separately, as a control for immunocytochemistry as it contained a mixture of endothelial and fibroblastic cells.

Five cell culture stocks from five different dogs were prepared using the above method, and frozen in the liquid nitrogen tank.

The growth of endothelial cells in monolayer culture was evaluated by plating 1×10^4 cells/cm² in 25 cm² Costar flasks, each containing 10 ml complete RPMI-1640 without Heppes. The medium was changed every three days. The number of viable cells was determined by counting the trypsin-dispersed cells in a hemocytometer using the trypan blue exclusion technique. Cells in



Fig. 2. Endothelial random cell branching in non - confluent culture. Anti - factor VIII. $\times 500$

triplicate tissue culture flasks were harvested and counted at daily intervals.

Knob-like structure assessment

Endothelial cell cultures were initiated with an inoculum cell density of 1×10^4 cells/cm² (4 petri dishes). The medium of one set of 2 was changed daily while the others every three days. The same was repeated with the higher cell density inoculum of 3×10^4 cells/cm². All these cell cultures were observed daily under the phase contrast microscope and the time knob-like structures appeared was recorded.

Immunocytochemistry, cytology and histology

Endothelial cell cultures grown in pyrex petri dishes, and smears of floating cellular debris were fixed in 50% ethanol and stained by the Papanicolaou method for cytological examination and PAS, PAS-diastase, Alcian blue (pH 2.5) for glycosaminoglycan detection. Endothelial confluent cell cultures containing tube-like structures were fixed in formalin, scraped with a rubber



Fig. 3. Endothelial cells capable of forming long rows in a horizontal plane. Anti-factor VIII. $\times 500$



Fig. 4. Knob-like formation stained dark red in the outer part and lighter at the centre. Papanicolau $\times 50$

policeman, and embedded in paraffin. Thin serial sections were stained with PAS, PAS-diacetate and Alcian blue (pH 2.5).

Non-confluent and post-confluent endothelial cell cultures, mixed endothelial-fibroblast cell culture from the first harvest during preparation, human skin fibroblasts (control culture) and smears of culture cellular debris were fixed in cold acetone for 10 min. and were immunostained for factor VIII (1:100, Dako), keratin (1:40, polyclonal Dako) using the peroxidase-antiperoxidase method (Delellis et al., 1979).

Electron microscopy

Cell cultures for ultrastructural examination were immediately fixed in a phosphate buffered mixture of 1% glutaraldehyde and 4% formaldehyde, postfixed in buffered 1% osmium tetroxide, dehydrated in graded ethanols and embedded in a mixture of Epon and Araldite. Semithin sections were cut with glass knives, stained with toluidine blue and used for light microscopic orientation. Ultrathin sections were cut with diamond knives, contrasted with uranyl acetate and

lead citrate and then examined under the electron microscope.

Results

Growth characteristics and cytology

Monolayer cultures of endothelial cells showed a 95% plating efficiency, grew exponentially with a mean generation time of about 37 hours (4.5 population doublings in 7 days), reaching a population density in the range of 2.2×10^3 cells/cm² before exhibiting contact inhibition of growth. Growth rate was reduced as cell density increased (Fig. 1).

Endothelial cells were highly vacuolated, mononucleated with polygonal fibroblastic morphology and basophilic granular cytoplasm. The nuclei were round to oval, each containing one to two large compact or 4 smaller basophilic nucleoli, and uniformly fine granular chromatin. The nuclear and cell membranes were very well defined.

During the non-confluent stage, the cells and their protoplasmic projections showed a random arrangement

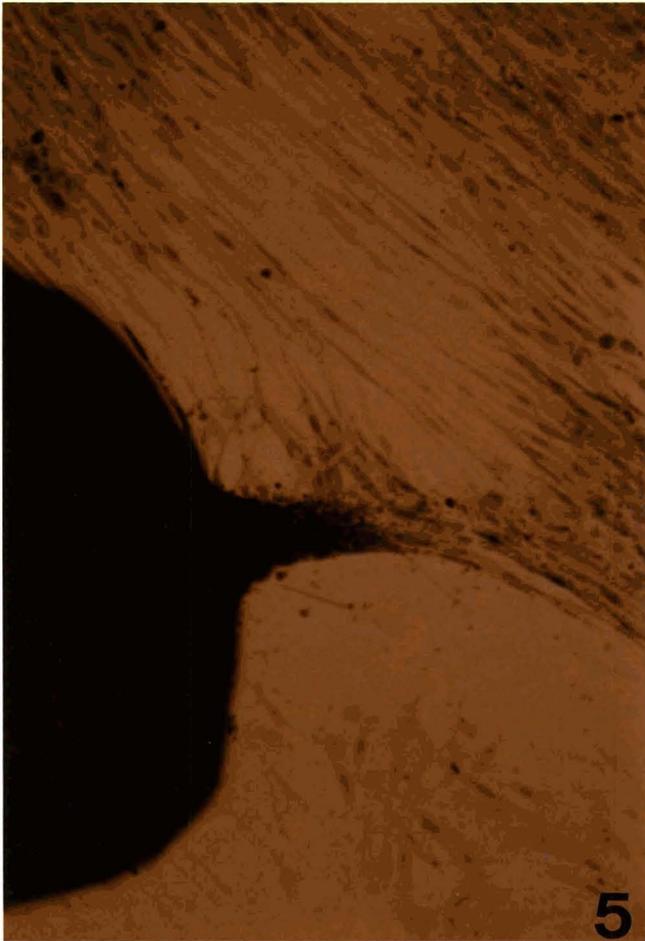


Fig. 5. Onset of capillary formation. The cells are oriented in parallel towards the circumference of the knob-like formation. Papanicolaou $\times 50$

(Fig. 2), while upon confluence they followed a multicentric pattern, lined in parallel, each cell extending only two protoplasmic projections, diametrically opposite. At this stage the nuclei became elongated and narrow and much debris appeared on the cells and culture medium.

Endothelial cells, in a horizontal plane, were capable of forming long rows in which every nucleus was overlapped by the thin protoplasmic projections of the previous and following cell (Fig. 3).

Capillary formation

A multicentric type of cell growth took place around accumulated amorphous material (day 7 of growth curve) and grew to an irregular knob-like formation outwards in space during post confluence.

By the tenth day of the growth curve, the knob's diameter ranged from 0.4 mm to 0.7 mm. The knob-like formations were stained dark red at the outer part and lighter at the centre, as if they contained a core (Fig. 4). The cells were oriented towards the circumference of the knob-like formations in a parallel pattern, extending,

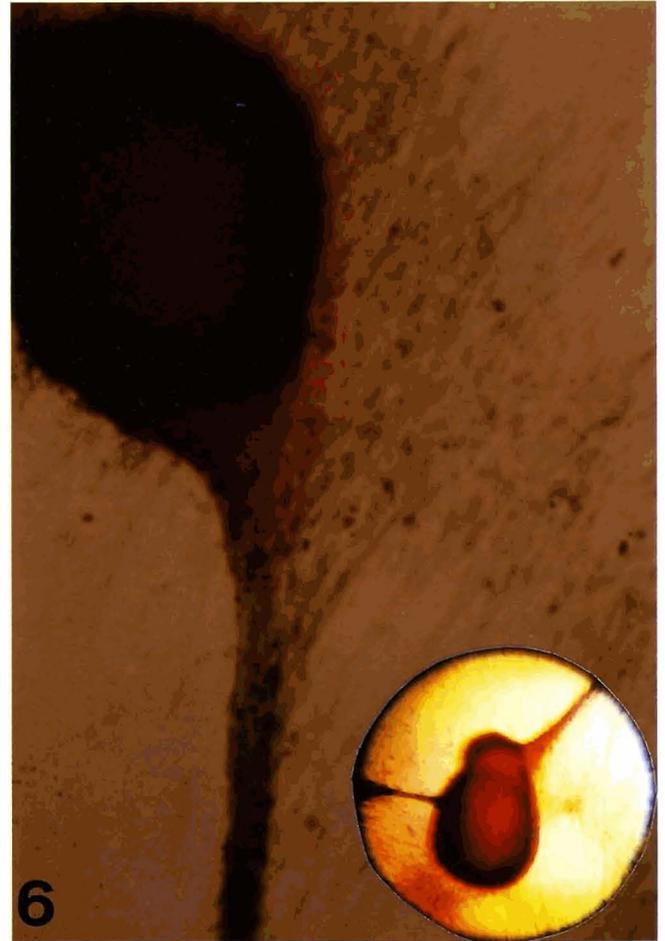


Fig. 6. A well-formed capillary and its association with knob-like formation. Papanicolaou $\times 50$. Inset: Knob-like formation with two tube-like structures opposite each other. Papanicolaou. $\times 10$

and starting capillary formation (Fig. 5).

Two capillaries were formed from most knob-like formations, usually opposite each other from the end of the circumference of each one. Capillary branching was observed, and associated with one knob-like formation, originating from common lumen at the point where knob-like material ended (Figs. 6, 7).

The capillary length ranged from 0.2 mm to 0.5 mm in 25 days of continued cultivation. By this time the knob-like formations and formed capillaries were observed in three dimensions with the knobs and capillaries detached from the culture vessel surface in space and only the other capillary end (opposite the knobs) was still attached on the vessel surface.

Control human skin fibroblasts formed whorls upon confluence without any knobs or tube-like structures.

Knob-like structure assessment

Endothelial cell cultures initiated with 1×10^4 cells/cm² and medium changes every three days exhibited knob-like structures scattered all over the dish surface on the

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Fig. 7. Capillary branching *in vitro* originating from common knob and lumen. $\times 50$. Inset: The whole structure. Papanicolaou. $\times 10$

seventh day of continuous cultivation, while those of higher cell density inoculum showed this on the fourth day. The daily change of the cell culture medium delayed knob-like structure appearance to 25 days with the high inoculum and 30 days with the lower cell inoculum.

Immunocytochemistry and histology

Endothelial cells were immunostained for factor VIII and negative for keratin. The knob-like formations, the capillaries, the cells, their protoplasmic projections (Fig. 8) and the floating cellular debris (Fig. 9) were positive for factor VIII. Incubation with non-specific serum as first layer instead of specific primary antibody resulted in no staining of the cells. Human skin fibroblasts were unstained for keratin and factor VIII.

Canine endothelial-fibroblast mixed cell harvest contained a lot of unstained fibroblasts and a few positive endothelial cells for factor VIII.

Eosin and hematoxylin stained paraffin sections of the culture tube-like structures showed a small segment of the capillary lumen filled with amorphous material

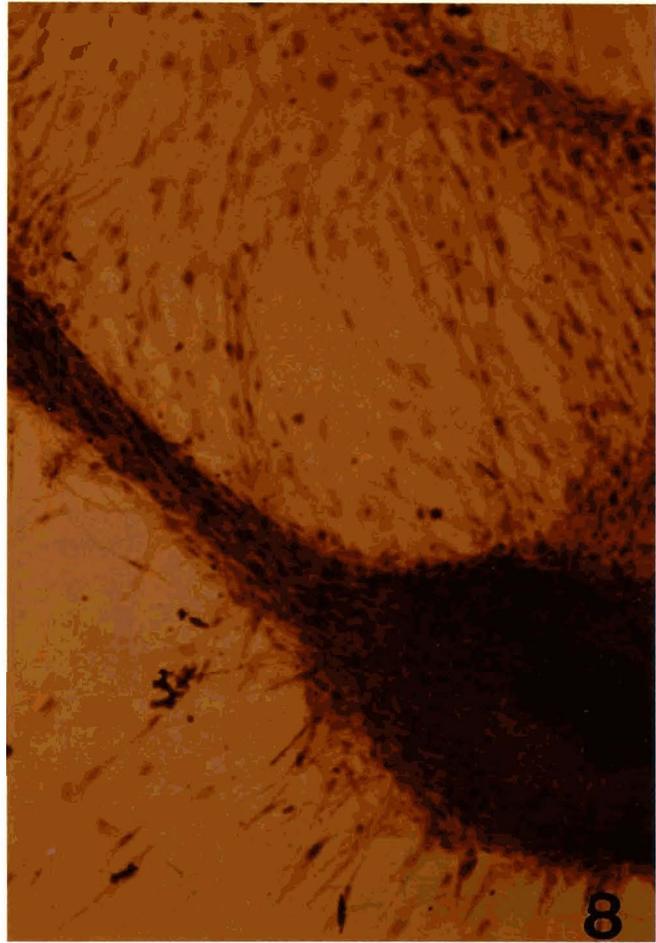


Fig. 8. Factor VIII immunoreactivity of knob- and tube-like structures. $\times 50$

(resembling plasma filled tissue capillary *in vivo*) while the rest were empty lumens of smaller diameter (Fig. 10).

Endothelial cell cultures and paraffin sections were PAS-positive even after pretreatment with diastase (Fig. 11). Alcian blue stained the core of the knob-like formations and the thin lining of the lumen facing endothelial cells in paraffin sections, indicating acidic glycosaminoglycans while the other region of the knob was PAS-positive, indicating neutral or basic glycosaminoglycans. PAS-positive granules were observed intracellularly and extracellularly, while Alcian blue-positive material was observed only extracellularly. PAS-positive GSG were observed in the lumen of the paraffin sectioned capillaries.

Cell identity

Endothelial cell identity was determined by phase-contrast microscopy and their strong immunoreactivity for factor VIII. Control human normal skin and canine fibroblast cultures were negative for factor VIII and formed whorls upon confluency. Endothelial cell cultures formed tubules upon confluency leaving vacant the



Fig. 9. Factor VIII immunoreactivity of floating medium cellular debris. $\times 500$

culture vessel surface. Moreover, lumen formation was documented ultrastructurally, while no collagen was found. In addition fibroblast cell contaminants appeared in the first cell harvest which was not used in the pure endothelial cell stock establishment.

Electron microscopy

The endothelial nature of the cells was confirmed by electron microscopy. The spindle shaped cells with long cytoplasmic extensions were attached by junctions, had abundant pinocytotic vesicles in cytoplasm along the cell membranes and had various amounts of cytofilaments accumulated in the cell periphery (Figs. 12, 13). Focal densities were only occasionally present. Weibel-Palade bodies were not seen.

Abundant lipid droplets and phagolysosomes were the most prominent features in cytoplasm and sometimes formed large intracytoplasmic vacuoles (Fig. 14). An amorphous, partially granular material was present in lumen.

Sometimes, larger masses of fibrillary, medium electron-dense material were present in the extracellular

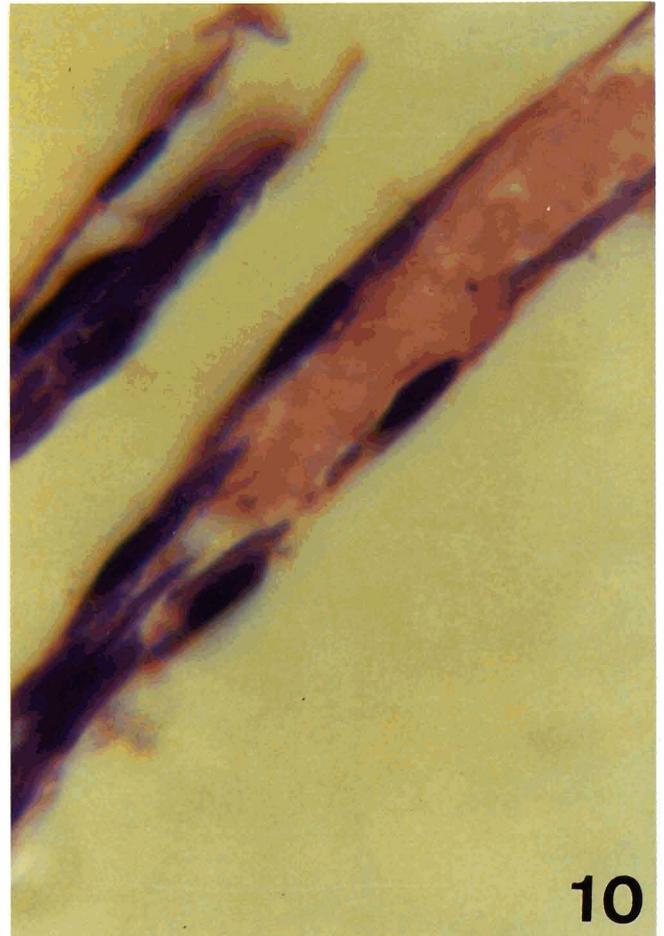


Fig. 10. Eosin and hematoxylin stained paraffin section of *in vitro* tube-like structure with GSG-filled segment. $\times 500$

matrix and surrounded by cells (Fig. 15).

Discussion

Microvascular endothelial cell cultures have become the focus of extensive research recently because of their fastidious nutritional growth requirements and extra-cellular matrix (Gospodarowicz and Ill, 1980). Moreover, their dynamic ability to form tube-like structures renders them an important *in vitro* system to study angiogenesis.

Tube-like structures have been formed by endothelial cells from bovine calf adrenal capillary, human foreskin capillary, human tumor biopsy capillary (Folkman and Haudenschild, 1980), human umbilical vein (Maciag et al., 1982), fetal bovine and bovine calf aorta (Feder et al., 1983) and bovine adrenal cortex (Milici et al., 1985). These cell systems, although significant differences have been reported on their growth requirements and substrata, all form similar tube-like and knob-like structures.

The first general mechanism of microvascular lumen formation is suggested by the formation of an autolytic vacuole within a capillary endothelial cell, the conversion

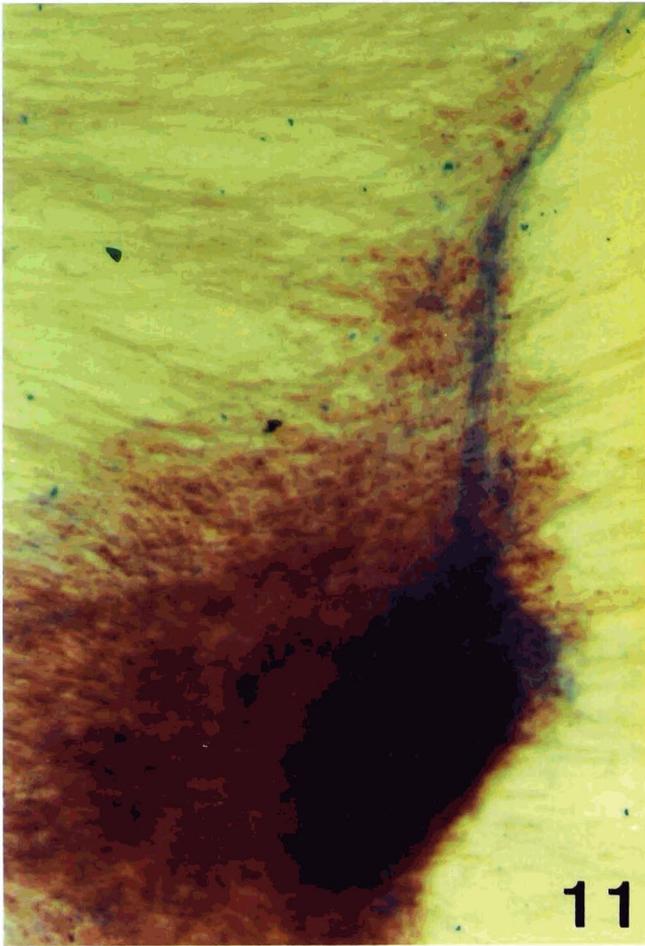


Fig. 11. PAS and Alcian blue staining of knob- and tube-like structure. $\times 50$

of this vacuole to a tube, the connection of one tube to another, the girdling of the tube by capillary endothelial cells that form special junctions with themselves and with each other and the ability of these cells to glide along the tube once it has been formed (Folkman and Haudenschild, 1980).

The biochemical composition and function of the knob-like structure remains unknown, although it is described as being formed of fibrillar, partially membranous and amorphous material (Folkman and Haudenschild, 1980; Feder et al., 1983; Milici et al., 1985).

In this report canine endothelial cell cultures were prepared for PTFE (polytetrafluorethylene) graft seeding and implantation in dogs. Although many investigators work with this system, there has not been a report on capillary formation by canine endothelial cells, originating from adipose capillaries.

The present work shows that canine adipose capillary endothelial cells grow without any special substrata or tumor-conditioned medium. The cells show high vacuolization, cell branching, colony formation and exhibit an additional mechanism of capillary lumen formation *in vitro*.

Knob-like formations are observed all over the culture vessel and precede the formation of tube-like structures. Knob-like formations and subsequent tubelike structures are found dependent on the initial cell inoculum density

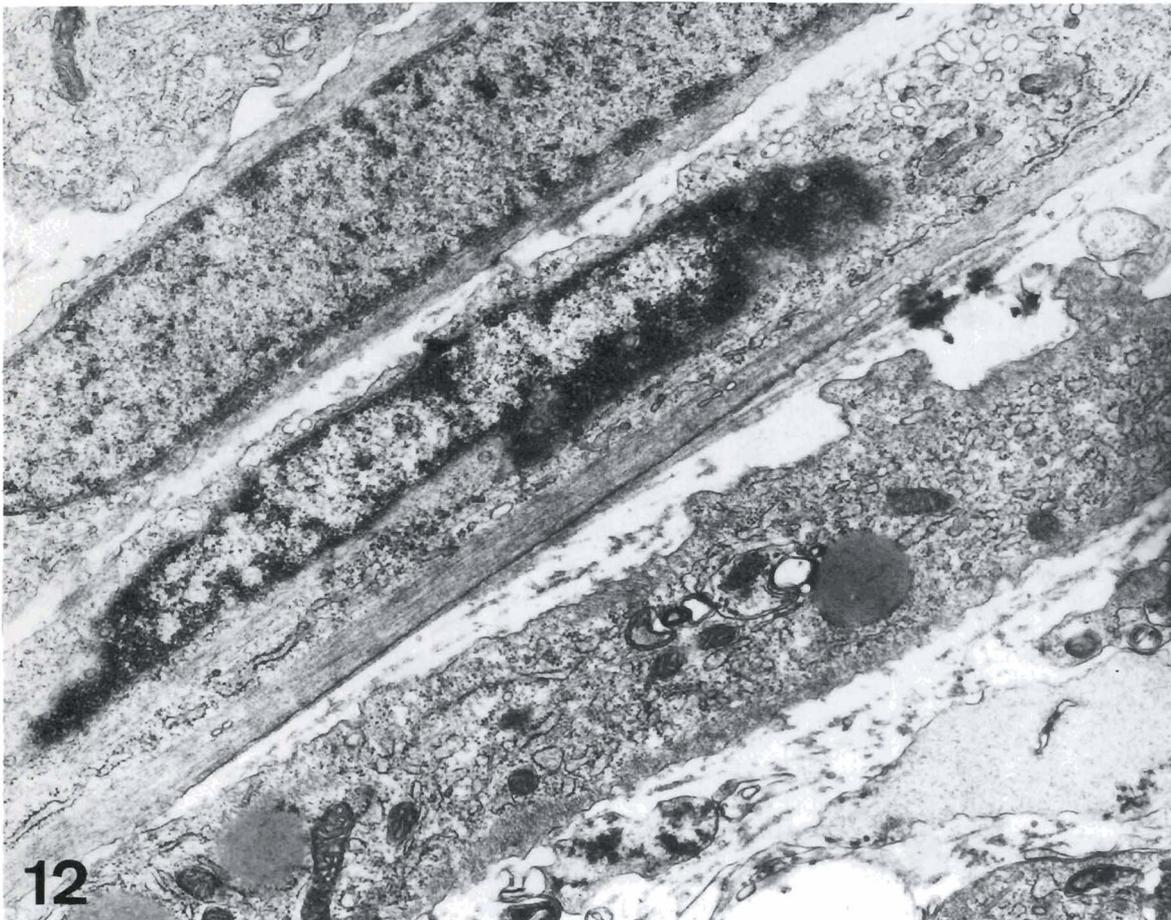


Fig. 12. Spindle shaped endothelial cells with abundant cytofilaments and pinocytotic vesicles. Uranyl acetate and lead citrate. $\times 17,600$

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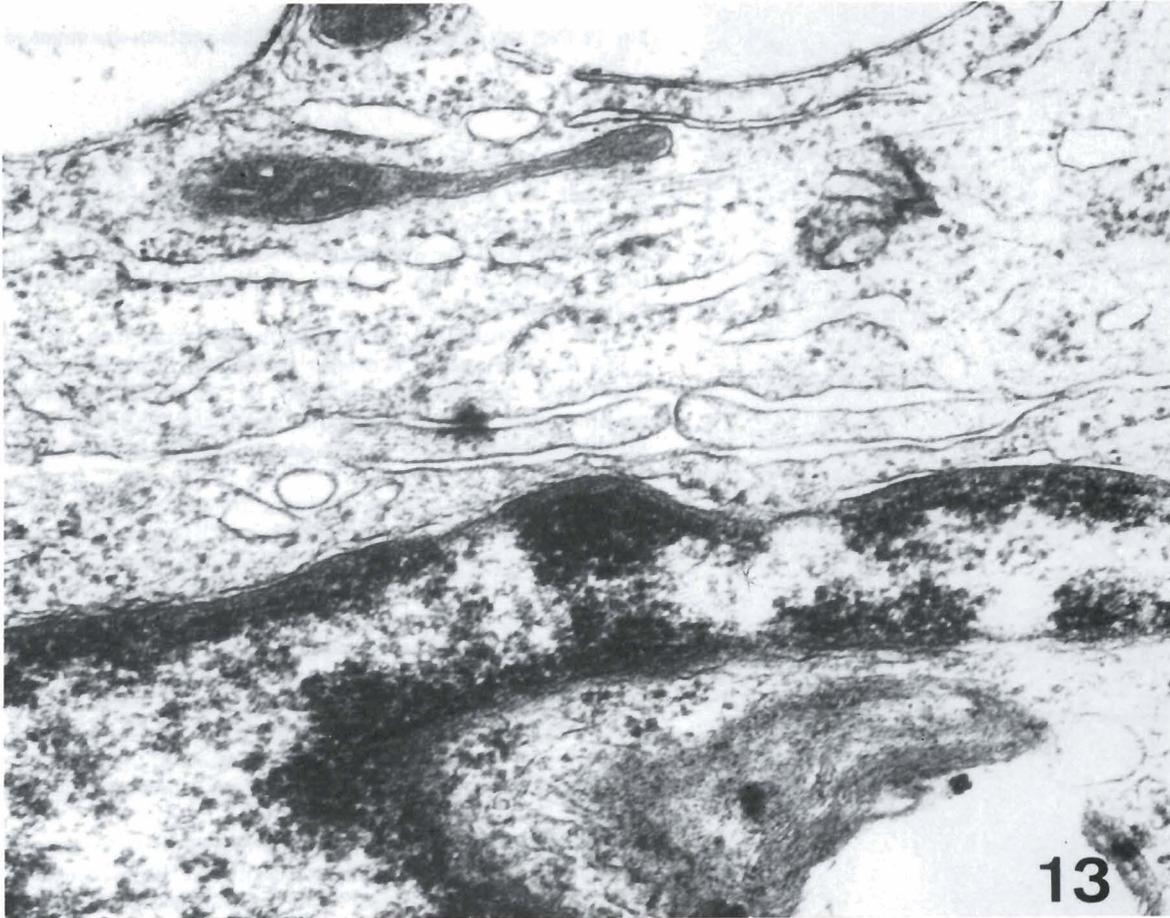


Fig. 13.
The cells
are linked with
desmosomes.
UA/LC. $\times 61,600$



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Fig. 14. Abundant lipid vacuoles and phagosomes are present in cytoplasm. UA/LC. $\times 8,800$

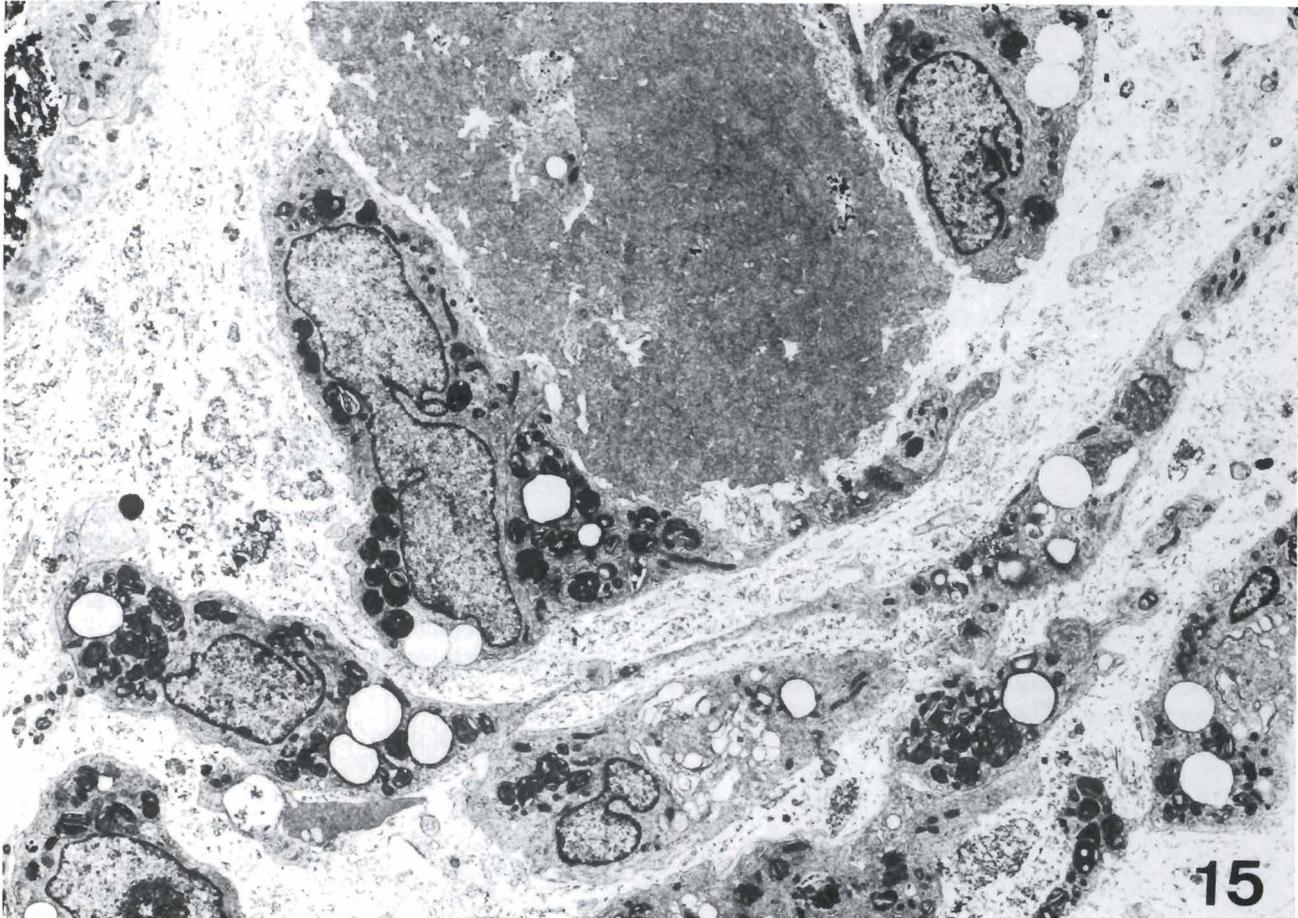


Fig. 15. Aggregate of extracellular fibrillary material surrounded by endothelial cells. UA/LC \times 3,960

and cellular extrudates. The higher the cell inoculum density the sooner the knob and tube-like structures are formed. The biochemical nature of the knob-like formation is found to be acidic and basic glycosaminoglycans arranged in order. GSG may be involved in capillary lumen formation in the following mechanism: endothelial cells produce basic and acid GSG intracellularly, which by cellular degeneration appear as floating debris in the culture medium. The floating GSG then, somehow, aggregate and form knobs, where the acidic ones are organized in the centre of the knob forming long rows with the basic GSG on top of them.

The cells show chemotaxis to the GSG knob, move onto it, line and extend forming lumen.

The observations that only a small segment of the capillary lumen is filled with GSG while the rest of the lumen continues empty, as well as the delayed knob-like formation with daily medium changes, strongly support that the GSG - knob-like structures take part in capillary lumen formation.

Capillaries take a long time to appear in culture because their lumen formation is dependent upon the knob-like structure formation which is built by endothelial cell extrudates. Histologically, *in vitro*

capillaries and lumen resemble the *in vivo* tissue capillaries containing plasma without the blood cells, information which may help to identify GSG-knobs and newly formed tissue capillaries *in vivo*.

Glycosaminoglycans produced by bladder cancer cell cultures have been reported to form tumor nodules and organize into membrane extensions (Logothetou-Rella et al., 1988a,b). It has also been reported that tumor-conditioned medium enhances endothelial cell formation of capillaries (Folkman and Haudenschild, 1980). These observations indicate that cancer cells may supply GSG or GSG precursors to the endothelial cells for knob-like formation in such a way that GSG may play the role of direct angiogenic factor.

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