

LYMPHATICS AND BLOOD VESSELS, LYMPHANGIOGENESIS AND HEMANGIOGENESIS: FROM CELL BIOLOGY TO CLINICAL MEDICINE

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ABSTRACT

The past 15 years have witnessed an explosion of knowledge about blood vascular endothelium due in large part to in vitro growth of endothelial cells from both large blood vessels and capillaries. In contrast, little comparable information has accumulated on endothelium of lymphatics, which lie in intimate contact with parenchymal cells and drain excess fluid, macromolecules, particles, and immunocompetent cells in a continuous recirculation between tissues and bloodstream. While structural and functional differences between the two vascular systems have been described in vivo, in tissue sections, and in isolated preparations, similarities are notable in ultrastructure, biochemistry, physiology, and pharmacologic responsiveness, and these may predominate under pathologic conditions. In 1984, three separate groups described in vitro culture of lymphatic endothelial cells from collecting ducts and cavernous lymphangiomas. Lymphatic, like blood vascular, endothelium grows in confluent monolayers, "sprouts", synthesizes Factor VIII-associated antigen and fibronectin, and ultrastructurally shows Weibel-Palade bodies; overlapping intercellular junctions and anchoring filaments typical of lymphatic endothelium are also found. Genetic, congenital, and acquired disorders such as strangulating fetal nuchal cystic hygromas (Down and Turner syndromes), vascular tumors and dysmorphogenesis (Maffucci

and Klippel-Trenaunay syndromes), Kaposi's sarcoma, lymphogenous and hematogenous spread of cancer, and parasitic infestations such as filariasis, share overlapping abnormalities in formation, growth, and/or neoplasia of lymphatics and blood vessels. In these and similar clinical disorders, confusion often exists as to the nature of the cell or tissue of origin, and insight into the role and control of hemangiogenesis and lymphangiogenesis is still in its infancy. Nonetheless, with the ever widening array of investigative techniques, it is not only timely but imperative to explore the endothelial biology underlying these inborn and acquired disorders.

Blood and lymphatic vasculatures are closely intertwined in embryonic development and respond to many similar stimuli in the microenvironment (e.g. ischemia, inflammation, and neoplasia). The two circulations work together in an integrated fashion in the uptake and transport of interstitial liquid and macromolecules such as extravasated plasma proteins and ingested lipids, which recirculate between lymph, blood, and tissue. Distinct migration streams of immunocompetent cells interchange at various points in the "blood-lymph loop." Anatomic connections exist or open up between the two as lymphatic-venous communications, which function normally (viz. thoracic duct-jugular venous junction) or become operational under physiologic and pathologic conditions (e.g. carcinomatous

venous or lymphatic obstruction or in portal hypertension from alcoholic cirrhosis). While lymphatics closely resemble blood vessels on tissue section, they are more thin-walled attenuated structures containing bloodless fluid, and they ultrastructurally exhibit overlapping intercellular junctional complexes, specialized anchoring filaments, and discontinuous or absent basal lamina (1). Permeability, surface charge distribution, vesicular macromolecular movement, lipid absorption and transport, intrinsic contractility, and vasoresponsiveness of the two vasculatures are distinct in some respects, vary from organ to organ, and also may overlap.

The vascular endothelium is the crucial interface between circulating blood or lymph and the tissues. Two decades ago only surmised by Lord Florey to be more than an inert passive membrane or in "nucleated cellophane", endothelium is now recognized as the biologically active mentor of the microcirculation and of tissue homeostasis--originating, receiving, translating, transducing, and transmitting physical and chemical messages to and from different parts of the body. The ability to culture large and pure endothelial cell populations not only from major blood vessels but since 1979, also from human capillaries has produced an explosion of knowledge (1-3). Despite differences among species and organs, blood vascular endothelium (BVE) exhibits remarkably consistent morphology and function *in vitro* mimicking its structural, synthetic, and transport properties *in vivo* and in isolated vascular preparations. In culture, endothelial cells grow as confluent monolayers with characteristic cobblestone appearance, which under appropriate conditions sprout and form tubules, that is, display "angiogenesis *in vitro*." Distinctive ultrastructural features mirroring those found in tissue section include intricate intercellular junctions, micropinocytotic vesicles, intermediate filaments, and Weibel-Palade bodies, which are thought to manufacture or store Factor VIII-associated antigen (Factor VIII_{AA}). On immunohistochemi-

cal examination, endothelial cells contain Factor VIII-associated antigen, angiotensin-converting enzyme, and extracellular matrix components such as fibronectin, all of which in addition to prostacyclin and many other vasoactive metabolites can be measured quantitatively after release into the supernatant overlying the monolayer.

Considerable attention has been directed to a search for angiogenic factors controlling blood vessel formation and thereby tumor and organ growth. The genetic code for one such substance, angiogenin, has recently been deciphered. Blood vascular endothelium interacts in a "symbiotic" relationship with immunocompetent cells, directing lymphocyte cell traffic and "homing" and also producing colony-stimulating activity differentiating hemopoietic stem cells into granulocytes and monocytes (4). Immunocompetent cells as well as fibroblasts and adipocytes in turn secrete angiogenic factors and share cell surface receptors with endothelium. Thus, in large part because blood vascular endothelium can now be isolated *in vitro*, its role as a structural barrier as well as active facilitator of small solute and macromolecular transport into and out of tissues, as a director of cellular migration, as a stabilizer of the coagulation cascade, and as a biosynthetic factory is now being unraveled. Blood vascular endothelial damage and/or repair have been implicated in processes as diverse as atherosclerosis, hypertension, inflammation, wound healing, ischemia, diabetes mellitus, and transplant rejection.

Yet, in part because of a lack of analogous *in vitro* models, only rudimentary information is available about the highly permeable vascular interface on the "dark side" of the blood capillary barrier, deep in the tissues, separating circulating "lymph" from extracellular matrix and liquid and parenchymal cells in lymphoid and non-lymphoid tissues. Within this oft forgotten sluggish lymphatic-tissue fluid circulation pass surplus liquid, macromolecules, particles, and migrating cells from the interstitium on their way

through regional and central lymph nodes before returning to the blood circulation (Fig. 1). In 1984 for the first time,

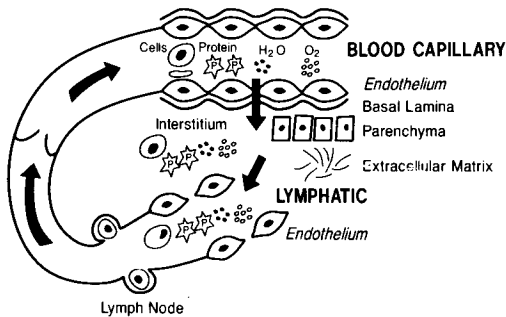
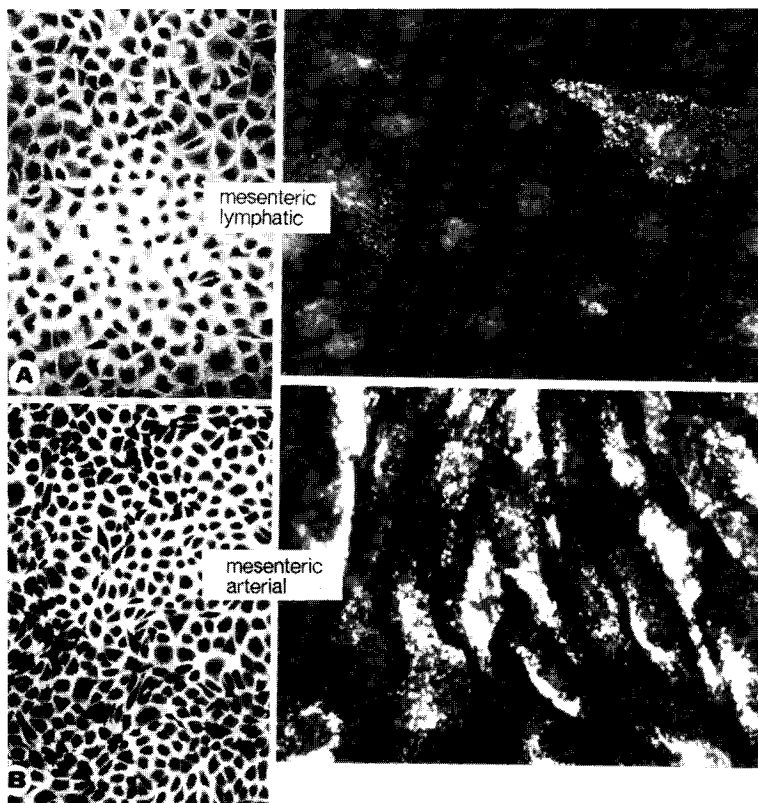


Fig. 1. Blood-lymph loop. There is a continuous circulation and recirculation of cells, particles, macromolecules, small solutes, and gases between blood, tissues, and lymph. Blood vascular and lymphatic endothelium represent crucial interfaces in this circulation. Lymph exiting parenchymal organs filters through lymph nodes on its way to return thru lymphatic-venous communications to the bloodstream.

small relatively pure populations of lymphatic vascular endothelium were isolated by our group from explants of a massive recurrent lymphangioma (5), by Johnston from normal bovine lymphatic ducts (6) and by Gnepp from canine and human cadaveric thoracic ducts (Figs. 2 and 3) (7). In culture, lymphatic vascular endothelium (LVE) from large ducts and microvasculature bears a strong resemblance to BVE, forming confluent "cobblestone" monolayers, sprouting (Fig. 4), and staining positively for endothelial markers, Factor VIII_A and Ulex lectin. Thus, CH3, our second nearly pure lymphatic endothelial cell line from a recurrent chylous retroperitoneal lymphangioma resembles blood vascular endothelium in staining positively for Factor VIII_A, F-actin, and fibronectin (Fig. 5), and exhibiting numerous Weibel-Palade bodies (Fig. 6L) (8). Nonetheless, LVE appears to possess some distinctive features: overlapping intercellular junctions and abundant intermediate anchoring-type filaments typical of lymphatic endothelium (Fig. 6R) (8). Although questions may be raised about stromal blood vessels giving rise to some of the endothelial cells found in this

lymphangioma cell line, the same question can be but has not been raised about designated blood vascular endothelial cell cultures from microvasculature in such standard sources as omentum and foreskin, tissues rich in lymphatics as well as blood vessels. At this point, there is every reason to believe that lymphatic like blood vascular endothelium is also a vast endocrine organ maintaining a lymph-fluid compatible surface and a changeable selective interface between the lumen and interstitium that is also the target for numerous perturbations affecting not only its intrinsic structure and function but also that of surrounding tissues.

The close interactions between the lymphatic and blood vasculature and lymphangiogenesis and hemangiogenesis on a cellular and organ level are further illustrated in the clinical manifestations of disease. Congenital lymphologic syndromes of genetic or intrauterine origin involving abnormal growth of lymphatics often include widespread blood vascular abnormalities as in Maffucci's and Klippel-Trenaunay syndromes (Figs. 7 and 8). These soft tissue hemangiomas and lymphangiomas are commonly accompanied by lymphedema, venous aplasia or hypoplasia as well as arteriovenous anomalies and striking soft tissue overgrowth such as limb hypertrophy and macrodactyly, likely closely linked to the circulatory disturbances (Figs. 7 and 8). On rare occasions malignant vascular transformation may take place. Blood vascular and lymphatic anomalies also coexist in Turner's XY gonadal dysgenesis syndrome where webbed neck from regressed fetal cystic lymphangiomas, extremity lymphedema associated with lymphatic hypoplasia and aplasia, and coarctation of the aorta are typical manifestations; variants of the syndrome also exhibit severe intracardiac anomalies. Down syndrome (trisomy-21) similarly may present *in utero* with strangulating cystic hygromas, cardiovascular anomalies and lymphedema or survive into adulthood with other variations of these vascular abnormalities. On the other hand, in acquired condi-



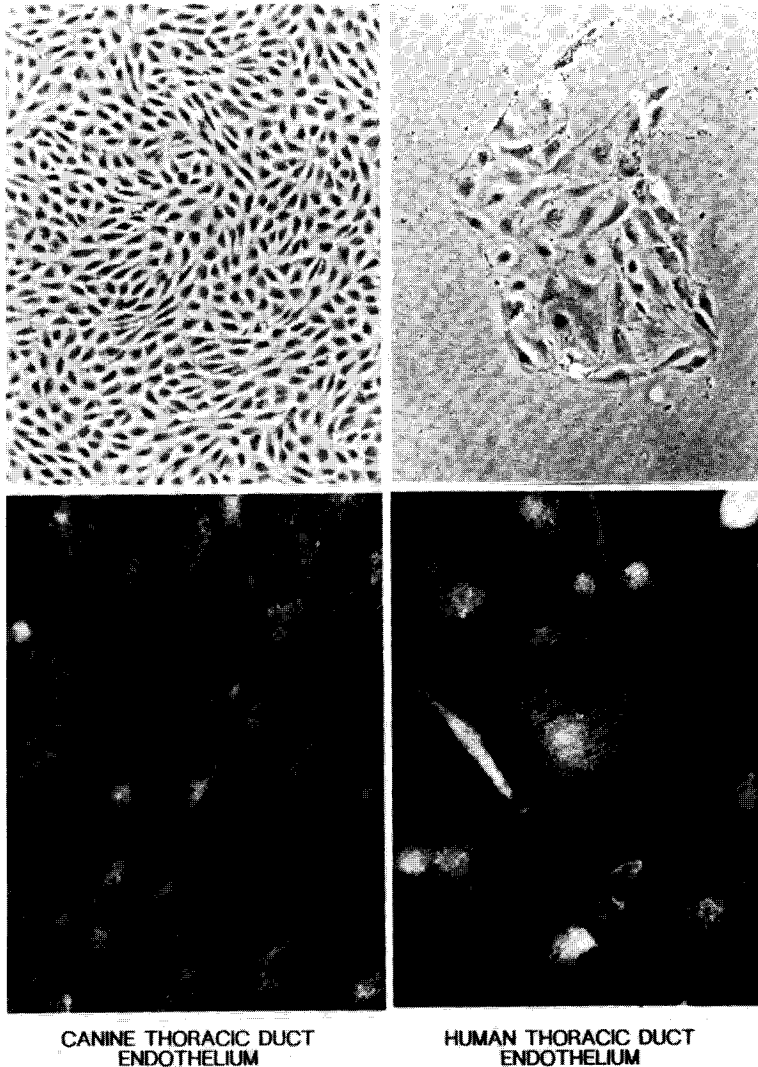
BOVINE MESENTERIC VASCULAR ENDOTHELIUM

(M.G. Johnston and M.A. Walker, In Vitro, 1984)

Fig. 2. Comparison of bovine lymphatic endothelial with bovine superior mesenteric arterial endothelial in tissue culture. The cells in A and B were stained with a Hemacolour stain kit (Harleco) as follows. The cells were washed twice with PBS and fixed for 5 min in methanol. The cells received an eosin solution (30s) followed by a thiazine solution (30s) and were then washed with water. A, Bovine lymphatic endothelial cells, Passage 9; (x141). B, bovine mesenteric artery endothelial cells, Passage 4. (x141); (6; modified by permission). C and D show assay for Factor VIII-related antigen. C, bovine lymphatic endothelium. D, Bovine mesenteric artery endothelium (antibody to human Factor VIII-related antigen diluted 1/10) (6; modified by permission).

tions such as classical or AIDS-associated epidemic Kaposi's sarcoma, abnormal lymphatic-venous communications may comprise or contribute to the multicentric tumor some of its peculiar morphologic and immunohistochemical properties as well as the associated lymphedema and hemorrhage. On rare occasions, malignant vascular tumors appear as Stewart-Treves syndrome after many years of lymphostasis associated with intense he-

mangiogenesis and lymphangiogenesis, and lymphangiomatoid changes superimposed on exuberant profuse scarring and fat deposition. The latter is well exemplified in filarial infestation, which leads to elephantiasis where thickening and piling up of the lymphatic as well as blood vascular endothelium, intraluminal blood or lymph clots, and exuberant deposition of underlying scar tissue characterize the pathologic process and the



(D. Gnepp and W. Chandler, In Vitro, 1985)

Fig. 3. L, upper, canine thoracic duct culture demonstrating a sheet of uniform contact inhibited non-overlapping endothelial cells with typical cobblestone morphology. (x131). L, lower, indirect immunofluorescence of canine thoracic duct endothelial cells demonstrating marked positivity of Factor VIII antigen (note granular cytoplasmic staining. (x189). R, upper, human thoracic duct culture demonstrating one nest of typical endothelial cells, Day 9. (x129). R, lower, indirect immunofluorescence of human thoracic duct endothelial cells demonstrating granular cytoplasmic and perinuclear fluorescence. (x189). (7; used by permission).

host response to the worm and its products. These interrelationships between lymphangiogenesis and lymphologic syndromes have been summarized by us recently (9), and an analogous intercon-

nected scheme can be postulated for hemangiogenesis and blood vascular syndromes.

Endothelial biologists working in tissue culture have opened up the pheno-

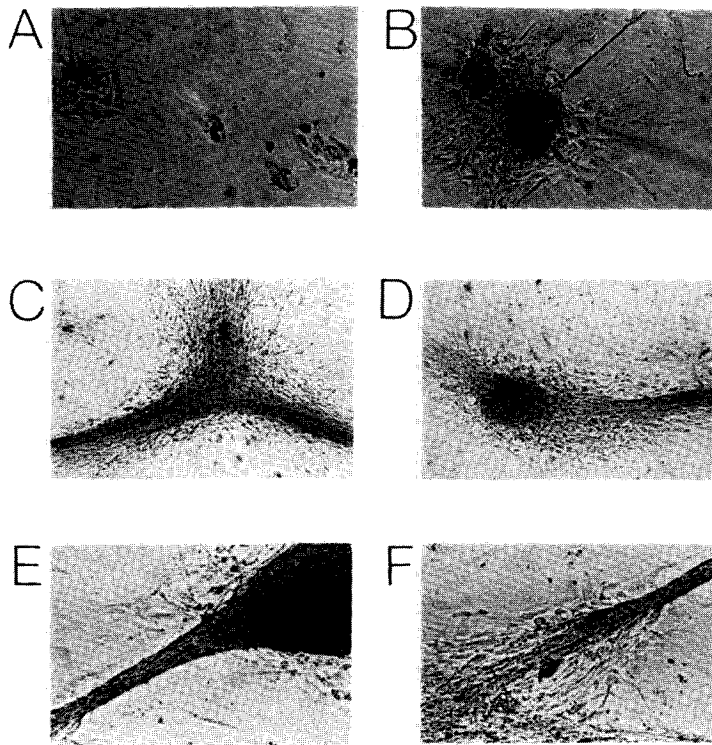


Fig. 4. Photomicrographs (inverted light) depicting in vitro evolution of lymphatic endothelial cell growth derived from a resected lymphangioma of the knee. An "early" phase displays a loose cluster of endothelium with occasional "sprouting" or branching (A: $\times 100$). With time, this sprouting pattern becomes more prominent (B; $\times 100$) and eventually evolves into a sheet-like cellular aggregate (C,D: $\times 40$) with intense "lymphatic-like" tumorous branching (E,F: $\times 100$).

menon of tumor angiogenesis to intensive inquiry. In 1972, Folkman (10) first proposed the concept that all tumors are angiogenesis-dependent and once tumor take has occurred, enlargement of the tumor cell population is preceded by growth of new blood capillaries converging on the tumor. Inhibition of angiogenesis, he proposed, might be a therapeutic approach to solid tumors. Interesting questions have arisen about the role of endothelial mitogens in normal tissues and natural mechanisms that restrain and inhibit formation of capillary and thereby tissue and organ growth. Lymphatic vessels have been scarcely mentioned in the context of "angiogenesis", and some workers have even suggested that tumors do not contain lymphatics. Nonetheless,

the parallel development and common response of the two vascular systems to varied physiologic and pathologic stimuli suggest, however, that hemangiogenesis and lymphangiogenesis go hand in hand and that the mysterious growth factors stimulating both vasculatures are self-generated as well as arise in or are delivered through the neighboring tissue matrix; that is, the stimuli are autocrine, paracrine, and endocrine.

Despite the mounting interest in tumor angiogenesis, investigation of a related process--what we have termed "angiotumorogenesis," i.e., the growth and development of blood and lymphatic vascular tumors--has been extremely limited despite their frequency as cosmetic imperfections or as disfiguring or even

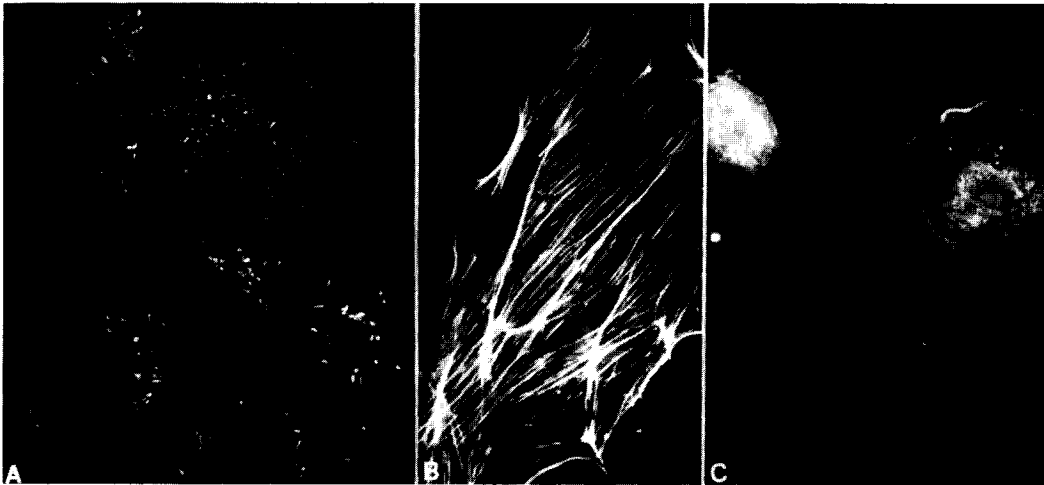


Fig. 5. Indirect fluorescent-antibody labeling using rabbit antihuman IgG shows the characteristic granular pattern of Factor VIII-related antigen (A) ($\times 168$), F-actin positive microfilament bundles forming a well-organized cytoskeleton stained with NBD-phalloidin ($\times 591$), cell surface-associated fibronectin using rabbit antihuman fibronectin IgG (C) ($\times 168$), which is also deposited extracellularly (8; used by permission).

life-threatening tumors of childhood. Genetic, congenital and environmental influences on endothelial growth, such as by hormones and drugs (e.g., estrogens, oral contraceptives), industrial carcinogens (e.g., vinyl chloride) and viral agents (e.g., human immunodeficiency virus and cytomegalovirus) are poorly understood but these agents are known stimulants of endothelial proliferation, DNA transformation, and neoplasia. Controversy continues about the nature of some vascular tumors, i.e., whether they are embryonic rests (hamartomas), true neoplasms or mere expressions of exuberant angiogenesis. As in Kaposi's sarcoma, different areas of the same lesion may appear strikingly heterogenous ranging from normal vessels to highly anaplastic or wildly aberrant structures indistinguishable as lymphatics or blood vessels. Moreover, it is unclear whether multiple tumors in separated or remote sites are multicentric in origin or metastatic. Occasionally, benign-appearing endothelial tumors may exhibit local invasion, recur, and even spread (e.g., "benign metastasizing lymphangioma"). On the other hand, benign and even malignant vascular tumors sometimes spontaneously regress. Unfortunately, immunohistochemical

studies to detect the presence of intracytoplasmic or cell surface endothelial markers (e.g., Factor VIII A or basement membrane components) have produced more confusion than clarification because of heterogeneity of staining, postulated but disputed differences between the blood and lymphatic vasculature, inconsistent staining techniques, neoplastic or non-neoplastic transformation to more primitive or aberrant cell types or pluripotential nature of the cells, and presence of mixed cell types including mesenchymal and lymphoid elements. Thus, in part because of the paucity of animal models and the lack of *in vitro* systems to study pure populations of tumor cells, classification of endothelial tumors of lymphatic or blood vascular origin remains largely based on morphologic criteria and clinical behavior. Although tumor modulation by hormones, immunoregulatory substances, and growth factors seems almost within our grasp, detection and treatment of these neoplasms has progressed little over the past several decades beyond refinement or extension of surgical resection.

Understanding the structural-functional interrelationships between the blood and lymphatic vasculatures *in vivo*

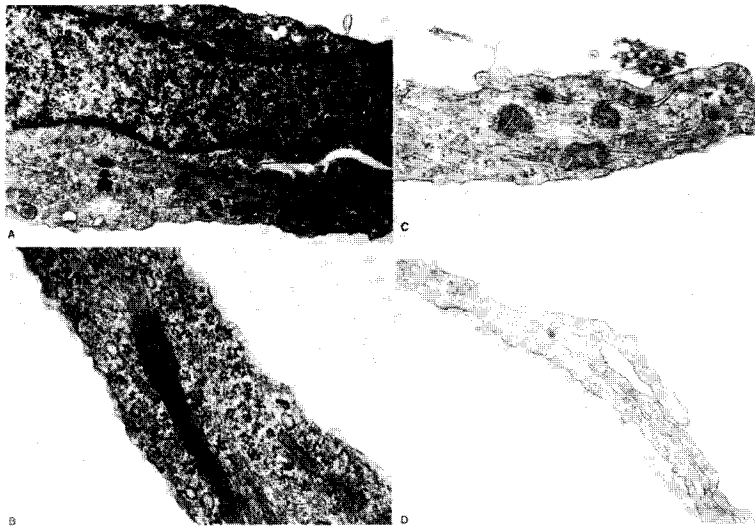
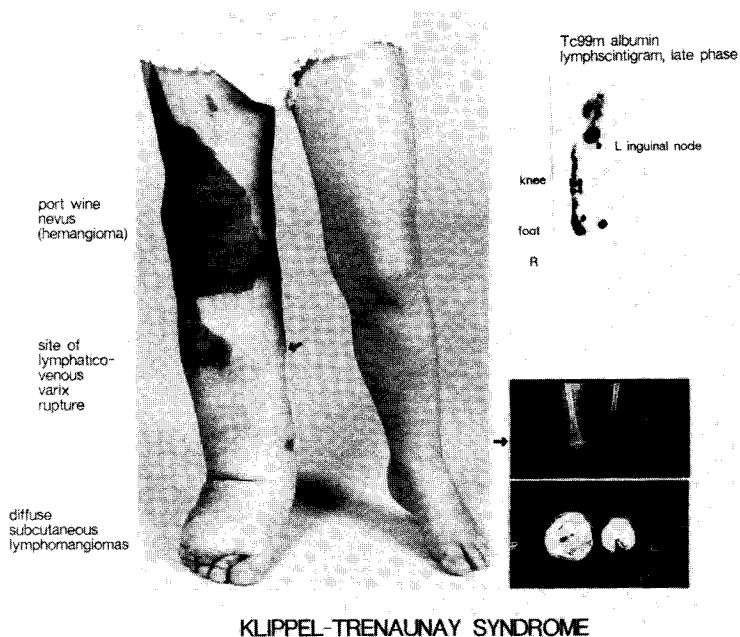


Fig. 6. Transmission electron microscopy (A-D) of cultured tumor cells reveals relatively smooth cell surfaces with few microvillous projections, numerous vesicles, and cytoplasm rich with Golgi and rough endoplasmic reticulum. Abundant Weibel-Palade bodies (A, arrow; B, higher magnification) are seen surrounded by bundles of intermediate filaments characteristic of lymphatic endothelium. Higher power detail of intermediate filaments can be best appreciated in C. Typical macula adherens, overlapping intercellular junctions are also shown (C,D). (A= $\times 8550$; B= $\times 41,800$; C= $\times 48,450$; D= $\times 26,650$) (8; used by permission).



KLIPPEL-TRENAUNAY SYNDROME

Fig. 7. 3-year-old girl with Klippel-Trenaunay syndrome involving the right leg. In addition to the characteristic port wine nevus and agenesis of the deep venous system, small pedal arteriovenous fistulae lymphoscintigraphy (^{99m}Tc albumin) shows aberrant lateral "pick-up" with a large, dilated truncal "lake" reaching the lateral groin only after many hours. The left leg is unremarkable. Computed tomography with contrast enhancement suggests diffuse lymphangiomatous change with little or no edema fluid in the right leg.

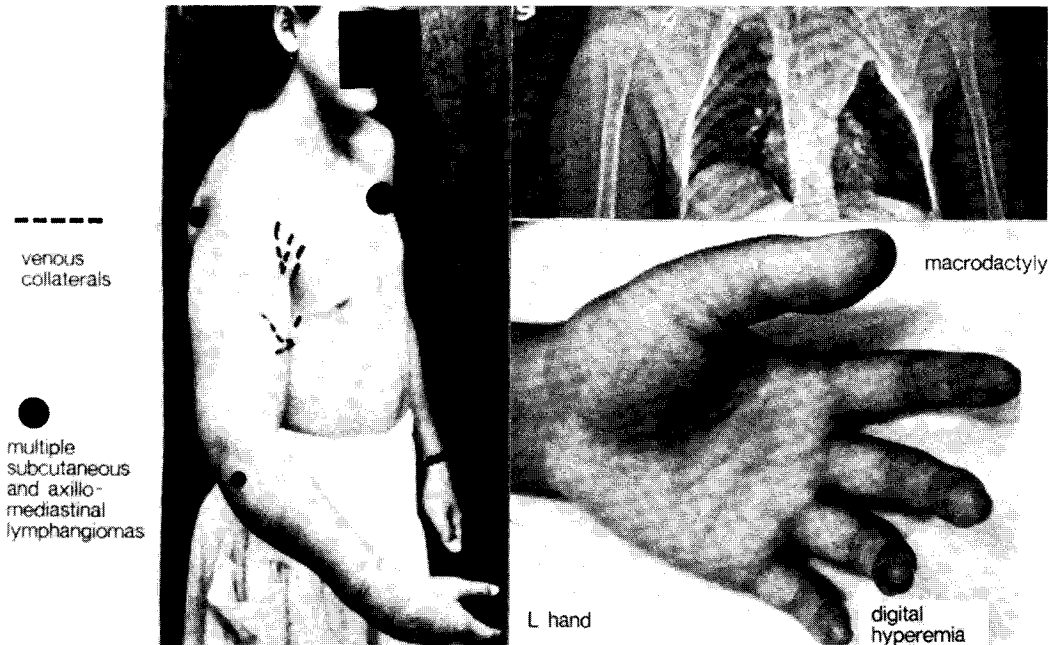


Fig. 8. 13-year-old girl with multiple lymphangiomas including of the back, mediastinum and left axilla. Also present are right arm hypertrophy, macrodactyly, digital hyperemia.

and *in vitro* is key to unraveling the control mechanisms and detailed steps of tissue growth and repair from embryonic life to senescence. Greater availability and variety of *in vitro* models of normal and neoplastic endothelium should provide clues to detection of abnormal lymphangiogenesis and hemangiogenesis (e.g., enhanced or inhibited release of distinctive endothelial products from neoplastic endothelium) as well as more effective treatment (such as by angiostatic and angioinhibitory agents including naturally occurring hormones for chemo- and radioresistant vascular tumors or excessive scar formation. Isolated in pure culture, released from central control and telescoped in time, such *in vitro* models combined with the tools of molecular biology can also be used to explore endothelial cell interactions with related and distant cell types thereby potentially shedding light on disorders as varied as transplant rejection, inflammation, hypoplastic lymphedema, scleroderma, diabetes mellitus, lymphogenous vs. hematogenous spread of cancer, and limb ischemia.

What these test tube models teach us about fundamental biology must then be returned to the body, validated, and applied to this bewildering array of human disorders characterized by defective, exuberant, or uncontrolled hemangiogenesis and/or lymphangiogenesis and a wide variety of interrelated and dependent phenomena.

ACKNOWLEDGEMENTS

Supported in part by Arizona Disease Control Research Commission Contracts #8277-00000-1-1-AT-6625, ZB-7492, and ZM-8036.

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