REVIEW ARTICLE

# THE VASCULAR ENDOTHELIUM—PATHOBIOLOGIC SIGNIFICANCE

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*A Review*

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THE WORD "ENDOTHELIUM" was introduced in 1865 by the German anatomist His,[1] combining the Greek words "endon," meaning within, and "thele," meaning nipple. Its literal meaning is thus: "within the nipple." It was proposed as a complementary anatomic term to "epithelium" ("over the nipple") first used by Ruysch around 1700 to describe the cell layer covering the tongue and other areas with nipple-like papillae.[2] Henle expanded the domain of the latter term to include all specialized cell surface layers, including those lining the respiratory, gastrointestinal, and urinary tracts.

As suggested in Altschul's pioneering monograph,[3] "endothelium" has been a controversial term, but currently there is agreement to use it to describe the cellular lining of the entire circulatory system, including lymphatic channels.[4] In this context the endocardium is both anatomically and embryologically a part of the endothelial lining.

This unique and strategic location at the interphase of the circulating blood or lymph with the vascular wall or surrounding tissues at all levels of the circulatory system imposes certain functional demands on the endothelium and calls for highly specialized properties. In recent years, it has become evident that the endothelial cell is metabolically highly active and plays an important role in the synthesis of several biologically important substances. Abnormalities in the structure and function of endothelial cells are, therefore, likely to contribute significantly to a number of disease processes, not only to overt vascular diseases such as atherosclerosis, thrombosis, disseminated intravascular coagulation (DIC), and defective hemostasis but also to the general fields of inflammation, distribution of immune complexes, and metastatic spread of tumors, each of which will be discussed following evaluation of the structural and functional characteristics of endothelial cells. Due to the extent of the subject and the rapidly increasing number of contributions being made to our under-

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standing of every aspect of endothelial function, this review, rather than being comprehensive, will attempt to highlight a constellation of diseases in which physiopathologic alterations of this tissue may play key roles.

## Histogenesis

During embryogenesis, endothelium constitutes the first layer organized at the wall of all segments of the circulatory system.[5-7] The cells originate from mesodermal cell clusters or cords, called "blood islands," that first appear in the yolk sac at the end of the third week of fetal development but soon are found throughout the fetal mesenchyme. Subsequently, fluid-filled spaces appear in the blood islands, separating peripheral cells from central ones. As these spaces expand, peripheral cells become flattened while remaining adherent to each other and eventually become the endothelial lining of the newly formed vessels. In contrast, cells entrapped in such spaces become separated from each other and develop into primitive blood cells.[6]

The newly developed endothelial tubes then become linked with one another and form a net of communicating vessels. The pattern of this network seems to be largely independent of hemodynamic factors since the endothelial lining can undergo considerable development even when circulation is prevented by experimental removal of the heart.[5] Wilt,[7] however, found that endoderm was necessary for the early differentiation of these blood islands in the chick embryo. If the endoderm was separated from the mesoderm of the area vasculosa of an early embryo, cellular clusters forming the blood islands underwent erythropoiesis but no endothelium was formed.

The relationship between extraembryonic and intraembryonic endothelia remains controversial.[5] According to the *angioblastic theory*, the extraembryonic endothelium extends into the body of the fetus. In contrast, the *theory of local origin*, currently favored, maintains that intraembryonic endothelium is formed from mesodermal cells *in situ* and secondarily becomes linked with the extraembryonic vascular network.

At these early stages of vascular differentiation there is no clear distinction between arteries, veins, or capillaries. Later, some of the mesenchymal cells surrounding the endothelial tubes differentiate into pericytes and further evolve into the medial and adventitial cells found in adult arteries and veins or into the myocardial and epicardial layers of the heart.

Among embryologists the origin of the lymphatic endothelium has been a matter of dispute for a long time. According to Kampmeier,[8] early in this century the subject completely dominated annual meetings of American anatomists, and the controversy still has not been resolved.[5] One school of

thought postulates that lymphatics develop as sac-like outgrowths from the embryonic venous system. The opposing viewpoint maintains that the lymphatic system arises directly from mesenchyme. Perivenous spaces coalesce to form larger ones and subsequently organize as distinct vessels which secondarily anastomose with the venous system. The undifferentiated mesenchyme lining the original lymphatic spaces flattens out and forms the lymphatic endothelium. Proponents of both theories agree that the lymphatic system develops concurrently with and in close relationship with the venous system.[5] In the adults, variations in the structure and complexity of the lymphatic drainage are found in different organs, reflecting important functional differences. Their description is beyond the scope of this review, and the reader may consult Kampmeier's excellent comparative monograph.[9]

## Structure

The vascular endothelium consists of a single layer of flattened, fairly uniform, polygonal, and elongated cells that are approximately 25 to 50 $\mu$ long and 10 to 15 $\mu$ wide.[10] Their long axis is oriented in the direction of the bloodflow. In the neighborhood of the nuclei, cells measure approximately 3 $\mu$ in thickness and thin at the periphery to a thickness from 1 $\mu$ (aorta) to less than 0.1 $\mu$ (capillaries and veins).[10,11] The luminal surface of the unstimulated aorta visualized by scanning electron microscopy is generally described as fairly smooth with longitudinal folds.[10,12-14] The endothelial lining of the pulmonary artery, on the other hand, is covered by a meshwork of irregular projections ("microvilli") (250 to 350 nm in diameter and 300 to 3000 nm long) which vastly increase the surface area of the cells.[15] Like all mammalian cells, the endothelium is "coated" with a carbohydrate-rich cell coat or glycocalyx. This coat was identified as the "endocapillary layer" by Luft in 1966,[16] utilizing a term coined in the 1940s by Chambers and Zweifach [17,18] to describe material similar to the "intercellular cement" that coated the intraluminal surface of the endothelium. Until Luft applied the ruthenium red technique to visualize this thin layer, electron microscopists had failed to demonstrate its existence because routinely it is removed during tissue processing. This substance was found to have histochemical features similar to those of other cell coats previously described.[19] Several other methods that are semispecific for carbohydrates have been used to demonstrate it.[20] Carbohydrates in the glycocalyx include the polysaccharide-rich side chains of the plasma membrane glycoproteins and glycolipids, as well as free polysaccharides and glycosaminoglycans (GAGs).[19-21] Its inner margin is indistinguishable from the outer leaflet of the plasmalemma and is generally considered an

intrinsic part of it.[19] The luminal boundary is indeterminate since the density becomes diluted toward the lumen. The thickness of the coat has the order of magnitude of several hundred Ångström units. A similar layer exists on the extraluminal side of the endothelial cell that meets the endocapillary layer at the lateral border of the cell, beyond cell junctions, thus enclosing the entire cell. The functional role of the endothelial glycocalyx will be discussed in a subsequent section.

In addition to a prominent, oval nucleus, the endothelial cell is equipped with all the usual cytoplasmic organelles [4,10,11,22-24]: smooth and rough endoplasmic reticulum, attached and free ribosomes, a moderate number of mitochondria,[25] Golgi zone, centrosphere with two centrioles, lysosomes, multivesicular bodies, glycogen granules, and thin and thick filaments containing actin and myosin.

In addition to a complement of common intracellular components, endothelial cells contain an unique structure, the Weibel–Palade body.[26] It is a rod-shaped cytoplasmic organelle, measuring approximately 0.1 $\mu$ in diameter and up to 3 $\mu$ in length, composed of a number of small tubules, approximately 150 Å thick, arranged in the long axis of the rod and surrounded by an outer membrane. The organelle was first described in detail in 1964 by Weibel and Palade on the basis of ultrastructural studies of numerous organs from humans and rat and of skin vessels of the *Ambystoma punctatum* (the spotted salamander). In 1958, Hibbs and coworkers [27] described "dense, rod-shaped granules, varying from 0.1 to 0.3 $\mu$ in diameter and 0.3 to 0.6 in length" in capillaries and arterioles of human dermis and subcutis, but ultrastructural detail was lacking. Subsequently, Weibel–Palade bodies have been identified in the vascular endothelium of numerous other species, including monkey, pig, dog, chicken, frog, and shark, as well as in cultured endothelial cells.[24] Steinsiepe and Weibel [28] carried out a systematic evaluation of the distribution of these organelles in the frog and found that their cytoplasmic volume density seemed to be more dependent on the distance from the heart than on vessel size, the thoracic aorta showing proportionally the highest content (8% of cytoplasmic volume) while the capillaries had the lowest (0.3%).

It seems to be a fair assumption that such endothelial specific organelles contribute to some function that is unique for this cell type. It has been suggested that the granule plays a role in blood coagulation,[29] but there is no experimental evidence to support that notion. The function of the Weibel–Palade body remains a mystery.

A characteristic feature of endothelial cells at all levels of the circulatory system are plasmalemmal vesicles measuring 600 to 700 Å in overall

diameter.[4,10,11,30,31] Their relative number varies greatly between segments of the circulatory system. In a systematic study of the microcirculation of the mouse diaphragm, Simionescu et al [31] observed that the arteriolar endothelium had a much smaller population of plasmalemmal vesicles than did the capillaries. The vesicles either lie entirely within the endothelial cytoplasm or open onto the cell surface at the luminal or the tissue front. Bruns and Palade [31] estimated that the frequency of plasmalemmal vesicles at the endothelial surface of muscle capillaries was as high as 120/sq $\mu$ and occupied up to 18% of the cytoplasmic volume. Also in muscle capillaries Simionescu and co-workers [32,33] have identified fused vesicles that bridge the endothelial cytoplasm, forming patent transendothelial channels. These channels were not present in the arterioles of the microcirculatory segments studied and have not been identified outside the muscular capillaries of rats and mice. The functional role of plasmalemmal vesicles, single as well as fused, will be discussed further while reviewing the barrier function of vascular endothelium.

Although endothelial cells are morphologically relatively homogeneous, there are distinctive regional differences, some of which have been mentioned above. They include differences in size and thickness (aortic cells thicker than those of capillaries and veins [10]), differences in relative number of certain organelles (Weibel–Palade bodies in highest number close to the heart [28]), and plasmalemmal vesicles more frequent in capillaries than in other segments of the circulation.[32]

Main regional morphometric and functional differences are found, however, in the interendothelial junctions. There are fundamentally two categories of endothelium: continuous endothelium (found in arteries, veins, and muscular capillaries) and the fenestrated endothelium of various forms of visceral capillaries.[10,34] By the use of freeze-cleaved preparations, Simionescu et al [34–36] provided further evidence that each segment of the circulation has a distinct, characteristic arrangement of interendothelial junctions. The arterioles have the most elaborate junctional system composed of a continuous network of tight junctions surrounding large communicating (gap) junctions.[35] Arteries also have a composite system of tight junctions and communicating junctions, which is considerably less complex than that of arterioles.[36] Capillaries, by contrast, differ in having less complicated general organization and no communicating junctions.[35] The venules have the least organized endothelial junctions with low ridges and shallow grooves, mostly devoid of junctional particles.

The above studies confirmed previous observations made on sectioned specimens by transmission electron microscopy that the endothelium of larger arteries has a particularly complex junctional arrangement.

Schwartz and Benditt [37] studied the aortic intima of Winston Firth/Mai rats and identified four types of interendothelial junctions varying in complexity and frequency. The simplest and less common form was a single end-to-end junction. The next step in complexity, a simple overlap junction, also was infrequent. The most common junction was a "mortice" junction formed by the intrusion of a tongue from one cell into a groove in the adjacent cell. The most complexly folded junctions, forming the fourth category, also were common. Robertson and Khairallah [38] found the mortice type junction to be the most common in the thoracic aorta of the Sprague–Dawley rat, and Hüttner et al [39] demonstrated the presence of communicating (gap) junctions in addition to "tight" junctions in the endothelium of large arteries.

Ultrastructural studies of lymphatic capillaries have revealed a variable pattern of interendothelial junctions.[40–42] In many areas, adjacent cells overlap or interdigitate but completely lack junctional elements. In other areas, there are large gaps between neighboring cells up to several microns, but at intervals specialized junctional devices are present, mostly consisting of desmosomes but also of periodic tight junctions. Gap junctions have not been described in lymphatics. The loose adherence of opposing, overlapping cell membranes allows them to slide past each other and form patent intercellular canals. The junctional arrangement in lymphatic endothelia thus represents an unique category among endothelial intercellular junctions, particularly well fitted to serve the unique role of lymphatics: a one-way drainage system capable of handling both large molecules and particulate matter.[42]

### Endothelium *In Vitro*

Much of the early work on endothelial tissue culture was reviewed by Altschul,[3] and recently Gimbrone [43] has provided an excellent and comprehensive summary of later developments. Although attempts had been made to grow *in vitro* endothelial cells since the early 1920s, particularly following Lewis' pioneering work,[44] the field was hampered for a long time by several frustrating problems.[43] There were and still are inherent difficulties in handling endothelial cells in culture. They are extremely sensitive to any shortcomings in environmental conditions; their viability after the donor's death is limited; and, worst of all, they are difficult to grow in homogeneous populations and reliable cytologic methods to identify them were lacking until recently. In 1963, Maruyama [45] was able to obtain large numbers of vascular cells by trypsin digestion of umbilical veins. However, they only remained viable for 14 to 21 days and did not grow. The cells were found to be morphologically heterogeneous and

there was a nagging doubt as to their exact identity since these observations were made before the discovery of a specific endothelial marker (Weibel–Palade body).

By substituting buffered collagenase for trypsin, reducing intraluminal digestion of umbilical veins from 45 to 15 minutes, and paying strict attention to the environmental conditions for their cultures, Jaffe and co-workers [46] and Gimbrone and co-workers [47] were almost simultaneously able to obtain homogeneous cultures of cells containing characteristic Weibel–Palade bodies that remained viable and grew *in vitro* for several months. Since then, several laboratories have applied this or similar techniques,[48–51] and the umbilical vein remains the most reliable source for human endothelial cells in high numbers. Arterial endothelial cells have been cultured successfully in large numbers from bovine aortas [52–54] and from the aortas of pigs,[55] rabbits,[56] and guinea pigs.[57]

Other specific endothelial markers recently have been identified. Of most practical value for tissue culture studies is Factor VIII antigen,[58,59] which can be demonstrated by indirect immunofluorescent microscopy and is shared only by platelets and megakaryocytes.

As a model system, endothelial tissue culture has most of the advantages and shortcomings of all tissue culture techniques. The advantages are simplified definable experimental conditions offering possibilities of dissecting complicated biologic phenomena and manipulation of single variables. In a relatively short time, tissue culture work has provided valuable information about various functional and biochemical properties of endothelial cells, which will be further discussed in subsequent sections. Major shortcomings include the unavoidable artificial conditions of all *in vitro* systems that raise important questions regarding the relevance of the experimental findings in relation to the *in vivo* realities.

## Functional and Biochemical Properties

### Selective Barrier

The ultimate purpose of the circulatory system is to allow continuous exchange of molecules between blood and tissues. Because of its location, the endothelial layer in most areas assumes the role of a barrier which selectively regulates transfer of substances of varied molecular size between the circulating blood and the surrounding tissues.

Plasmalemmal vesicles and intercellular junctions provide the pathways for water-soluble molecules from the vascular lumen.[10] Substantial progress has been made during the past few years in clarifying questions about capillary transport. The methods used have in general depended on either

direct or indirect ultrastructural demonstration of the passage of tracer molecules of known molecular size. The effects of these tracers on endothelial permeability *per se* have been questioned, and it is possible that their transport is not fully representative of transport of physiologic substances of similar molecular weight. Structural equivalents of the two-pore system postulated by physiologists on the basis of permeability studies with graded molecules [60] have been located with fair certainty in visceral capillaries, the small-pore system in the diaphragms of fenestra,[61] and large pores in both plasmalemmal vesicles and fenestra with compliant diaphragms or no diaphragms.[61,62] There also is evidence that the large-pore system in muscular capillaries with continuous endothelium resides in the plasmalemmal vesicles.[63] Results concerning the location of the small-pore system in these capillaries are, however, conflicting. Some investigators have favored passageways between cells,[64] but more recently Simionescu et al [33] have made a strong case for their theory that fused plasmalemma vesicles bridging the endothelial cytoplasm serve this role. In the postcapillary venule, the loosely organized interendothelial junctions [35] have been found to be highly permeable to tracers such as heme-undecapeptide (HIIP) [32] and provide a morphologic basis for the leakiness and lability of the venule [65,66] that is of particular importance in the pathophysiology of inflammation,[67] as will be discussed further.

The transport through the more complicated arterial endothelium is, however, far from being fully understood. The concept that the endothelial lining of large arteries provides a barrier against plasma proteins has been challenged. Smith and co-workers have, in a series of studies of the chemical composition of the human arterial intima, shown that the "normal" intima in persons older than 30 years contains plasma proteins in concentrations that are linear functions of their molecular weight and concentration in plasma.[68-71] The concentration of LDL with a molecular weight of $2 \times 10^6$ daltons is thus practically identical to the plasma concentration, whereas on the other end of the spectrum, the intimal concentration of albumin is much lower. This has given rise to the concept that the endothelium always is highly permeable to plasma proteins, and the final concentration in the intima is mainly the result of variable retention or rates of egress.[70,72]

Bell and co-workers also showed that both $^{131}$I-albumin [73] and $^{131}$I-fibrinogen [74] cross normal aortic endothelium in young pigs studied *in vivo*. There were, however, regional as well as focal differences in the uptake of both these plasma proteins with the areas of higher permeability correlating roughly with areas of increased tendency to develop atherosclerosis, raising questions about how "normal" these areas of increased

permeability actually were. Klynstra et al [75,76] also found site-dependent permeation of trypan blue into the intima of apparently normal thoracic aortas of normal pigs. The sudanophilic streaks of older pigs, however, were found exclusively within the blue areas.

Bondjers and Björkerud found that the uptake of free and esterified cholesterol into rabbit aorta was higher in regions with defective endothelium (blue areas after staining with Evans blue) compared with that in intact endothelium (white areas) [77] and that net incorporation of $^3$H-cholesterol from blood is 15 times higher in mechanically denuded areas of rabbit aorta compared with that in endothelialized arterial tissue (re-endothelialized or uninjured).[78] In contrast, Minick et al [79] found in hypercholesterolemic rabbits that, following mechanical denudation of aortic endothelium by ballooning, lipid accumulation was increased in re-endothelialized areas.

Electron microscopically detectable tracers have been used in attempts to resolve the questions about the route of macromolecules through the arterial endothelium but have not given unequivocal answers. Most tracers studied, including ferritin (diameter $\approx$ 110 Å), have only been detected in plasmalemmal vesicles of unstimulated endothelium, whereas horseradish peroxidase (diameter $\approx$ 50 Å) has been reported to migrate through both plasmalemmal vesicles and intercellular junctions.[80,81] The disagreement centers around the transport route of horseradish peroxidase. Some investigators have found that the junctions are not penetrated by HRP under normal conditions,[82,83] while others maintain that the junctions are normally penetrated by the tracer.[81,84] Schwartz and Benditt [37] suggested the possibility of vesicular transport from the lumen to the junctional cell surface or, alternatively, that the occluded intercellular regions were only partial permeability barriers.

Of particular interest are the reports of Hüttner et al, who not only found regional differences in endothelial permeability to HRP (transported through plasmalemmal vesicles and interendothelial clefts) and ferritin (vesicular transport) in several large arteries of Sprague–Dawley rats [85] but also found differences in transport at different blood pressure levels.[86] Compared with normotensive controls (blood pressure, 100 to 120 mmHg), peroxidase reaction products appeared earlier in the subendothelial space of rats with blood pressure of 140 to 200 mmHg but were not detected following periods of low blood pressure (60 to 70 mmHg). At very low blood pressure levels (20 to 30 mmHg), both tracers, however, entered the subendothelial space in striking amount, indicating that barrier function of some sort had broken down.

Robertson and Khairallah [38] found that injection of several other va-

soactive agents led to a transient increase in endothelial permeability of the aortas of Sprague–Dawley rats, not only to HRP but also to ferritin, colloidal carbon (diameter ranging from 250 to 500 Å), LDL, and VLDL. Intracardiac injection of 10 to 100 ng of these agents into 200- to 220-g rats resulted in permeability changes coinciding with temporary ultrastructural alterations in the endothelial lining, comprising opening of intercellular gaps and nuclear pinching, suggesting active contraction. Previously it has been shown that endothelial cells contain contractile proteins (actomyosin).[87,88] Recent studies utilizing high-voltage electron microscopy to allow the direct study of full-thickness aortic endothelial cells [89] have shown development of short-lived sinusoidal tracts between endothelial cells without morphologic changes of existing "tight junctions" following intracardiac injections of subpressor doses of octapeptide angiotensin II. These preliminary findings suggest that arterial interendothelial junctions may be functionally and locally altered by a variety of stimuli without permanent structural changes or overt endothelial cell contraction.

Shimamoto et al [90] studied uptake of colloidal carbon with the same diameter as LDL and VLDL (200 to 700 Å) in aortas of several species. They found focal areas where the endothelial cells were capable of phagocytizing these particles and reported that these cells were exclusively found in areas susceptible to atheromatous changes.

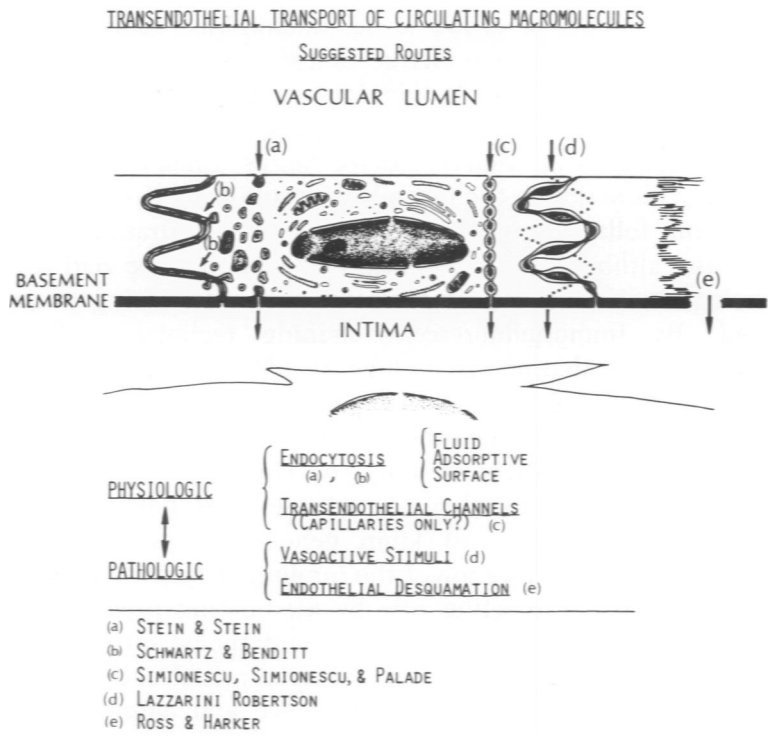
All the above findings demonstrating regional and focal variability as well as selectivity in endothelial penetration of plasma proteins support the notion that the arterial endothelium serves a complicated regulatory and perhaps protective function in the transport of macromolecules.[76–80,86,91] Figure 1 is a schematic summary of current concepts regarding macromolecular transport through vascular endothelium.

Some of the questions still remaining are as follows: What are the relative contributions of plasmalemmal vesicles and interendothelial clefts in macromolecular transport in different areas of the vascular tree? How effective a barrier is normal arterial endothelium to lipoprotein passage into the vessel wall?

#### Thromboresistant Surface

One of the basic functional characteristics of intact, normal endothelium is that it does not promote platelet or leukocytic adherence nor activation of intrinsic or extrinsic coagulation systems.[92–94]

Although this nonthrombogenic property has been known since the days of Virchow,[95] its nature is still not fully understood. Spaet [96] suggests that it may be a nonspecific property of a living cell, resulting from continuous renewal of cell membranes. He points out that normal leuko-




TEXT-FIGURE 1—Schematic representation of current concepts regarding macromolecular transport through vascular endothelium. (From Robertson AL: The spectrum of arterial disease. Atherosclerosis Reviews, Vol 3. Edited by AM Gotto, M DeBakey. New York, Raven Press, Publishers, 1978)

cytes and erythrocytes are equally nonreactive with platelets and coagulation factors as are endothelial cells. Activation of platelet adherence and aggregation, as well as coagulation systems, in his scheme would be solely the result of exposure of extracellular molecules of connective tissue, not absence of endothelial cells as such.

A theory of long standing has assigned the major role in thrombus prevention to the carbohydrate-rich cell coat, or glycocalyx.[97] Long before the visualization of the cell coat, the view was expressed that surface charge on the luminal surface of vascular endothelium played a role in the adherence of blood cells, particularly leukocytes, to the endothelium.[96] Later it was shown that the negative charge of both platelets and the endothelial cells was in large part contributed by sialic acid associated with the glycoproteins and glycolipids of the cell coat.[97] The concept was developed that the mutual repulsion between the two negatively charged elements prevented platelets from adhering to the endothelium. Danon and Skutelsky,[97] however, have shown that the thrombogenic sub-

endothelial elements of rabbit aorta, microfibrils, collagen fibers, and internal elastic lamina have charge density comparable to that of the normal endothelial surface. It therefore seems unlikely that the critical difference between the endothelial lining and subendothelium in terms of nonthrombogenicity is based on surface charge. Chen and Barnhart [99] did not find any reduction in the thromboresistant property of umbilical vein endothelium following perfusion with either neuraminidase or glycohydrolase, although both chemical evidence of sialic acid release and morphologic evidence of loss of ruthenium red staining property was obtained. By immunofluorescent staining techniques, Becker and Harpel [100] showed that $\alpha^2$-macroglobulin ($\alpha^2$M) is located at the luminal surface of human blood vessels and lymphatics. In that study, antibodies to $\alpha^2$M did not stain the endothelial cell cytoplasm, suggesting that this protein may not be synthesized by the endothelial cells. $\alpha^2$M has an inhibitory effect on a number of proteases that belong to the blood coagulation, fibrinolytic, and kinin generating systems. Becker and Harpel suggest that its location at the luminal surface of endothelial cells may serve a broad protecting role by modulating protease generating reactions that take place adjacent to the endothelial surface.

In contrast to these concepts of a "passive" role of endothelium and its glycocalyx in preventing platelet adherence and aggregation, a more active contribution by endothelial cells to thromboresistance has also been suggested and recently received strong experimental support.

Endothelial cells, particularly in the pulmonary microcirculation, have been shown to actively remove prostaglandin F1$\alpha$,[101] serotinin,[53,102] adenine nucleotides,[103] bradykinin,[103] and angiotensin I [103,104] (with subsequent conversion to angiotensin II), which all promote platelet aggregation. There is also evidence that endothelium binds thrombin [105] and contains an activator of plasminogen,[106] which is likely to serve an important role in the prevention of thrombosis.[107] Loskutoff and Edgington [108] have shown that endothelial cells derived from rabbit vena cava synthesize and secrete plasminogen activator in culture. Fibrinolysis only was detected when plasminogen was present in the culture medium, indicating that no plasminogen-independent fibrinolytic enzymes were present. Heynes et al [109] found that intimal extracts from human aorta have ATP-ase activity. Lieberman and co-workers [110] demonstrated similar activity in microsomes from rabbit aorta. These authors suggest that ADP degradation with concomitant formation of AMP and adenosine may limit the size of platelet aggregates *in vivo* by combining their antiaggregatory properties.

The discovery of prostacyclin ($PGI_2$) has opened a new chapter in

endothelial and platelet physiology. Moncada and associates [111] discovered that microsomes prepared from pig and rabbit aortas do not form thromboxane $A_2$ ($TXA_2$) from endoperoxides as platelets do; instead they contain an enzyme that converts the endoperoxides to an unstable prostaglandin that prevents or reverses platelet aggregation and relaxes several kinds of blood vessels. As an antiaggregating agent it is 30 times more active than $PGE_1$. The material was first named "prostaglandin X," but the name was changed to "prostacyclin" after structural determinations had shown that it contains a ring not found in other prostaglandins.[112] Most recently, the compound has been designated "$PGI_2$" in accordance with the nomenclature scheme used for the prostaglandins as a whole.[113]

Moncada and associates [114] have shown that $PGI_2$ is synthesized in human arteries and veins and in pig aortas. They provided evidence that the intimal portion is more active in $PGI_2$ production than are the other elements of the vascular wall.[115] They separated rabbit aortas into layers, intima (scraped off with a scalpel blade), internal elastica, media, and adventitia (dissection). The ability to generate $PGI_2$ was highest in the intimal fraction and decreased to the adventitia but was present in all layers of the vascular wall. More recently, Weksler et al [116] demonstrated that rigorously isolated endothelial cells derived from human umbilical veins or bovine aortas synthesize $PGI_2$ from $^3$H-arachidonate in culture.

The spontaneous production of prostacyclin as well as its generation from exogenous arachidonic acid is inhibited by aspirin and indomethacin, while production from prostaglandin endoperoxides is not. A lipid peroxide, 15-hydroperoxyarachidonic acid, inhibits prostacyclin production in all situations.

On the basis of these findings it has been suggested that the capacity of the endothelial lining to produce $PGI_2$ is the fundamental mechanism explaining the nonthrombogenic properties of intact vascular endothelium.[111,114,115,117] Consequently, where the endothelial layer is damaged there would be a lack of $PGI_2$-synthesizing enzyme and thrombus formation could occur. Vane and co-workers [117] also suggested that the lack of dramatic results in clinical trials testing aspirin-like drugs for the prevention of thrombosis may be linked with the site of action of these drugs. Since they inhibit endoperoxide production, they not only inhibit $TXA_2$ production in platelets but also $PGI_2$ production in the vascular wall, thus paralyzing the entire regulatory system.

This theory obviously has not been proved and it seems likely that some of the other mechanisms reviewed above, active as well as passive, play a role in the thromboresistance of intact vascular endothelium. However, the presence of $TXA_2$ in the platelets, with strong proaggregating and

vessel constricting properties, and of $PGI_2$ in the endothelial layer, with opposite properties on both counts, provides a powerful regulatory system. Considering that the prostacyclin synthetic enzymes in the vascular wall can use platelet-derived endoperoxides as a substrate, it seems evident that the vascular wall, particularly the endothelium, contains a powerful surveillance system to counteract any unnecessary or excessive platelet activation.

#### Synthetic and Metabolic Functions

The synthesis of $PGI_2$ is an example of the biochemical versatility of endothelial cells. Another example, also of importance for the interaction of platelets with the vascular wall, is the synthesis of factor VIII antigen and Von Willebrand factor. Jaffe and co-workers [59] showed that endothelial cells *in vitro* contain factor VIII antigen, as do endothelial cells *in vivo*. Cultured cells secrete factor VIII antigen and Von Willebrand factor into their media and incorporate labeled amino acids into factor VIII antigen, which has the same subunit structure as plasma factor VIII.[59,118-120] Factor VIII procoagulant activity has, however, not been demonstrated in material secreted by endothelial cells or in material released from them by freeze-thawing.[59]

Recent *in vitro* work has conclusively shown that endothelial cells have the capacity to synthesize basement membrane. Jaffe et al [121,122] found that human endothelial cells from umbilical veins stain brightly with antiserums to human glomerular basement membrane and, when fed $^3$H-proline and $^3$H-glycine, they incorporate these amino acids into two types of proteins which on SDS–polyacrylamide gel electrophoresis have the properties of Type IV and Type III collagen. These investigators also identified ultrastructurally microfilamentous structures and elastin in the subcellular membrane. Their conclusion was that the endothelial cells can contribute several components, including basement membrane, to the subendothelial collection of extracellular macromolecules.

Howard and co-workers [54] studied collagen synthesis by calf aortic endothelial cells. They found that $^{14}$C-proline and $^{14}$C-lysine were almost exclusively incorporated into protein with the properties of Type IV collagen. Immunofluorescence study showed positive staining of the endothelial cells with antiserum to Type IV collagen from bovine lens capsule basement membrane.

While reviewing endothelial thromboresistance, several biochemical properties of vascular endothelium were discussed that relate to the removal of fibrin and substances with proaggregating potential from the circulation.[53,101-107] Cytochemical and biochemical studies have revealed a

high number of enzymes that participate in various important anabolic and catabolic reactions, eg, oxydoreductases ($NADH_2$ and $NADPH_2$ tetrazolium reductases, lactate dehydrogenase, lipoic and malate dehydrogenases, aldolase, glucose-6-phosphate dehydrogenase), hydrolases (aryl-sulfatase, ATP-ase, $\beta$-glucuronidase, naphthyl esterase, aminopeptidase, 5-nucleotidase),[123] adenylate cyclase,[124] histidine decarboxylase,[125] monoamine oxidase,[126] and cholinesterase.[127] Buonassisi and Venter [56] reported that an established cell line from rabbit aorta, which they think is endothelium, contains receptors to a variety of vasoactive agents, including norepinephrine, acetylcholine, 5-hydroxytryptamine, phenylephrine, propranolol, phentolamine, angiotensin II, and histamine.

#### Growth Properties

Under normal *in vivo* conditions, vascular endothelium has a low turnover rate. Mitotic figures are rarely seen in tissue sections, and thymidine uptake *in vivo* has consistently been found to be low. Schwartz and Benditt [128] studied the replication of aortic endothelium in rats using $^3$H-thymidine autoradiography on Haütchen preparations. They found that approximately 0.3 to 1.5% of the endothelial population of the aorta of 3-month-old rats enter DNA synthesis each day, while the thymidine labeling index in newborn rats is 10 to 20%. The distribution of labeled cells was uneven. In the thoracic aorta, the dorsal surface showed a higher labeling index than did the ventral surface, but no such pattern was found in the abdominal aorta, although irregularly distributed labeling clusters separated by unlabeled areas were present.

Wright [129] used similar techniques to study the pattern of thymidine uptake in aortic endothelium of normal guinea pigs. Labeled nuclei were found with highest frequency around the openings of intercostal arteries. Of interest is the fact that these areas correspond to the areas of increased permeability to both fibrinogen and albumin in young pigs and are areas with predilection for the development of atherosclerosis.[130]

Tannock and Hayashi [131] studied proliferation of capillary endothelial cells in a number of mouse tissues by autoradiographically measuring uptake of $^3$H-thymidine. Labeling indexes were found to be in the range of 0 to 2.4%. In the regenerating cells of a fractured femur, however, the labeling index increased to 10%. A number of other physiologic and pathologic stimuli have been found capable of stimulating endothelial replication and will be further discussed in relation to endothelial reaction to injury.

The recent refinement of *in vitro* models for study of endothelial properties offers new possibilities for careful evaluation of a complicated,

multifactorial process. Gimbrone and co-workers [47] described the general growth behavior of endothelial cells derived from human umbilical veins. They found that primary cultures as well as subcultures reached and maintained confluent density of $1 \times 10^5$ cells/sq cm. In recently confluent cultures, labeling indexes after 12-hour pulsing with $^3$H-thymidine were found to be 2.4% in central closely packed regions and 53.2% in peripheral, still actively growing areas. At 3 days after confluence, labeling became uniform: 3.5% in central areas and 3.9% in peripheral areas. In the log-phase of growth, doubling time was estimated at approximately 42 hours. Jaffe et al,[46] also studying endothelial cells from human umbilical veins, estimated the doubling time of their cultures to be approximately 92 hours, while Booyse and co-workers [52] found 32 to 34 hours to be the doubling time of bovine aortic endothelial cells in culture. In our own studies of primary or low passage cultures of human endothelial cells from umbilical veins, the generation time (time of one complete cell cycle) was approximately 36 hours. The fraction of cells traversing the cell cycle has varied from 20 to 40%, and the calculated doubling time has consequently varied from 70 to 140 hours.

Haudenschild and co-workers [132] compared DNA synthesis of primary cultures of human umbilical vein endothelial cells with human foreskin and BALB/c-3T3 mouse fibroblasts. Unlike transformed or tumor cells, all these cell types show density-dependent inhibition of growth at confluence. The "contact inhibition" of the human fibroblasts and 3T3 cells is, however, not absolute, and they can be readily stimulated to synthesize DNA by addition of fresh serum or proteases to the media. Endothelial cells at confluence, however, cannot be stimulated to proliferate or synthesize DNA by any serum concentration. This stringent form of growth control seems to be a unique characteristic of endothelial cells; the reason for this is not known. Haudenschild et al suggest that the endothelial cells lack ability to make use of growth factors in serum.[132]

Our own findings,[51] as well as those of Wall and co-workers,[49] that endothelial cells, in contrast to vascular smooth muscle cells, grow independently of platelet factors, supports this notion. Other investigators, however, including Saba and Mason,[48] Maca et al,[133] and D'Amore and Shepro,[134] reported that platelets promote *in vitro* growth of human endothelial cells obtained from umbilical veins [48,132] as well as bovine arterial endothelium.[134]

Gospodarowicz and co-workers [135] have shown that bovine vascular endothelial cells are sensitive to the fibroblast growth factor (FGF) they have isolated from bovine brains and pituitary glands. In the presence of FGF the endothelial cells, which otherwise are dependent on high seeding

densities for survival and growth,[43] can be stimulated to grow at extremely low seeding densities (1 cell/sq cm), allowing cloning of the cells.

It can be concluded that endothelial cells have more stringent growth control than do most other cell types. They respond to some growth factors in serum, including FGF, but details of the control mechanisms are unknown. Gimbrone and Fareed [136] found that $SV_{40}$-transformed endothelial cells lost their characteristic topoinhibition of growth as well as anchorage dependence. Transformed cells had markedly increased growth potential, requiring less serum for growth, but specific endothelial markers such as Weibel–Palade bodies or factor VIII antigen could not be demonstrated.

### Endothelial Response to Injury

From a pathologic viewpoint, questions about endothelial replication assume particular significance due to the possible role of endothelial injury in the pathogenesis of disease processes of extreme socioeconomic importance, such as thrombosis and atherosclerosis.

As previously noted, vascular endothelium has a low turnover rate under basal conditions but responds vigorously to a number of physiologic and pathologic stimuli. Gaynor [137] found 3-fold, 7.7-fold, and 9-fold increases in $^3$H-thymidine labeling of endothelial nuclei in rabbit pulmonary capillaries, renal capillaries, and aorta, respectively, following injection of a single dose of *Escherichia coli* endotoxin. Fallon and Stehbens [138] demonstrated a marked elevation of endothelial mitotic index in the inferior vena cava of rabbit subjected to the hemodynamic stress of experimental aortocaval fistulas. Folkman and co-workers [139] isolated a factor from both human and animal tumors that is mitogenic for capillary endothelial cells.

As will be further discussed in relation to atherosclerosis, a number of mechanisms and experimental tools have been used to study the reaction of arterial endothelium to injury. Various results have been obtained with respect to the rapidity of re-endothelialization, depending on the extent of the injury. Extensive injury, both in depth and surface area, has required up to 60 weeks for re-endothelialization [140] and up to 71 weeks to regain normal endothelial morphology. Poole et al [141] described two phases in the re-endothelialization of rabbit aorta: a phase of rapid endothelial replication followed by a slow phase. Hirsch and Robertson [12] observed a complete re-endothelialization in 72 to 96 hours of selective endothelial injury tracts of rabbit aortas, which were produced with loaded flexible catheters of uniform weight and external axial vibration.

Sholley, Gimbrone, and Cotran [142] suggested that postconfluent endo-

thelial cultures provide an exceptionally good *in vitro* analog to naturally occurring vascular endothelium, since it is organized as a single cell layer, has a low replication rate at confluence with strict contact inhibition, and is able to regenerate an intact monolayer after mechanical denudation. They recently used this model to study cellular migration and replication during endothelial regeneration. After standardized mechanical wounding of postconfluent endothelial monolayers, they assessed migration of cells from the edges of the wound and $^{3}$H-thymidine labeling at different intervals. Their findings suggest distinct differences in the timing of these events: migration was clearly detectable at 12 hours while increase in $^{3}$H-thymidine labeling in the injury tract was detected only after 36 hours. Following irradiation of the cultures (1500 rad) thymidine uptake was totally inhibited, but cellular migration persisted undisturbed and resulted in repopulation of the "wound" similar to that observed in replicating cultures. The investigators concluded that replication and migration are separable functions which can be influenced by different factors and that small endothelial defects may be repaired by migration alone.

Recently we used this model to explore the role of platelet factors in the *in vitro* proliferative response of endothelium to injury.[51] We did not find any differences in the rate of cellular migration or in $^{3}$H-thymidine uptake in the wound margins of cultures that were grown in the presence of serum derived from platelet-poor plasma compared with whole blood serum. These findings are in agreement with *in vivo* observations that experimental thrombocytopenia, while inhibiting myointimal thickening that normally follows endothelial injury, has no effect on endothelial regeneration as assessed by the Evans blue exclusion test.[143-145]

## Role of Endothelium in Pathologic Processes

### Atherosclerosis

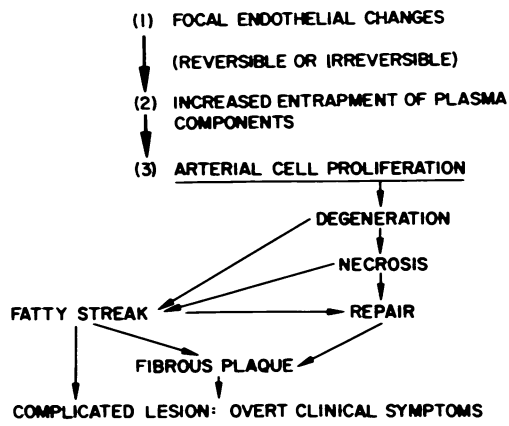
Endothelial injury has been implicated as a possible initiating event in atherogenesis since Virchow's days. Several versions of the response-to-injury hypothesis have been the spark to important experiments.[91,146] The current revival of this theory is based on the finding of morphologic similarities between the initial stages of atherosclerosis and the response of arteries to experimental injury resulting in smooth muscle cell proliferation, *de novo* synthesis of connective tissue, and intracellular and extracellular deposition of lipids in variable amounts.[147] A variety of injurious mechanisms have been found to lead to similar vascular tissue response, including mechanical trauma,[140,148-150] desiccation,[151] immune injury,[152] homocystinemia,[153,154] hypercholesterolemia,[155] and hemodynamic stress.[156]

In the last instance, atherosclerotic changes have even been described in veins subjected to arteriovenous shunts and subsequent hemodynamic stress.[157]

It should be pointed out, however, that hardly any of the injurious mechanisms stated above are likely to act specifically against the endothelial layer. Species susceptibility may vary since some of the experimental models proposed have not proved reproducible in other animals. For example, Ross and Harker [155] reported that chronic hypercholesterolemia produced focal loss of up to 5.0% of endothelial cells in the aortas of pigtail monkeys. Clowes et al,[158] on the other hand, did not find in rats any evidence that such hypercholesterolemia induced chronic endothelial injury, smooth muscle cell proliferation, or subsequent development of fibrous plaques.

Other observations compatible with the possible role of endothelial injury in atherogenesis include evidence that endothelial changes are found in highly predictable spontaneous pigeon's atheroma.[159] During the induction of experimental aortic atherosclerosis in hypercholesterolemic rabbits,[160] Jørgensen and co-workers [161] described focal injury areas in the endothelial lining of aortas from young persons at branching points that are the usual anatomic sites of increased risk for the development of atherosclerosis. Spontaneous atherosclerotic lesions in humans and experimental animals occur most frequently in areas which would be expected to be subject to hemodynamic stress.[156] Recently, Stemerman et al [140] have shown that in the rabbit, following endothelial denudation by Baumgartner's original technique, reendothelialization not only is associated with loss of reactivity to platelets and decreased permeability to Evans blue but also seems to accelerate regression of the reactive intimal thickening. Surprising, however, was their finding that the lesion regression started before re-endothelialization was complete, suggesting that other unknown mechanisms played a role. Fishman et al [151] also found a correlation between the duration of endothelial denudation and the extent of myointimal thickening.

Several mechanisms have been postulated by which endothelial injury could lead to atherosclerosis (Table 1). If normal endothelium acts as a selective barrier to the passage of blood constituents into the artery wall,[38,78,80,81,91] as described while reviewing the functional properties of the endothelium, breakdown in this regulatory mechanism would lead to changes in the extracellular milieu, eg, intracellular and extracellular lipoprotein entrapment in the deeper layers of the vascular wall. Based on recent studies in cell culture, secondary lipoprotein-induced proliferation of smooth muscle cells and/or deposition of free cholesterol and choles-




TEXT-FIGURE 2—Schematic representation of possible pathologic evolution of focal endothelial changes into atherosclerotic lesions. (From Robertson AL: The spectrum of arterial disease. Atherosclerosis Reviews, Vol 3. Edited by AM Gotto, M DeBakey, New York, Raven Press, Publishers, 1978)

terol esters could thus occur, involving both myogenic and lipogenic mechanisms of atherogenesis.[162]

The discovery by Ross and co-workers[163,164] of the platelet growth factor has opened up new vistas in the study of arterial reaction to injury. Following up on Balk's original observation that plasma did not replace serum in promoting growth of chicken fibroblasts, they compared the growth stimulating effects of whole blood serum with those of platelet-poor plasma serum on monkey arterial smooth muscle cells. They found that serum derived from platelet-poor plasma lacked the growth-stimulating capacity of whole blood serum. Addition of isolated platelets or platelet factors restores the growth-stimulating capacity of the medium. More recently, these observations have been extended to human arterial and venous smooth muscle cells.[51] *In vivo* experiments have shown that platelet survival in monkeys decreases proportionally with the amount of experimentally removed aortic endothelium.[153-155] Several groups also have found that induction of thrombocytopenia or administration of the platelet inhibitor dipyridamole inhibits the myointimal proliferation that otherwise follows endothelial injury.[143-145,156]

On the basis of these observations, Ross and co-workers proposed a modified version of the reaction-to-injury hypothesis. They suggested that focal endothelial desquamation leads to adhesion and aggregation of platelets exposed to subendothelial connective tissue, local release of platelet constituents, including a platelet mitogenic factor, and proliferation of intimal smooth muscle cells, probably derived from the media.

Burns and associates,[165] however, have noted that, whereas platelet factors are capable of initiating smooth muscle cell proliferation after

arterial intimal injury, they are incapable of sustaining it. They have also found that thrombocytopenia only has a protective effect when it is produced around the time of injury itself. Clowes and Karnovsky,[166] on the other hand, found that aspirin, flurbiprofen, or reserpine had no effect on myointimal thickening in the rat following endothelial injury sustained by air infusion. Platelets from the treated rats showed markedly decreased aggregation in response to collagen *in vitro*, slightly diminished response to ADP and thrombin, and slight prolongation of mesenteric bleeding times, suggesting inhibition of platelet functions. No differences, however, were detected between control and treated rats with respect to platelet adherence to injured areas or the ultrastructure of adherent platelets. This lack of pharmacologic inhibition of intimal proliferation is contradictory to findings reported by Harker *et al*,[154] Friedman et al,[143] and Moore et al [144] but does not necessarily speak against a role of platelets in myointimal proliferation, since these agents showed minimal effects on platelet function *in vivo* without affecting platelet adherence.

Although many experimental and pathologic observations of atherosclerosis can be explained in terms of the reaction-to-injury hypothesis, all its versions fail to explain entirely some important properties of the atherosclerotic plaque. Benditt and Benditt [167,168] made the original observation subsequently confirmed by Pearson et al [169] that a large proportion of discrete, raised aortic atherosclerotic plaques in black females are of single phenotype with respect to G6PD isoenzymes, while only very small patches of the normal aorta are monotypic with respect to this enzyme. They postulated that the plaque originated from a single cell, ie, was monoclonal, as many neoplasms have been shown to be. They also pointed out that clonal selection [170] was unlikely since both forms of the enzyme were found in different atheromatous lesions in the same vessel but only very rarely within the same lesion. It is obvious that the reaction-to-injury hypothesis alone does not explain these features of the lesion. It should, however, be emphasized that the two concepts are not mutually exclusive. If cellular mutation is one of the initiating events in atherosclerosis, the reparative processes that follow injury may provide the cells involved with a chance to express selective growth advantages they have so acquired (promotion). In this context, arterial cell reaction to injury could assume a key pathogenetic role in this multifactorial disease.

#### Thrombosis—Disseminated Intravascular Coagulation (DIC)

As already discussed, the nonthrombogenic property of normal endothelium, although well-known, is not fully understood. The question of

platelet adherence to injured endothelial cells, however, remains controversial. Several authors have published evidence showing that platelets adhere to damaged endothelium [171-173] while others have concluded that, even following injury, the endothelium remains unreactive.[174-176] It has been suggested that rheologic conditions, extent of injury, and other factors influence the reactivity of platelets to altered endothelium and account for some of this discrepancy.[93] The basic problem, however, is centered around the difficulty of excluding the possibility that some of the subendothelial structures actually are exposed when platelets adhere to the vessel wall and can be held responsible for the platelet activation. In spite of uncertainty regarding the adherence of platelets to the altered endothelial cells themselves, there seems to be little doubt that following endothelial separation or denudation platelets adhere to the vessel wall. As already noted, endothelial injury may cause focal defects in $PGI_2$ production by the vascular wall with disturbance in the equilibrium of the forces that regulate platelet activation and subsequent thrombus formation.[114] Exposed subendothelial connective tissue elements have platelet-stimulating properties. In capillaries, the basement membrane is the reactive surface.[177] In the complex subendothelium of larger vessels, the relative contribution of the various components of the intima to platelet activation is less clear. Stemerman [178] has listed these components in a descending order with respect to affinity to platelets: collagen fibers, "intact" subendothelium, subendothelial microfibrils, basement membrane, and subendothelial elastin. Conflicting results have been published about the platelet reactivity of basement membranes in different segments of the vasculature. Suresh et al [179] found the basement membrane of cardiac valve to have low reactivity, whereas Baumgartner and Haudenschild [180] found markedly diminished platelet accumulation on the subendothelium of everted rabbit aorta when the basement membrane was digested away.

The reaction of platelets to stimulation involves a complex process of shape change, internal transformation or contraction, and release of granular constituents.[181] Some of these substances, especially ADP, recruit other platelets to aggregate and form a thrombus. Simultaneously, the coagulation system is activated through several mechanisms to form fibrin strands.[182] The initial injury provides tissue factor throboplastin to set off the extrinsic coagulation pathway.[183] Maynard et al [184] have shown that endothelial cells in culture, although 20 times less active than smooth muscle cells and fibroblasts, have enough tissue factor activity to markedly accelerate coagulation. Undisturbed monolayers showed little activity, while trypsinization or mechanical disruption increased the coagulant

activity 10- to 20-fold. Following endothelial injury, the exposed subendothelial collagen activates factor XII (Hageman factor) to initiate the cascade of the intrinsic coagulation pathway. The platelet aggregate itself may activate factor XI, bypassing factor XII.[185] Platelet membrane lipoproteins also contribute to the activation of factor X.

The initiation of these events, however, does not always lead to occlusive thrombosis. Several investigators have observed in rabbits,[140,186-189] rats,[151,190,191] monkeys,[192] and swine [193] that after experimental de-endothelialization, only a single layer of platelets or small, transient clumps adhere to the denuded surface. Although a well-established phenomenon, its nature is poorly understood. To explain, Poole et al [186] simply concluded that the hemodynamic conditions in an otherwise normal artery did not allow a thrombus to form. Baumgartner and associates [187,194,195] also studied *in vivo* and *in vitro* the various factors that affect platelet adherence to the vascular wall and thrombus formation. These authors found that 30 seconds after removal of the endothelial layer of a rabbit aorta by a balloon a few single platelets were seen at the surface. Mural thrombi were detected 1.25 minutes after ballooning and reached a peak of approximately 15% surface coverage at 10 minutes. By 40 minutes, however, almost all of the platelet thrombi had disappeared, leaving behind a thin carpet of spread platelets. If the denuded subendothelium was treated with $\alpha$-chymotrypsin, stable thrombi formed and were anchored to the wall by collagen fibrils. This would indicate that although both basement membrane and microfibrils in the subendothelium can stimulate platelet adherence and thrombus formation, the amount of collagen exposed is critical for the stability of the mural thrombus. A logical corollary of this prediction would be that the extent and depth of vascular injury would be important determinants of the fate of the thrombus. Considerable experimental evidence supports this postulate.[196,197] Hirsch and Robertson [12] found that, following selective removal of endothelium from small, longitudinal segments of rabbit aortas, platelet adhesion was short-lived and followed by attachment of circulating leukocytes before endothelial regeneration ensued in the absence of mural thrombosis.

Another factor of importance for the stabilization of thrombi in general is the amount of fibrin deposited. The binding effect of fibrin is necessary to prevent deaggregation and washing away of the platelets by the flowing blood. It has recently been shown that, in contrast to platelet adhesion and aggregation, fibrin deposition on subendothelium predominates at low shear rates.[198] Normal arterial flow conditions are thus favorable for platelet adherence and aggregation but not for the stabilization of the

aggregate. The converse is true on the venous side of the circulation. In arteries, pathologic deviation from normal streamlined blood flow may induce fibrin deposition that in turn would favor thrombus stabilization.[199]

The research of the modern scientific era seems to confirm the conclusions reached by Virchow some 100 years ago, ie, that thrombus formation results from a complex interplay between hemodynamic factors, blood components, and condition of the vascular wall.

Disseminated intravascular coagulation (DIC) constitutes a series of syndromes resulting from intravascular activation of the coagulation system, with resultant consumption of coagulation factors and platelets.[200] The number of underlying diseases are legion, and its pathogenesis is extremely complex. There is considerable evidence that the basic mechanism by which a number of diseases cause intravascular coagulation is endothelial injury, with subsequent activation of both intrinsic and extrinsic coagulation pathways, as discussed above. Colman et al [200] concluded that 40% of the cases in their series were caused by diseases that were associated with endothelial injury. These fell into two major categories: a) infections and b) prolonged hypotension and acidosis or hypoxia. The most common infectious agents were *Neisseria meningitidis* and other gram-negative organisms, but gram-positive bacteria and viruses also were represented.

#### Defective Hemostasis

Damaged capillary endothelium is assumed to be the pathologic basis of a number of bleeding disorders that usually are associated with normal platelet count (nonthrombocytopenic purpuras), although platelet function may be either normal or abnormal.[201] As a rule, the bleeding problems caused by these diseases are not severe. In many instances the exact vascular defect is incompletely understood in terms of cellular pathology or biochemistry. Both congenital or familial diseases, as well as acquired diseases, fall into this category.

Von Willebrand's disease or vascular hemophilia, a familial disease with autosomal dominant inheritance, is considered a primary endothelial disorder with secondary platelet dysfunction,[202] as will be further discussed below. In hereditary hemorrhagic telangiectasia (Sutton–Rendu–Osler–Weber syndrome), the basic defect is in the walls of capillaries and arterioles [201] but is also found in larger arteries and veins,[203] leading to dilatation of the vessels and, sometimes, formation of arteriovenous aneurysms.

In the category of acquired disorders of vascular hemostatic mechanisms, infections comprise the largest group. Most common are me-

ningococcemia, septicemia, typhoid fever, and subacute bacterial endocarditis.[201] The bleeding problems in these cases, however, often are caused by a complex combination of DIC with associated thrombocytopenia and disordered coagulation and a true vascular defect. The most severe cases of purpura (purpura fulminans) frequently have pathologic features that resemble the Shwartzman phenomenon: widespread obstructive thrombi resulting in ischemic necrosis with hemorrhage. Anaphylactoid of Schönlein–Henoch purpura is associated with hypersensitivity reactions to food, bacteria, and a number of drugs and involves the host's endothelium.[201] Pathologically, there is acute inflammatory reaction of the capillaries and small arterioles with exudation and hemorrhage into the surrounding tissues.

Macroglobulinemia, multiple myeloma, and cryoglobulinemia can cause vascular purpura by directly injuring endothelial cells as well as causing disturbances in platelet and coagulation function.[201] In contrast, purpura in patients with Cushing's syndrome and senile purpura, rather than being endothelial defects, are due to loss of perivascular supporting tissue.

A theory of long standing holds that platelets support the integrity of the vasculature by forming plugs that close any gaps that normally develop in the endothelial lining. The petechial and purpuric hemorrhages that characterize clinical and experimental thrombocytopenia are easily explained on the basis of open gaps in the vasculature in the face of a deficiency of plugging material. Considerable experimental evidence, however, has accumulated supporting the notion that, in addition to this plugging function, platelets also may directly support endothelial cells. Roy and Djerassi [204] attempted to differentiate between the effects of platelets on preservation of vascular endothelium and on bleeding as a result of mechanical injury of vessels. In experiments on thrombocytopenic dogs, these authors found that numbers of infused platelets too small to result in detectable increases in the peripheral platelet counts could decrease by 50% the number of red blood cells found in thoracic duct lymph without affecting the bleeding time. Only with much larger platelet infusions did the peripheral platelet count increase and the bleeding time decrease. Aursnes [205] reported that red blood cells in ear lymphatics of irradiated thrombocytopenic rabbits decreased markedly one day prior to the increase in the platelet count during their spontaneous recovery. Both observations were interpreted as consistent with the theory that only a small number of platelets is needed to maintain vascular integrity.

In search of mechanisms of the platelet support of vascular integrity, Cronkite and associates [206] transfused $^{35}SO_4$-labeled platelets into normal

and irradiated rats and by autoradiography demonstrated deposition of the radioactive label in the controls. Johnson and co-workers [207-210] studied the mechanism of thrombocytopenic bleeding and restoration of vascular integrity after platelet transfusion in guinea pigs made thrombocytopenic by radiation or by platelet antiserum. Their ultrastructural studies were interpreted as showing that thrombocytopenic bleeding occurs by trans-endothelial passage of red blood cells rather than through intercellular junctions. Platelet transfusion led to actual incorporation of platelets into endothelial cells and coincided with restoration of the ability of endothelial cells to resist erythrocytic penetration. These observations, however, have not been confirmed, and it has been repeatedly pointed out that published electron micrographs supporting the transcellular route are difficult to interpret.[211]

The mechanism for the suggested endothelial supporting function of platelets remains unclear, but experimental observations consistent with a direct supporting role continue to be made. Gimbrone and co-workers [212] perfused thyroid glands from dogs with platelet-rich and platelet-poor plasma and evaluated vascular integrity by transmission electron microscopy. They found that although normal endothelial structure was maintained in the presence of platelets, their absence frequently led to endothelial disruption.

More recently, Kitchens and Weiss [213] examined the endothelium of capillaries and postcapillary venules in tongues of rabbits maintained thrombocytopenic (platelets <20,000/cu mm) up to 24 hours by injections of guinea pig antirabbit platelet serum or administration of busulfan. Within 6 hours these authors found striking morphologic changes with endothelial thinning and effacing of projections and folds of the luminal surface. Appearance of occasional pores and membranous diaphragms were observed, with concomitant increase of permeability to colloids. Prednisone administration ameliorated these endothelial changes and reduced the bleeding despite persistent thrombocytopenia.[214]

Related to the concept that platelets support the integrity of endothelial cells is the finding that the latter produce substances that are necessary for normal platelet function. As discussed before, Jaffe and co-workers [59,118-120] demonstrated that endothelial cells in cell culture synthesize and secrete factor VIII antigen as well as von Willebrand factor, considered to be two different properties of the same molecule.[215,216] Von Willebrand factor is essential for normal platelet function. In classic von Willebrand's disease, factor VIII antigen, antihemophilic factor, and von Willebrand factor are all decreased. The idea that this disease is a vascular disorder first was proposed by McFarlane [217] in 1941, and it has gained further support by

the work of Jaffe et al, referred to above, and the demonstration by Holmberg and co-workers [218] that tissue samples from patients with severe von Willebrand's disease show no factor VIII antigen in endothelial cells. It also has been shown [118] that medium from endothelial cultures corrects defective retention in glass bead columns of platelets from patients with von Willebrand's disease and that when antiendothelial factor VIII antibody is added to the blood of normal subjects, platelet retention in glass bead columns decreases from 90% to 20%.[118]

On the basis of all these findings, Jaffe and co-workers proposed an attractive working hypothesis that brings into a coherent scheme what is presently known about the so-called platelet–endothelial-cell–factor-VIII axis [120,219]: Endothelial cells synthesize a protein that contains the properties of both factor VIII antigen and von Willebrand factor (VWF). These molecules circulate in plasma, some bound by platelets and/or megakaryocytes. The VWF that binds to platelets supports their function. Some of the molecules, on the other hand, are either converted to antihemophilic factor with procoagulant activity or combined somewhere in the body with molecules possessing antihemophilic factor. The hypothesis further suggests that in von Willebrand's disease, endothelial synthesis is deficient and both these properties are low. In variant von Willebrand's disease, the molecule seems to be abnormal, possibly in carbohydrate content. In contrast, in classic hemophilia, the system that provides procoagulant activity to factor VIII is deficient, but the endothelial cells are able to synthesize normal amounts of factor VIII antigen.

#### Inflammation

Ryan and Majno [67] recently have written a comprehensive review of acute inflammation, emphasizing the active role played by the endothelial cells in the microcirculation.

One of the hallmarks of acute inflammation is increased vascular permeability. Since the endothelial layer in all but the glomerular capillaries serves the function of the main permeability barrier, increased vascular permeability has logically been attributed to endothelial changes. It is by now well-established that in acute inflammation the main site of permeability changes is the postcapillary venule.[65,67] As discussed in the section on endothelial structure, the intercellular junctions in venules have looser organization than any other segments of the circulatory system.[34,35] Tracer studies have shown this structural feature to correlate with much higher permeability than prevails in other parts of the microcirculation, even under normal conditions.[82] Several vasoactive substances, including well-known mediators of inflammation such as hista-

mine, have been shown to cause the formation of interendothelial gaps in venules, accounting for the dramatic permeability changes caused by these mediators.[220] Majno and associates [221,222] have made a strong case for their theory that the endothelial cells respond to inflammatory mediators by an active contraction, thus pulling apart from each other and causing gaps between the cells. This concept is based on the demonstration of actomyosin filaments in the endothelial cytoplasm as well as the following ultrastructural changes in the endothelial cells: nuclear shape change from oval to round, appearance of pinched folds in the nuclear membrane and abluminal cell membrane, and bulging of the cell body into the lumen.

In addition to this functional response of venular endothelium to inflammatory mediators resulting in immediate and transient permeability change, it also has been shown that noxious agents can cause direct injury to endothelial cells, resulting in cell death.[223-226] The subsequent increase in permeability is more sustained and is thought to account, at least in part, for prolonged vascular leakage observed during inflammation either immediately following the noxious insult or several hours later.[224-226]

Endothelial cells also are involved in another aspect of the acute inflamatory response: leukocytic infiltration. On their route to the battleground, leukocytes stick to the endothelial lining before they leave the circulation. It is not known what type of change occurs in either endothelial cells or leukocytes that results in such interaction. Chamber and Zweifach [18] suggested that the endocapillary layer (endothelial glycocalyx) was responsible. Although no morphologic changes have been identified in this layer during inflammation, the concept still seems to be valid in view of the general idea that interactions between cells to a large extent are attributable to their cell coats.[19] Bangham,[227] on theoretic grounds, suggested that local protuberances with small radii of curvature would allow close approach of leukocytes and endothelial cells, resulting in successful bonding by calcium bridge. That calcium and/or magnesium may play a role in such cell behavior is strongly supported by observations in several experimental models that EDTA inhibits leukocyte sticking, including the rabbit ear chamber,[228] mouse mesentery, and the hamster cheek pouch.[229] Local anesthetics such as lidocain also have an inhibitory action, possibly through their effect on membrane stability, but the exact mechanism is still unknown.[230]

Marchesi and Florey [231] unequivocally demonstrated that leukocytes actively migrate between endothelial cells when they leave blood vessels during inflammation. The main site of leukocytic diapedesis again is the postcapillary venule.[232] How this process is induced remains a mystery.

Mediators so far shown to have the capacity to initiate the process are chemotactic substances, and it has been suggested that they may leak into the interendothelial junctions and direct leukocytes out of the bloodstream.[67]

Recently it was shown by Carr [233] that macrophages migrate into lymphatic vessels in granulomas by reverse diapedesis, ie, by an active movement between lymphatic endothelial cells.

In several experimental models it has been demonstrated that endothelial cells in the microvasculature start proliferating relatively early in the course of acute inflammation.[234-236] Active research is being aimed at questions that relate to the stimulatory mechanisms of microvascular proliferation. In recent years particular interest has been focused on the possible role of neutrophils and circulating mononuclear cells. Fromer and Klintworth [237] found that leukocytic infiltrates, particularly of neutrophils, preceded vascular ingrowth of the inflamed cornea. When a delay occurred in the leukocytic invasion, the vascular ingrowth also was delayed. Rapid and extensive leukocytic infiltration, on the other hand, correlated with enhanced corneal vasoproliferative response. This neovascularization was inhibited in rats depleted of leukocytes by 1500 or more rad of whole-body irradiation.[238] Injection of neutrophils or their extracts stimulated vascular ingrowth in the irradiated animals, while lymphocytes failed to do so.[239] In contrast, Sholley et al [240] found that while combined radiation treatment (800 rad) and antineutrophil serum injections in rats reduced peripheral leukocyte counts to nearly zero and prevented corneal infiltration by monocytes and neutrophils, neovascularization was not prevented. Since in the latter, however, the mean vascular length was reduced, it suggested that, although neutrophils might facilitate vascular growth, they are not necessary for its initiation. These authors also found [241] that infiltration of blood-borne mononuclear cells is not a necessary stimulus for vascular regeneration following thermal injury of rat skin.

#### Immune Disorders

Immune mechanisms affect vascular endothelium in a variety of ways, eliciting both functional response and endothelial injury. As discussed in the previous section, venular endothelial cells react to histamine and slow-reacting substance of anaphylaxis (SRS-A) that are released from mast cells during immediate hypersensitivity reactions.[242] Several diseases of immediate type hypersensitivity consequently are associated with increased venular permeability, resulting in edema formation as well as influx of leukocytes from the marginated cell pool. These disorders in-

clude anaphylaxis, urticaria, angioedema, and allergic rhinitis. Also, there is evidence [243] that complement mediators such as anaphylotoxin may open endothelial junctions so that soluble immune complexes can deposit in basement membranes.

As pointed out by Ryan,[244] immune mechanisms can cause endothelial injury at three levels:

1. Primary, *specific* injury when antibodies are formed specifically against endothelial cells or they are attacked by sensitized T lymphocytes
2. Primary, *nonspecific* injury caused by circulating immune complexes that have no specific affinity for endothelial cells but are deposited in adjacent tissues
3. Secondary injury when endothelial cells suffer from immune attacks aimed at neighboring tissues such as basement membranes

Some patients with the syndrome of anaphylactoid purpura, briefly discussed in the section on defective hemostasis, probably fall into the first category [243]; others involve immune complex deposition and belong to the second group. Vetto and co-workers [245] were able to raise antibodies in rabbits against isolated dog aortic endothelial cells. The antibodies showed cytotoxic activity against endothelial cells but not against lymphocytes. Lindqvist and Osterland [246] detected specific antibodies to human endothelial cells in the serums of patients with a wide variety of diseases. They were, however, also present in a few healthy individuals, and no direct relationship to clinical disease was postulated. Poimelli et al [247] concluded, on the basis of their studies with specific rabbit antiserums against human intima, that most cases of vasculitis are due to deposition of nonspecific immune complexes.

Vascular lesions have been recognized as a common component of allograft rejection since the earliest days of transplantation.[248,249] Vetto and co-workers [245,250] detected transplantation antigens on canine vascular endothelium and suggested that the endothelial cells of a transplanted organ are the original source of sensitizing antigen. Rosenberg and associates [251] demonstrated that the endothelial cell is the prime target of antigen–antibody–complement interaction in the hyperacute rejection process. In a large series of human renal allografts, Busch et al [252] found frequent evidence of endothelial injury associated with thrombus formation, fibrinoid necrosis, intimal thickening of cortical arteries, and marked deposition of IgG, complement, and fibrinogen.

Moraes and Stasny [253] provided evidence that endothelial antigens are different from previously known HLA antigens and are located in separate molecules from the products of HLA-A, -B, and -C. Similar antigens were detected in mononuclear cells from the blood, whereas Ia-like antigens

found in B cells were distinctly different. It is suggested that these endothelial antigens may play a role in kidney allograft rejection.

The list of immune-complex diseases is steadily growing and represents a variety of etiologic agents. The pathogenetic mechanism, however, is similar in all cases: immune complexes activate complement components, leading to formation of vasoactive agents and chemotactic factors. Neutrophils phagocytize the immune complexes, and lysosomal enzymes are released that cause tissue injury, including endothelial injury. A number of glomerular diseases and vasculitides have all the hallmarks of immune complex diseases, but the antigens involved are not known and the disorders consequently are frequently classified as "probable" immune complex diseases.[254] The mere presence of immune complexes in vessels does not prove a primary pathogenic role but can be the result of passive trapping due to increased permeability in the microvasculature.[255]

The third group of immune mediated endothelial injury, ie, secondary injury resulting from immune attacks at neighboring tissues, has similar mechanisms with respect to the endothelial cells as do immune-complex diseases. The immune complex in this instance is formed *in situ*, eg, antibody–basement-membrane antigen, and initiates a similar cascade of events, activation of complement, leukotaxis, and eventual release of lysosomal enzymes resulting in tissue injury.

#### Vascular Neoplasia and Metastasis

Endothelial cells give rise to both benign and malignant neoplasms.[256] Benign hemangioendothelioma most commonly is found in the skin and subcutaneous tissue of children but can occur in adults and involve any organ. It arises from active proliferation of endothelial cells that pile up into several layers around narrow and frequently inconspicuous blood spaces. Sometimes they form solid aggregates or sheets of cells.*

Malignant hemangioendothelioma (hemangiosarcoma) is a rare tumor of great variability that most frequently occurs in bones, muscles, liver, spleen, subcutaneous tissue, and the retroperitoneal space.[256] Recently, a number of such tumors have been found in the livers of industrial workers exposed to vinyl chloride monomers.[257] The microscopic characteristics of the malignant tumor include blood spaces that are enclosed by proliferating anaplastic endothelial cells that vary in size and shape and sometimes form syncytial masses and giant cells.[256]

In addition to giving rise to tumors of its own, vascular endothelium

* The term "hemangioblastoma" is reserved for a benign endothelial tumor occurring in the cerebellum.

also, and much more frequently, assumes a role in the pathogenesis of metastatic spread of other tumors. The development of metastasis involves a complicated interaction between the host and the tumor cells.[258] Zeidman [259] divided the development of tumor metastasis into three major steps: a) tumor invasion into blood vessels and lymphatics; b) escape of tumor emboli into the circulation with subsequent entrapment in the microcirculation of distant organs; and c) invasion of the vessel wall and infiltration of the surrounding tissues by cells of the arrested embolus. Concurrently, a vascularized stroma grows into the newly formed tumor.

The mechanisms by which tumor cells are arrested in the microvasculature and how they attach and penetrate the vascular wall are of interest for this discussion. The main determinants were listed by Warren [260] in several categories: a) tumor cell survival within the vasculature; b) adherence of tumor cells to the luminal side of the vascular wall; c) movement of tumor cells within the attached embolus; d) penetration of the vessel wall; and e) the survival of the tumor cells in the extravascular tissue.

It is generally assumed that vascular endothelium affects this process,[259,260] but the significance and exact mode of its contribution are not known. Through their ability to affect fibrinolysis they could affect the active attachment of the tumor cells to their surface. Wood,[261] using direct microscopic observation of an ear chamber, observed tumor cells becoming fixed in a clump with fibrin and then penetrating the vessel wall. Clifton and Grossi [262] found that formation of fibrinogen and fibrin clots enhances tumor cell attachment and survival in the microvasculature. Fibrinolytic agents and anticoagulants [262] have been found to reduce metastases in several animal models. Chew and Wallace [263] recently confirmed in a study of Walker 256 tumor in rats that fibrin formation is a common or constant event in tumor embolization and adherence to pulmonary vessels. Fibrin formation, however, occurred in small amounts early and disappeared while the tumor cells still were intravascular.

Tissue damage can enhance local experimental metastasis.[264] Endothelial damage is probably at least in part responsible.[258] Further support for the notion that endothelial injury promotes metastasis came from the observations of Warren and co-workers.[265] They observed that *in vitro* HeLa cells adhered more firmly to venous walls where the endothelium was damaged rather than where it was intact. Similar observation was made *in vivo* using suspensions of mice thymic lymphomas and rat Walker 256 carcinoma.

The final aspect of endothelial participation in tumor metastasis to be discussed here is the formation of independent blood supply. This subject

has been intensively studied by Folkman and co-workers [139,266-268] and was recently reviewed by Folkman and Cotran.[269] In short, these authors have shown that a wide variety of tumors or extracts from tumors induce neovascularization *in vivo*. Recently, endothelial growth *in vitro* also has been found to be stimulated by simultaneous culture with sarcoma 180 cells in diffusion chambers.[270] An extremely important implication of these observations is the possibility that solid tumors, including metastases, can be rendered dormant by inhibition of the capillary proliferation induced by the tumor angiogenesis signals.[271]

## Future Developments and Summary

As predicted earlier by Florey,[11] application of new techniques and experimental probes is rapidly increasing our knowledge of both the physiologic and pathologic roles of the vascular endothelium.

Perhaps one of the most important contributions of current research is increasing experimental evidence of important regional differences in the structure and function of the endothelial cell. Undoubtedly, such degree of cellular specialization will require painstaking individual evaluation of each segment of the vascular system, from main arteries to arterioles, capillaries, venules, and large veins as well as within individual organs.

On the other hand, the endothelial cell has emerged as a rather complex and metabolically highly active structure serving a number of critical functions, particularly those of a relative blood "barrier" and an effective thromboresistent surface.

When this cell fails in these roles, it participates in a long list of pathologic processes of such clinical importance as atherosclerosis, thrombosis, disseminated intravascular coagulation, defective hemostasis, inflammation, immune disorders, and metastatic spread.

Since each of these entities constitutes a rather specialized research field, an attempt was made in this review to provide illustrations of recent advances in our understanding of some of these functions rather than presenting an exhaustive appraisal of the problems.

Many unanswered questions remain. They will undoubtedly continue to provide topics for future biologic research. As examples, we could mention the relationship between intraembryonic and extraembryonic endothelium in fetal development; the specific function of the Weibel–Palade body; the relative contributions of intraendothelial and interendothelial transport of circulating macromolecules in different vascular segments; the relative role of specific substances synthesized by endothelial cells such as the recently discovered $PGI_2$ or prostacyclin on vascular thromboresistance; the regulation of endothelial regeneration by pro-

liferation and/or migration and the significance of repetitive luminal injury and repair in the development of vascular disease; and the regulation of the inflammatory response. The list is very long and, as is true of all scientific endeavors, will continue to increase as new data becomes available. Significant advances from His' original description have been made, however, and future investigations may close many gaps in our understanding of this cell monolayer.

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