Overview

Homeostasis in the cardiovascular system is maintained by a hierarchy of interacting genetic and epigenetic programs, the disequilibrium of which contributes to the pathogenesis of complex disease processes such as atherosclerosis and its life-threatening complications, myocardial infarction, and stroke.
Endothelial cell dysfunction (ECD; see Note), manifested in lesion-prone areas of the arterial vasculature, results in the earliest detectable changes in the life history of an atherosclerotic lesion—the focal permeation, trapping, and physicochemical modification of circulating lipoprotein particles in the subendothelial space. This sets into motion a complex pathogenic sequence, initially involving the selective recruitment of circulating monocytes from the blood into the intima, where they differentiate into macrophages and internalize modified lipoproteins to become foam cells (the hallmark of early fatty streak lesions); multiple chemokines and growth factors elaborated by activated endothelium and macrophages then act on neighboring smooth muscle cells (or their precursors) to induce their proliferation and synthesis of extracellular matrix components within the intimal compartment, thus generating a fibromuscular plaque. Progressive structural remodeling of developing lesions results in the formation of a fibrous cap, overlying a lipid-rich, necrotic core consisting of oxidized lipoproteins, cholesterol crystals, and cellular debris, and is accompanied by varying degrees of matrix remodeling and calcification. The lateral edges of these complicated plaques contain a rich population of inflammatory cells (activated macrophages and T cells, natural killer T cells, and dendritic cells), which further modulate the endothelial proinflammatory phenotype and contribute to structural instability of the plaque through the proteolytic modification of its extracellular matrix components. In unstable or vulnerable plaques, this may result in a catastrophic transition in the life history of an atherosclerotic lesion—frank plaque rupture, with luminal release of the highly thrombogenic contents of the necrotic core, triggering an atherothrombotic occlusion. Alternatively, significant clinical sequelae also can result from superficial intimal erosions, without evidence of plaque rupture. The latter critical transition seems to be a consequence of endothelial cell apoptosis, with localized endothelial denudation and the triggering of thrombus formation. These superficial erosions typically occur on the surface of lesions containing abundant smooth muscle cells and proteoglycans, but few macrophages, and characterizedly are associated with regions of disturbed blood flow. Other stable lesions, characterized by a thick fibrous cap, and less lipid and inflammatory cell content, can progressively encroach on the vessel lumen causing ischemic symptoms, but typically do not precipitate atherothrombotic events. In a given individual, multiple atherosclerotic plaques can coexist—each at its own stage of pathobiologic evolution.

Given its multiple, mechanistically important roles throughout this complex series of events, ECD thus seems to constitute a pathogenic sine qua non for ACVD. In this review, we will trace the evolution of the concept of ECD, focusing on recent insights into the cellular and molecular mechanisms that underlie its pivotal roles in atherosclerotic lesion formation and progression; explore its relationship to classic, as well as more recently defined, clinical risk factors for ACVD; consider approaches to the detection of ECD; and outline some promising new directions for its treatment.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>ACVD</th>
<th>atherosclerotic cardiovascular disease</th>
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<tr>
<td>ECD</td>
<td>endothelial cell dysfunction</td>
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<td>EDRF</td>
<td>endothelial-derived relaxing factor</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>hs-CRP</td>
<td>high-sensitivity C-reactive protein</td>
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<td>IL</td>
<td>interleukin</td>
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<td>KLF2</td>
<td>Kruppel-like factor-2</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>MEK3</td>
<td>mitogen-activated protein kinase/extracellular-regulated kinase-3</td>
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<td>miRNA</td>
<td>micro-RNA</td>
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<td>Nrf2</td>
<td>nuclear factor erythroid 2-related factor-2</td>
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<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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Note

Endothelial cell dysfunction: in the lexicon of modern Cardiovascular Medicine, the term endothelial dysfunction typically is used to refer to abnormalities in the production or bioavailability of endothelial-derived nitric oxide and resultant deleterious changes in vascular reactivity. For the purpose of this Compendium on Atherosclerosis, we use the term endothelial cell dysfunction to encompass all of the maladaptive changes (multi-factorial, localized and systemic, and acute and chronic) in the functional phenotype of endothelial cells, which are associated with atherosclerotic cardiovascular disease.
an ideal container for blood; its luminal surface does not activate the intrinsic coagulation cascade or promote platelet adhesion, and actually exhibits anticoagulant and fibrinolytic properties.1 Examined from a bioengineering perspective, the individual endothelial cells comprising the lining of various parts of the cardiovascular system are seen to function as local biomechanical transducers, sensing and translating the diverse forces imparted by the pulsatile flow of blood into biological responses.2,3 Finally, when challenged with certain proinflammatory cytokines or bacterial products (eg, endotoxins), endothelial cells undergo a coordinated program of gene activation, which (reversibly) alters many of these vital functional properties, presumably serving as an adaptive response to potentially noxious stimuli.4 When viewed from these different perspectives, the vascular endothelium can be variously characterized as a distributed organ, a dynamically adaptable interface, and, at the individual cell level, an integrator of the local pathophysiological milieu. Thus, dysfunction of the endothelium, in the broadest sense, would encompass various nonadaptive alterations in its normal functional phenotype, with important implications for the regulation of hemostasis and thrombosis,5 local vascular tone6 and redox balance,7 and the orchestration of acute and chronic inflammation.8

Although the term endothelial dysfunction has clearly found its place in the lexicon of modern Cardiovascular Medicine,9-11 the evolution of this working concept has a rich history dating from the early practice of Anatomic Pathology, in the 1850s, and continuing through the development of the modern field of Vascular Cell Biology, in the latter half of the 20th century. This conceptual evolution has contributed in important ways to our present day understanding of the cellular and molecular mechanisms of ACVD. Its origins can be traced to the writings of Virchow,12 who called attention to the localized accumulation of circulating lipids and other macromolecular components of plasma at sites of early lesion formation (lipid insudation), which was detectable at autopsy and presumably reflected a localized change in endothelial permeability in life. Subsequently, the focal, nonrandom distribution of this permeability change was graphically illustrated by the mapping of blue and white areas, en face, in the aortas of experimental animals after injection of the plasma protein-binding azo dye Evans Blue.13,14 Blue areas were also associated with an increased rate of endothelial cell turnover in normal animals,15 suggesting that they represented areas of chronic intimal injury, which, in animals made hypercholesterolemic by dietary alterations, were correlated with developing atherosclerotic lesions.16 When examined ultrastructurally at the prelesional stage, these lesion-prone areas exhibited alterations in endothelial morphology, striking changes in the amount and composition of extracellular matrix, and the accumulation of extracellular liposomes, derived from plasma lipoproteins, which had become trapped in the subendothelial space and undergone complex biochemical and physicochemical alterations.17,18 Although these detailed morphological descriptions of early intimal changes strongly supported the notion of localized dysfunction of the endothelial lining, the pathophysiological stimuli, underlying mechanisms and consequences for atherogenesis, remained to be established.

Early characterizations of ECD, in the context of atherogenesis, focused on a loss of anatomic integrity of the intima, as exemplified by the seminal Response-to-Injury Hypothesis of Ross and Glomset,7 which stimulated much fruitful activity in the field. As originally formulated, the Response-to-Injury Hypothesis postulated that the initiating event in the atherogenic process was some form of overt injury to the intimal endothelial lining, induced by noxious substances (eg, oxidized cholesterol, constituents of cigarette smoke, hyperhomocysteinemia, hyperglycemia, etc.) or altered hemodynamic forces (eg, blood flow disturbances generated by hypertension). In particular, focal endothelial desquamation was envisioned as an inciting stimulus for platelet adhesion and the localized release of platelet-derived growth factors, which then would elicit the migration, proliferation, and phenotypic modulation of medial smooth muscle cells, thus generating a fibromuscular plaque.9 The experimental demonstration that this sequence of events could be induced simply by balloon catheter denudation of the intima lent support to this hypothesis10 and catalyzed an abundance of studies of the potential roles of growth factors and injury-repair mechanisms in the atherogenic process. However, the direct relevance of this form of endothelial injury to the development of natural atherosclerosis was unclear, given that careful morphological examination of the earliest fatty streak lesions in diet-induced animal models failed to demonstrate overt intimal injury or platelet adhesion.11,12 With the expanded awareness of the repertoire of vital functions of endothelial cells and their capacity for dynamic phenotypic modulation, the term endothelial (cell) dysfunction was introduced into the mainstream of atherosclerosis research.13-15 Various pathophysiologic stimuli of ECD (Table) soon became the focus of fruitful investigation by multiple groups, and the molecular manifestations of ECD began to be characterized in detail.11,15,19,30,35

Nitric Oxide and ECD

A major conceptual advance in the field of Vascular Cell Biology was the demonstration that the endothelial lining is a source of various autocoid factors that can influence the behavior of other types of cells within the vessel wall (pericytes and smooth muscle) and in the circulating blood (platelets and leukocytes). Using cultured vascular cells, as well as bioassays with isolated organs and vascular strips, several endothelial-derived autocrine and paracrine mediators were identified.16-23 In particular, endothelial-derived prostacyclin and platelet-derived thromboxane A2 were characterized as mutually antagonistic components of a dynamic hemostatic/thrombotic balance at the vessel–blood interface, and thus as potential targets for therapeutic modulation. In fact, pharmacological manipulation of this aspect of arachidonic acid metabolism remains an important clinical strategy in the treatment and prevention of ACVD.24 Collectively, these studies documented an active role of the vascular endothelium in the maintenance of blood fluidity and the regulation of vascular tone and provided the experimental tools and conceptual framework to further define the roles of endothelium in cardiovascular physiology and pathophysiology.

In 1980, Furchgott and Zawadzki25 demonstrated that vasodilation induced by acetylcholine was dependent on the
presence of an intact endothelium and seemed to be mediated by a potent humoral factor, later known as endothelium-derived relaxing factor (EDRF). Several studies documented that EDRF was a labile substance with a half-life of only seconds in oxygenated physiological media.55 Release of EDRF from blood vessels was observed under basal conditions, as well as after stimulation with acetylcholine.56 The biological effects of EDRF were shown to be inhibited by hemoglobin 57 and to be mediated by stimulation of a soluble guanylate cyclase with the consequent elevation of intracellular cyclic GMP levels in vascular smooth muscle cells.58 On the basis of similarities in the pharmacological properties of EDRF and nitric oxide (NO), EDRF subsequently was identified as NO or a labile nitroso species.59,60 A detailed comparison of the biological actions of EDRF and NO on vascular smooth muscle strips61 and platelets62 also showed that EDRF and NO were indistinguishable. In 1988, l-arginine was shown to be the precursor for the synthesis of NO by vascular endothelial cells.63 Endothelial cells metabolize L-arginine via the endothelial isoform of NO synthase (eNOS)64,65 to form NO, with L-citrulline as a byproduct (Figure 1). This overall process is subject to both transcriptional and complex post-translational regulation.66,67 A basal level production of NO by endothelial cells contributes to regulating vasomotor tone and preserving the nonthrombogenic behavior of the vascular lining. The synthesis of NO can be stimulated by receptor-dependent agonists (acetylcholine and bradykinin), nonreceptor-dependent agonists (calcium ionophores), and also fluctuations in blood flow.68 In addition, transcription of the eNOS gene is differentially regulated by fluid mechanical forces, such that endothelial cells in arterial geometries exposed to undisturbed laminar flow exhibit enhanced NO-forming capacity.2,33 Once NO is produced by eNOS, it can rapidly diffuse across cell membranes to act as a potent paracrine mediator, but it can also react with superoxide to form peroxynitrite anion, which leads to its inactivation. Particularly relevant for vascular homeostasis are the actions of NO on adjacent smooth muscle cells and circulating blood platelets and leukocytes. Many of these physiological actions are mediated through activation of soluble guanylate cyclase, which catalyzes the conversion of GTP to cGMP. Other actions of NO are mediated by S-nitrosylation, the addition of an NO group to a Cys thiol to form an S-nitrosothiol.69 S-nitrosylation has been shown to modify the function of proteins in a reversible manner, which is analogous to phosphorylation.70 In the cardiovascular system, S-nitrosylation of various target proteins has been demonstrated to modulate important cellular physiological processes including cell proliferation,69 apoptosis,71,72 exocytosis,73 and ion channel activity,74 as well as blood flow and systemic oxygen delivery.75,76

Figure 1. Endothelial-derived nitric oxide: production and biological actions. Endothelial cells rapidly produce nitric oxide (NO) via a unique isoform of NO synthase (eNOS) in response to agonists (eg, acetylcholine and bradykinin) and fluctuations in blood flow. Once generated, NO rapidly diffuses through the endothelial plasma membrane to activate guanylate cyclase in several cell types present in the blood (platelets and leukocytes) and also within the vessel wall (smooth muscle). Activation of guanylate cyclase in platelets results in inhibition of activation, adhesion, and aggregation; in leukocytes, decreased adhesivity; in smooth muscle cells, dephosphorylation of myosin light chain and vasorelaxation. NO also reacts with hemoglobin in erythrocytes, enhancing oxygen delivery to tissues. Chronic exposure of endothelial cells to laminar flow results in transcriptional upregulation of eNOS, thus increasing their NO-forming capacity (Illustration Credit: Ben Smith).
relax in response to sodium nitroprusside or papaverine.\textsuperscript{78,79} Furthermore, these eNOS-deficient mice were found to display increased endothelial–leukocyte interactions, platelet aggregation, and thrombosis,\textsuperscript{80,81} and, when crossed with ApoE-deficient (hypercholesterolemic) mice, developed accelerated atherosclerosis.\textsuperscript{82}

In a classic study, the clinical significance of EDRF for human ACVD was first demonstrated by Ludmer et al.\textsuperscript{83} using coronary angiography. The coronary arteries of individuals with advanced atherosclerotic disease showed a dose-dependent, paradoxical vasoconstriction in response to graded concentrations of acetylcholine, in contrast to the vasodilation observed in individuals with angiographically normal coronary arteries. Importantly, coronary vessels with minimal disease also constricted in response to acetylcholine, whereas coronary vessels from all 3 patient groups dilated in response to nitroglycerin. Hypercholesterolemia-dependent alterations in endothelium-dependent vasodilation were also documented in animal models of atherosclerosis.\textsuperscript{84–86} Taken together, these observations suggested that a deficiency in endothelial NO production or its bioavailability, in both humans and animals, might actually precede the formation of clinically significant atherosclerotic lesions. Impairment of flow-mediated vasodilation, measured noninvasively in peripheral vessels such as the brachial artery, seems to correlate with direct measurements of altered vasoreactivity in the coronary circulation.\textsuperscript{87} Peripheral manifestations of endothelial dysfunction have also been shown to be an independent predictor of ACVD events.\textsuperscript{88} This has stimulated the development and application of various Food and Drug Administration–approved devices to measure flow-mediated arterial dilation as a clinically useful index in the assessment of cardiovascular disease. However, as considered in greater detail below, the pathogenesis of ECD and its pathophysiological consequences extend beyond endothelial nitric oxide metabolism.

**Endothelial Proinflammatory Activation and ECD in Atherogenesis**

The involvement of the endothelial lining of small blood vessels (e.g., postcapillary venules) in acute inflammatory responses, induced by injury or infection, has long been appreciated.\textsuperscript{25,26} Indeed, the cardinal signs of inflammation (rubor, calor, tumor, and dolor) elicited by the action of histamine and other phlogistic agents can be mechanistically related to their effects on microvascular tone, permeability, and leukocyte diapedesis. These acute responses, which have been referred to as endothelial stimulation, or type I activation,\textsuperscript{88} are rapid in onset, self-limited in nature, and typically do not result in sustained morphological or functional changes. In contrast, endothelial cells can undergo a dramatic modulation in their functional phenotype in response to certain bacterial products, such as Gram-negative endotoxins, and other pathogen-associated molecular patterns (PAMPs), modified lipoproteins, and other damage-associated molecular patterns (DAMPs), or cytokines, such as interleukin-1 (IL-1), tumor necrosis factor, and IFN-γ.\textsuperscript{89} The hallmark of this form of endothelial response, termed type II activation,\textsuperscript{88–90} is the activation of pleiotropic transcription factors, such as nuclear factor-κB (NF-κB), resulting in the expression of various effector proteins with important pathophysiologic implications. Originally described as endothelial activation antigens,\textsuperscript{91} detectable on the surface of cultured human endothelial cells in vitro after their incubation with cytokines or endotoxin, these effector proteins include class II major histocompatibility antigens, involved in antigen presentation; inducible endothelial–leukocyte adhesion molecules, such as endothelial–leukocyte adhesion molecule-1 (E-selectin) and vascular cell adhesion molecule-1 (VCAM-1); procoagulant molecules, such as tissue factor; and secreted chemokines, such as IL-8 and monocyte chemoattractant protein-1.\textsuperscript{92–95} Together, this activation program confers a localized, temporally coordinat-ed, proinflammatory endothelial phenotype (Figure 2), which is detectable in vivo, at sites of inflammation, in the microvasculature of humans and experimental animals,\textsuperscript{96,97} and has become a keystone in our understanding of the active role that endothelial cells play in acute and chronic inflammatory responses and related disease processes.\textsuperscript{90}

A critical link between the activation program manifested by microvascular endothelium during inflammatory reactions and arterial ECD in atherogenesis came with the discovery of the atherosclerosis-associated endothelial–leukocyte adhesion molecule (VCAM-1).\textsuperscript{98} Initially characterized as a cytokine-inducible activation antigen in cultured endothelial cells which exhibited a sustained pattern of expression (in contrast to acute endothelial–leukocyte adhesion molecules such as E-selectin), VCAM-1 was found to have selective adhesivity for mononuclear leukocytes and lymphocytes, via their expression of its counterreceptor very late antigen-4.\textsuperscript{99–101} Importantly, its expression in vivo, in human coronary atherosclerotic plaques and in both dietary and genetic animal models of hypercholesterolemia, is localized to the intact endothelium overlying atherosclerotic lesions, and actually precedes the earliest recruitment of mononuclear leukocytes to nascent lesions.\textsuperscript{102–105} Additional studies implicated components of oxidized lipoproteins, such as lysophosphatidylcholine, as potent stimuli for its expression, thus mechanistically linking VCAM-1 induction to the atherogenic process.\textsuperscript{106} In addition, its spatial pattern of expression in vivo in various animal models of atherosclerosis is localized to those regions of the arterial vasculature that are most susceptible to lesion formation,\textsuperscript{103,104} and the detection of its soluble isoform in circulating plasma is correlated clinically with disease severity (lesion burden) in patients with atherosclerosis.\textsuperscript{107} Finally, its genetic deficiency in mouse models of atherosclerosis results in a reduction in lesion formation.\textsuperscript{108} These findings thus highlighted the pathogenic significance of VCAM-1 in the context of atherogenesis, and also implicated it as a molecular marker of ECD in the clinical setting.\textsuperscript{35}

A second important link between atherogenesis and proinflammatory endothelial activation is the intrinsic capacity of activated vascular cells (endothelium and smooth muscle) to secrete chemokines, such as IL-1, monocyte chemoattractant protein-1, granulocyte-monocyte stimulating factor, and IL-8, thus generating localized, intercellular autocrine and paracrine signaling loops within the vessel wall.\textsuperscript{53,90} Coupled with the influx of various classes of T-lymphocytes and
monocytes/macrophages,109 each contributing their particular repertoire of cytokines (part triggered by the recognition of antigens on native and oxidized lipoproteins), a complex immunoregulatory network is thus established within the developing atherosclerotic lesion, which has both local and systemic consequences.15,110–112 Locally, the balance of pro- and anti-inflammatory mediators (eg, IL-1 and IL-11), as well as agents that promote the resolution of inflammation (eg, resolvins),113 can contribute to lesion progression or regression, whereas systemic manifestations, such as the induction of acute phase reactants (eg, C-reactive protein [CRP]) by endothelial-derived IL-6, can serve as useful diagnostic and prognostic indices.114

It is noteworthy that NF-κB seems to play a central role in the proinflammatory activation of endothelium in atherogenesis. Various of the pathophysiologic stimuli of ECD have been implicated in NF-κB signaling in arterial endothelium115; expression of many of the effector molecules associated with ECD in atherogenesis (eg, VCAM-1 and monocyte chemotractant protein-1) is under NF-κB control, and certain flow-sensitive endothelial genes (eg, platelet-derived growth factor-B) have noncanonical NF-κB binding sites (shear stress response elements) in their promoters.116 Interestingly, in atherosclerosis-prone arterial geometries, endothelial NF-κB seems to be primed for enhanced activation in response to systemic stimuli (Figure 2), such as bacterial endotoxin or hypercholesterolemia.117 Recent studies suggest that NF-κB activation may also result in fundamental changes in endothelial chromatin structure, through the formation of superenhancer complexes, which can confer an epigenetic level of regulation to the proinflammatory endothelial phenotype in atherogenesis.118

This expanded appreciation of the roles of inflammatory and immunoregulatory mediators and cellular processes in atherogenesis has led to a reformulation of the original Response-to-Injury Hypothesis into what is now termed the Inflammatory Hypothesis of Atherothrombosis.11,13–15 In addition to providing a unifying view of the pathophysiologic mechanisms involved in lesion initiation, progression, and critical transitions, which are linked to many of the known risk factors for ACVD, the basic premise that atherosclerosis is a chronic inflammatory disease has important and timely clinical implications. These include the recent revision of the traditional clinical risk factors119 to include systemic indices of inflammatory status,120,121 and, as considered below, the initiation of randomized placebo-controlled clinical trials of anti-inflammatory treatments for ACVD.122 Importantly, the evolution of the concept of ECD has provided the conceptual framework for these paradigm-shifting, basic to translational advances.

Figure 2. Endothelial proinflammatory activation. In lesion-prone regions of the arterial vasculature, the actions of proinflammatory agonists (eg, interleukin [IL]-1, tumor necrosis factor [TNF], and endotoxin), oxidized lipoproteins (ox-LDL) and advanced glycation end products (AGE), as well as biomechanical stimulation by disturbed blood flow, leads to endothelial activation. These biochemical and biomechanical stimuli signal predominantly via the pleiotropic transcription factor nuclear factor-κB (NF-κB), resulting in a coordinated program of genetic regulation within the endothelial cell. This includes the cell surface expression of adhesion molecules (eg, vascular cell adhesion molecule-1 [VCAM-1], secreted and membrane-associated chemokines (eg, monocyte chemotractant protein [MCP]-1 and fractalkine), and prothrombotic mediators (eg, tissue factor [TF], vonWillebrand Factor [vWF], and plasminogen activator inhibitor [PAI]-1). These events foster the selective recruitment of monocytes and various types of T lymphocytes, which become resident in the subendothelial space. The concerted actions of activated endothelial cells, smooth muscle cells, monocyte/macrophages, and lymphocytes result in the production of a complex paracrine milieu of cytokines, growth factors, and reactive oxygen species (ROS) within the vessel wall, which perpetuates a chronic proinflammatory state and fosters atherosclerotic lesion progression. IL-R, TNF-R indicates receptor(s) for IL-1, TNF; Ox-LDL-R, receptor for oxidized LDL; RAGE, receptor for AGE; and TLRs, Toll-like receptors (Illustration Credit: Ben Smith).
Hemodynamics, ECD, and the Focal Nature of Atherosclerosis

An intriguing aspect of the pathobiology of atherosclerosis is the observation that the earliest lesions, in both humans and various experimental animal models, characteristically develop in a distinctive, nonrandom pattern, the geometry of which correlates with arterial branch points and other regions of altered hemodynamics.

Detailed studies of the blood flow in these lesion-prone regions have revealed complex, disturbed laminar flow patterns (flow separation, recirculation, and reattachment), which create significant temporal and spatial gradients over relatively short distances, resulting in a high oscillatory index and a low time-averaged wall shear stress. Initial hypotheses suggested that these conditions might result in longer dwell times for lipoproteins, favoring their increased permeation into the vessel wall, or that disturbed flow might physically disrupt endothelial integrity, creating foci of injury/repair reminiscent of the Response-to-Injury Hypothesis of Atherosclerosis. As discussed above, however, the latter explanation was challenged by detailed morphological studies documenting the presence of an intact endothelial lining in atherosclerosis-susceptible regions during early lesion formation.

The subsequent development of experimental fluid mechanical devices that could reproduce defined laminar, turbulent, and disturbed flows over the surface of cultured endothelial cell monolayers in vitro revealed that the fluid mechanical forces generated by arterial blood flow could also act directly on endothelial cells to alter their morphological and functional properties. For example, exposure of cultured endothelial cells to undisturbed laminar shear stresses induces changes in cell shape, alignment, surface glycocalyx, and cytoskeletal organization that mimic the morphology of aortic endothelium in vivo in lesion-resistant geometries. In contrast, the exposure to disturbed flows induces endothelial cell turnover and senescence and increases oxidative stress, as well as alterations in cell shape and the organization of cytoskeletal and intracellular junctional proteins changes. Similar to those seen in lesion-prone areas of the arterial vasculature in vivo, these observations suggested that distinct hemodynamic forces might constitute a local risk factor for ECD in atherogenesis.

A fundamental link between hemodynamic forces and atherogenesis came with the demonstration that the expression of various endothelial genes important in hemostasis and thrombosis, growth regulation, and proinflammatory activation was transcriptionally regulated by defined fluid mechanical stimuli. Because several of these genes were directly involved in endothelial adhesion biology (eg, intercellular cell adhesion molecule-1), these observations suggested a novel paradigm linking biomechanical stimulation with endothelial activation.

Further mechanistic analyses revealed the existence of shear stress response elements in the promoters of pathophysiologically relevant genes, such as the platelet-derived growth factor, eNOS, and VCAM-1, that acted to up- or downregulate gene transcription. Interestingly, clusters of multiple genes seemed to be differentially responsive to the spatial and temporal properties of the applied shear stresses, thus suggesting a complex, dynamic system of biomechanical endothelial gene regulation. In an early study, Topper et al used high-throughput molecular biological techniques to define the effects of undisturbed laminar shear stresses, similar to those present in lesion-resistant arterial geometries, on the expression of endothelial genes that might have special relevance for atherogenesis. Notably, several pathophysiologically important endothelial genes showed a sustained, differential upregulation with undisturbed laminar flow when compared with turbulent flow. These included COX-2 (the inducible isofrom of cyclo-oxgenase), eNOS, and manganese-dependent superoxide dismutase—enzymes that exert potent antithrombotic, antiadhesive, anti-inflammatory, and antioxidant effects, both within the vascular lining and in interacting cells, such as platelets, leukocytes, and vascular smooth muscle. These findings, together with the observation that, in the face of potent systemic drivers of cardiovascular risk (eg, hypercholesterolemia), certain regions of the arterial vasculature remain relatively resistant to the development of atherosclerotic lesions, led to the formulation of an alternative hypothesis for the selective distribution of atherosclerotic lesions, the Atheroprotective Gene Hypothesis. This hypothesis postulated that undisturbed laminar shear stresses upregulate the coordinated expression of a set of atheroprotective genes in endothelial cells, which then act locally in lesion-resistant areas to offset the effects of systemic risk factors in ACVD. The critical testing of this hypothesis entailed a 4-step experimental approach—first, a detailed characterization of the actual near-wall shear stress profiles in atheroprone and atheroprotected arterial geometries; second, the recreation of these complex flows, with spatial and temporal fidelity, over cultured human endothelial monolayers; third, a genome-wide comparative analysis of the resultant transcriptomes and their correlation with known pathogenic mechanisms in atherosclerosis; and fourth, the validation of these genetic expression programs in endothelial cells in atherosclerosis-resistant and atherosclerosis-susceptible vascular geometries in vivo. The combined efforts of several groups subsequently produced a robust body of experimental observations linking specific patterns of hemodynamic stimulation with the transcriptional regulation of functional phenotypes in endothelium in vitro and in vivo.
focused on transcriptional regulators. Among the transcription factors most responsive to hemodynamic stimuli, the zinc finger transcription factor, Kruppel-like factor 2 (KLF2), seemed to show the strongest differential upregulation by atheroprotective waveform stimulation. The expression of KLF2 had been previously demonstrated in the endothelium of atheroresistant regions of human arteries specimens, using in situ hybridization. When experimentally introduced into cultured human endothelial monolayers, via viral vectors, KLF2 coordinately upregulated a large number of the genes that were associated with atheroprotective hemodynamic stimulation. Taken together, these observations focused attention on KLF2 as a potential critical regulatory node in the endothelial homeostatic network. Several studies documented that the expression of KLF2 in endothelial cells promotes an anti-inflammatory, antithrombotic endothelial phenotype, which is explained, at least in part, by its antagonism of the NF-κB pathway. In addition, the expression of KLF2 was found to regulate other endothelial cell functions important for atherogenesis, including endothelial barrier function, and the release of micro-RNAs (miRNAs) via the shedding of endothelial microvesicles. Endothelial KLF2 expression also stimulates the production of several autocoids, including NO and C-type natriuretic peptide, which have been shown to be deficient in dysfunctional endothelium in vivo. Furthermore, mice genetically deficient in KLF2 display enhanced atherosclerotic plaque formation when compared with wild-type controls.

Importantly, the statins, a widely prescribed class of cardio-protective drugs, were found to upregulate KLF2 expression in cultured human endothelial cells at pharmacologically relevant doses. This class of statins was originally designed to reduce the biosynthesis of endogenous cholesterol, and thus treat hypercholesterolemia—a major risk factor in ACVD. Biochemically, they block production of mevalonate, which...
forms 2 major downstream products: farnesyl pyrophosphate and geranylgeranyl pyrophosphate. Statin-mediated upregulation of KLF2 in human endothelial cells is dependent on the depletion of geranylgeranyl pyrophosphate, which is known to prenylate several members of the Rho superfamily. Notably, upregulation of KLF2 is critical for many of the statin-dependent transcriptional changes in endothelial cells, thus implicating KLF2 in the so-called pleiotropic, nonlipid-lowering, beneficial cardiovascular effects of this class of cardiovascular drugs.

The atheroprotective flow-mediated expression of KLF2 in endothelial cells is dependent on the activation of the mitogen-activated protein kinase/extracellular-regulated kinase kinase kinase-3/mitogen/extracellular signal-regulated kinase 5/extracellular signal-regulated protein kinase 5/myocyte-specific enhancer binding factor-2 signaling pathway, and several characterized modifiers including 5/AMP-activated protein kinase (AMPK), sirtuin 1 (SIRT1), protein kinase C zeta (PKCζ), SUMO specific protease 2 and histone deacetylase 5.2,180 The activation of mitogen/extracellular signal-regulated kinase 5 and extracellular signal-regulated protein kinase 5 per se leads to endothelial vasoprotection in both a KLF2-dependent and KLF2-independent manner, suggesting that additional targets of these kinases may be involved in mediating the beneficial effects of flow.181,182 One such target is the transcription factor KLF4. KLF4 also is upregulated by atheroprotective flow in cultured endothelial cells via mitogen/extracellular signal-regulated kinase 5/myocyte-specific enhancer binding factor-2 signaling.181,183 KLF4 expression promotes vasoprotective gene expression in cultured endothelial cells, via several downstream transcriptional targets that are also activated by KLF2.181,183,184 Using endothelial-selective gain-of-function and loss-of-function approaches, an important role for KLF4 in atheroprotection has been documented in a mouse model of atherosclerosis.184

Another noteworthy flow-mediated transcriptional regulator of atheroprotection is nuclear factor erythroid 2-related factor-2 (Nrf2). Nrf2 is activated by atheroprotective flow in cultured endothelial cells, via the phosphoinositol 3-kinase/Akt and extracellular signal-regulated protein kinase 5 pathways and controls a series of downstream target genes that play a role in the regulation of intracellular redox balance, as well as resistance to extracellular oxidant stresses.285–287 In vivo, Nrf2 is differentially expressed in those regions of the vasculature that are relatively atherosclerosis-resistant, thus further suggesting its pathophysiological relevance.285,286 Recent studies have documented that KLF2 and Nrf2 act independently in the activation of flow-mediated gene expression, but suggest a requirement for KLF2 expression for full activity of Nrf2 in mediating antioxidant vasoprotection.289,190 Together, these 2 transcription factors account for the activation of ≈70% of the atheroprotective flow-induced endothelial transcriptome,190 thus pointing to their critical role as master regulators of the vasoprotective endothelial phenotype (Figures 3 and 4).

Beyond their major effects at a transcriptional level, atheroprotective and atheroprone flow stimulation seem to influence endothelial gene expression via 2 additional mechanisms: miRNAs and epigenetic modifications.191,192 (Figure 4). Initial in vivo miRNA profiling studies, comparing arterial regions in normal adult swine, identified high expression of miR10a in atheroprotective regions.193 In cultured endothelial cells, a major action of miR10a is to downregulate the NF-κB proinflammatory pathway. Atheroprotective flow also has been shown to upregulate the expression of miR19a–b,194,195 miR-23b,195 and miR101196 in cultured endothelial cells, leading to the suppression of endothelial cell proliferation. In contrast, the expression of miR92a,197 miR663,198 miR712,199 and miR34a200 is downregulated by atheroprotective flow and upregulated by atheroprone flow in cultured endothelial cells. The atheroprotective flow-dependent suppression of miR92a expression results in the upregulation of KLF2 and KLF4 and certain of their downstream transcriptional targets in vitro and in vivo.197,201 Inhibition of miRNA-92a in endothelial cells modulates endothelial activation in response to shear stress and oxidized low-density lipoprotein (LDL) and limits the development of atherosclerosis in LDL receptor–deficient mice at least, in part, by increasing the expression of KLF2 and KLF4, thus suppressing endothelial activation.202 Inhibition of the expression of miR663 suppresses atheroprotective flow-mediated endothelial activation.198 miR712 downregulates the expression of tissue inhibitor of metalloproteinase 3 leading to the stimulation of endothelial inflammation, permeability, and atherosclerotic lesion formation in mice.199 Similarly, the downregulation of miR34a contributes to the atheroprotective flow-mediated suppression of endothelial inflammation by downregulating NF-κB signaling.200 Finally, atheroprotective flow also induces the secretion of the miR143–145 cluster via a KLF2-dependent pathway. These secreted miRNAs can act on neighboring vascular smooth muscle cells to regulate their turnover and phenotype and reduce atherosclerotic lesion size in ApoE-deficient mice.202 The broad reaching implications of miRNAs for the diagnosis and treatment of ACVD are the subject of another review in this Compendium on Atherosclerosis series.

Recently, it has become apparent that hemodynamic forces can modulate endothelial gene expression at an epigenetic level. For example, 2 independent studies have shown that atheroprone flow significantly modulates endothelial DNA methylation patterns via alterations in DNA methyltransferase activity, in particular, DNA methyltransferase-1.203,204 In addition, at the single gene level, disturbed flow was shown to increase methylation of the proximal promoter of KLF4 in endothelial cells leading to the suppression of its expression by blocking a myocyte-specific enhancer binding factor-2 binding site and by regulating DNA methyltransferase 3A. This myocyte-specific enhancer binding factor-2 site was also hypermethylated in swine aortic endothelium isolated from atherosclerosis-susceptible regions of the aorta where KLF4 expression is low.205 In light of the recent awareness of the global role of epigenetic changes in proinflammatory endothelial cell activation, this new area of flow-mediated gene regulation may offer interesting opportunities for targeted pharmacological interventions.

In addition to the basic role that the anatomic configuration of large arteries plays in defining distinct hemodynamic
environments, physiological factors that alter systemic and local blood flow also can significantly influence endothelial functional phenotype. For example, aerobic exercise seems to exert some of its well-described beneficial cardiovascular effects by modifying flow patterns in the vicinity of arterial branch points, thus altering the wall shear stresses experienced by the endothelial lining. A transition to more disturbed laminar flow in lesion-prone geometries should favor the increased expression of certain atheroprotective genes (e.g., eNOS), the inhibition of arterial stiffness, and the restoration of a vasoprotective endothelial phenotype.

Multiple atherosclerotic lesions in a given human subject can progress, regress, or remain stable, each behaving in an autonomous fashion, thus suggesting that local factors are important determinants of their pathobiology. Using a combination of intravascular ultrasound, biplane angiography, and blood flow measurements, Stone et al undertook a detailed assessment of endothelial wall shear stresses and local arterial wall morphology in the coronary arteries of patients with acute coronary syndromes—at the time of intravascular intervention (stenting) and 6 months later. In addition to providing fresh insights into the natural history of coronary atherosclerotic disease, these and other vascular profiling studies suggested that endothelial shear stresses were a potentially useful predictor of subsequent pathobiological behavior of a given lesion. These observations were then extended in the Prediction of Progression of Coronary Artery Disease and Clinical Outcome Using Vascular Profiling of Shear Stress and Wall Morphology (PREDICTION) study, which demonstrated a strong correlation between localized, low (disturbed) endothelial shear stresses and subsequent lesion progression to a clinical event. Taken together, these studies suggest that vascular profiling, combined with biological imaging techniques, potentially could be used to guide more targeted interventional therapies in acute coronary syndromes.

Clinical Assessment of ECD and Its Relationship to Cardiovascular Risk

Given its involvement at all stages of ACVD progression, and its predictive significance for cardiovascular events, there has been considerable interest in the detection and monitoring of ECD in the clinical setting. Although direct measurement of impaired endothelial-dependent dilation during coronary angiography remains the historical “gold-standard,” various indirect approaches, such as the measurement of brachial artery diameter via noninvasive ultrasound imaging in response to flow-mediated reactive hyperemia, have found their way into general practice. The concordance of these measurements of peripheral arterial vasoreactivity with coronary artery endothelial-dependent vasodilation has been established in controlled comparative studies, lending further support to their use for cardiovascular risk evaluation in a given patient. Because ECD also has been implicated in the pathophysiology of various disease states in addition to ACVD, e.g., hypertension, diabetes mellitus, chronic heart, and renal failure, these indirect indices of ECD may have broader clinical significance.

In light of the expanded appreciation of the multiple roles that endothelium plays in cardiovascular homeostasis, the search for clinically useful measures of ECD, which more directly reflect the pathobiology of ACVD at the cellular and molecular level, has burgeoned. These efforts encompass a broad spectrum of activity—including the validation of circulating plasma biomarkers that reflect pathogenic events occurring within developing lesions per se (e.g., the secretion of inflammation-associated cytokines, such as IL-1 and IL-6, or the shedding of induced cell surface adhesion receptors, such as soluble VCAM, sE-selectin), as well as the measurement of systemic indices of the inflammatory state, such as CRP. Efforts in biomarker discovery now extend well beyond the validation of individual candidate molecules of interest and are using the power of high-throughput genomics, proteomics, and metabolomics, coupled with bioinformatic analyses. Complementary to these blood-based analytic approaches has been the emergence of novel imaging strategies to assess the clinical significance of individual atherosclerotic lesions, at a given moment, in a patient at risk.

At the present time, considerable attention is focused on the application of high-sensitivity CRP (hs-CRP) as a robust, clinical useful biomarker in the assessment of ACVD in individual patients and populations at risk. CRP is one of a class of acute phase reactants elaborated by the liver in response to systemic inflammatory stimuli such as IL-6. In the context of atherogenesis, the proinflammatory activation of endothelial cells would be an important source of IL-6 generation. Significantly, in prospective epidemiological studies of initially healthy men and women, hs-CRP has been shown to predict future risk for heart attack and stroke, independent of plasma LDL-cholesterol-associated risk. Multiple studies also have shown that statins reduce hs-CRP in a manner largely independent of their action in reducing blood LDL-cholesterol levels, thus suggesting that these widely prescribed agents are exerting their potent cardiovascular protective effects via both lipid-lowering and anti-inflammatory actions. The demonstration that statins can act directly on endothelial cells to activate KLF2, resulting in a vasoprotective endothelial phenotype (Figure 4), provides an important mechanistic insight into the interrelationships of statin therapy, hs-CRP measurements, and ACVD risk. The ability of arterial endothelial cells, in a lesion-susceptible geometry, to resist multifactorial pathophysiological stimuli of ECD (Table), through the coordinated expression of multiple atheroprotective genes, in effect...
reflects their reduced proinflammatory set-point. Thus, hs-CRP might be viewed as a systemic index of this local pathophysiologic state. This awareness has led to the development and validation of improved algorithms for the assessment of cardiovascular risk—The Reynolds Risk Scores for Women and Men,\textsuperscript{120,121} which incorporate indices of systemic proinflammatory reactivity.

Taken together, these considerations provide cogent epidemiological support for the hypothesis that atherosclerosis is indeed a chronic inflammatory process. The critical testing of this hypothesis is the goal of 2 ongoing prospective clinical trials assessing different anti-inflammatory approaches to ACVD prevention—Cardiovascular Inflammation Reduction Trial (CIRT), which is evaluating low-dose methotrexate, and Canakinumab Anti-inflamatory Thrombosis Outcomes Study (CANTOS), which is evaluating IL-1β inhibition.\textsuperscript{122}

Opportunities and Future Directions

What does the future hold for our understanding of the role(s) of ECD in the pathogenesis of ACVD, and, importantly, its more effective detection, treatment, and prevention in the clinical setting? At present, 3 areas of opportunity warrant consideration: first, understanding the various roles of endothelial metabolism in health and disease; second, characterizing the clinical consequences of genetic variations in atheroprotective genes; and third, targeting ECD via novel therapeutic strategies.

Endothelium is both the largest distributed organ in the human body (with an aggregate mass comparable with other vital organs such as the kidney), and an integral part, anatomically and functionally, of each of the body’s composite tissues and organs.\textsuperscript{227} As we have considered in detail in this review, ECD in response to specific pathophysiological stimuli (eg, hypercholesterolemia and other dyslipidemias, diabetes mellitus, obesity, hypertension, and aging) has important local manifestations within the walls of arteries in lesion-susceptible regions, but also can be detected in nonlesion regions of the vasculature.\textsuperscript{36} A systemic context for ECD becomes especially relevant when considering the inter-relationships of type 2 diabetes mellitus, insulin resistance, the metabolic syndrome, and ACVD risk.\textsuperscript{228,229} One important mechanistic link seems to be the capacity of lipid-laden, dysfunctional adipocytes to generate a variety of proinflammatory adipokines (eg, tumor necrosis factor), which can act locally within adipose tissue to activate macrophages and microvascular endothelium and also systemically to stimulate NF-κB–dependent pathways in other target tissues, including the NF-κB–primed arterial endothelial cells in lesion-susceptible regions.\textsuperscript{14,120,231}

Within the endothelium, per se, the nuclear receptor peroxisome proliferator-activated receptor gamma (PPARγ), an important transcriptional regulator of lipid metabolism, seems to play a central role in controlling metabolic and vascular responses to a high-fat diet, as well as the pharmacological effects of certain antiabetic agents.\textsuperscript{235} Furthermore, there is increasing evidence that a broad spectrum of basic metabolic pathways in endothelium is modulated by specific pathophysiologic stimuli associated with ECD.\textsuperscript{171,176} In contrast, relatively little effort has been given to applying state-of-the-art metabolomic approaches to characterize endothelial metabolism in the context of ACVD. Such studies offer much potential for enriching our understanding of the role endothelial metabolism in maintaining vascular homeostasis and pointing the way to biomarkers useful for the diagnosis of early ECD in individuals at risk.\textsuperscript{234}

Currently, there is a growing awareness that cardiovascular medicine is poised to become more personalized and precise through the translation of genome-based discoveries into clinical practice.\textsuperscript{235} Indeed, several of the reviews in this Compendium on Atherosclerosis series are related to this theme. These efforts are deeply rooted in the fundamental insights into ACVD pathogenesis that the study of familial hypercholesterolemias and other dyslipidemias has provided. Recent translational activities centered on the association of mutations in the gene encoding proprotein convertase subtilisin/kexin type 9 with alterations in LDL receptor intracellular trafficking and hypercholesterolemia provide a vivid example of continued progress in this realm.\textsuperscript{231} Given the state of our current understanding of the endothelial expression of atheroprotective genes and their critical roles in the generation of a vasoprotective phenotype (Figure 4), detailed analyses of the genetics of ECD in ACVD would seem to be a timely undertaking. The ACVD association of specific alterations in the genes encoding key transcriptional regulators, such as KLF2, or major downstream effectors, of the vasoprotective endothelial phenotype, would not only further substantiate the Atheroprotective Gene Hypothesis, but also enable better definition of cardiovascular risk in a given patient or population.

ECD seems to be a reversible process. However, to date, treatment of ECD has been focused largely on ameliorating known risk factors for ACVD rather than specifically targeting endothelial-based mechanisms.\textsuperscript{36} Looking to the future, the development of pharmacomimetics of the natural, flow-mediated vasoprotective endothelial phenotype would seem to be a potentially fruitful strategy. One can envision such drugs acting on endothelial cells in atheroprotective regions of the arterial vasculature to reprogram their expression of a vasoprotective phenotype, thus offsetting the effects of systemic risk factors, such as hypercholesterolemia, and slowing the progression of atherosclerotic lesion development. Novel biomarkers of ECD, directly related to intrinsic, vessel wall processes, could serve as a guide in the application of these endothelial-targeted agents. As detailed above, the statins may indeed be exerting some of their well-recognized pleiotropic (ie, nonlipid lowering) beneficial effects via endothelial KLF2 activation.\textsuperscript{175,176} However, members of this class of statins were not optimized around this endothelial-targeted action, thus highlighting the opportunity for the future development of selective therapies for ECD in atherogenesis (Figure 4).

In a monograph entitled Endothelium: Its Development, Morphology, Function, and Pathology, published in 1954 and totaling 155 pages, Dr Rudolf Altschul, Professor of Histology at the University of Saskatchewan, began his state-of-the-art review with the statement—"While working on problems of atherosclerosis, I have realized not only how little I knew about endothelium, but also how much I ought to know for the proper understanding of atherosclerosis."\textsuperscript{236} Several decades
later, this insightful comment continues to motivate and illumine our understanding of ACVD.

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None.

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