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Forum Review

Therapeutic Angiogenesis in Diabetes and Hypercholesterolemia: Influence of Oxidative Stress

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Abstract

Despite significant improvements in the medical, percutaneous, and surgical management, numerous patients are first seen with non-revascularizable coronary artery disease (CAD). The growth of new blood vessels to improve myocardial perfusion (*i.e.*, therapeutic angiogenesis) is an attractive treatment option for these patients. However, the successes of angiogenic therapy, observed in preclinical studies, have not been realized in clinical trials. Increasing evidence suggests that this discrepancy between animal and human studies may be due to the nature of the substrate, or the molecular and cellular environment within which the angiogenic agent acts. Antiangiogenic influences, including endothelial dysfunction, hypercholesterolemia, and diabetes, are present in virtually all patients with advanced CAD. Recent studies have better characterized the abnormalities associated with these disease states, providing novel targets for intervention. These substrate-modifying interventions can potentially enhance the response to protein-, gene-, or cell-based angiogenic therapy. In this review, we discuss key aspects of the angiogenic process and the pathophysiologic and molecular mechanisms that contribute to an impaired angiogenic response in the setting of endothelial dysfunction, hypercholesterolemia, and diabetes, with a focus on the role of oxidative stress. Last, we briefly explore substrate modifying agents that have been evaluated in preclinical and clinical studies to improve the angiogenic response. *Antioxid. Redox Signal.* 11, 1945–1959.

Introduction

Cardiovascular disease not only is the leading cause of death, disability, and health care expenditure in the United States, but also is the leading cause of mortality around the world. The principal cardiovascular disorder responsible for increases in cardiovascular mortality is no longer rheumatic disease, but rather ischemic cardiovascular disease (10). The prevalence of its risk factors (*i.e.*, physical inactivity, obesity, diabetes, hypercholesterolemia and smoking) continues to increase worldwide. It is not surprising, therefore, that despite improvements in the management of these cardiovascular risk factors and advances in percutaneous and surgical revascularization methods, coronary artery disease (CAD) affects more than 13 million people in the United States and is responsible for one of every five deaths (44). In a large number of patients, CAD can be of such a diffuse and severe nature that repeated attempts at catheter-based interventions and surgical bypass may be unsuccessful at restoring normal myocardial blood flow. Up to 20–37% of patients with ischemic heart disease cannot undergo coronary artery bypass surgery (CABG) or percutaneous coronary intervention (PCI), or they receive incomplete revascularization with these standard revascularization strategies (35, 50, 67, 69, 92). Furthermore, incomplete revascularization has been associated with increased mortality and poorer clinical outcome (32, 55).

The goal of therapeutic angiogenesis, with growth factor– or cell-based therapies, is to restore perfusion to chronically ischemic myocardium without intervening on the epicardial coronary vasculature. Early experiments in myocardial angiogenesis with recombinant growth factors or gene-based delivery led to great enthusiasm about their therapeutic potential. However, subsequent application in phase I to III

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Table 1. Placebo-controlled Clinical Trials of Growth-factor Therapy

Author	*Year*	N	*Growth Factor*	*Vehicle*	*Delivery*
Laham (58)	1999	24	FGF-2	Protein	IM (surgical)
Unger (113)	2000	25	FGF-2	Protein	IC
Pecher (82)	2000	40	FGF	Protein	IM
Vale (115)	2001	6	VEGF	Plasmid	IM (perc)
Simons (98)	2002	337	FGF-2	Protein	IC
Losordo (60)	2002	19	VEGF	Plasmid	IM (perc)
Grines (37)	2002	79	FGF-4	Adenovirus	IC
Grines (38)	2003	52	FGF-4	Adenovirus	IC
Henry (45)	2003	178	VEGF	Protein	IC + IV
Hedman (43)	2003	103	VEGF	Plasmid + adenovirus	IC
Tio (108)	2004	23	VEGF	Plasmid	IM (perc)
Kastrup (53)	2005	80	VEGF	Plasmid	IM (perc)
Ruel (86)	2008	19	VEGF ± l-arginine	Plasmid	IM (surgical)
Total		984			

IM, intramyocardial; IC, intracoronary; IV, intravenous; perc, percutaneously delivered; year, year of publication.

clinical studies has demonstrated limited clinical benefit, and therapeutic angiogenesis remains an experimental treatment for patients for whom conventional therapies have failed (Table 1).

The discordance between successful preclinical studies and disappointing clinical trials may be explained by a number of factors (99). First, angiogenesis is a complex process that involves interactions between a number of pro- and anti-angiogenic mediators, the endothelium, and the extracellular matrix. It is therefore not surprising that single-agent growth-factor therapy has not led to large functional improvements in patients. Second, patients with end-stage coronary disease are vastly different from the young and healthy animals in whom preclinical testing is typically conducted. The presence of diabetes, hypercholesterolemia, and endothelial dysfunction can significantly limit the effect of growth factors on the angiogenic response (87, 118). Third, the optimal delivery strategy, one that provides local delivery and prolonged exposure to a sufficient dose of growth factor without causing unwanted effects, remains to be discovered. Last, the lack of sensitive assays of myocardial angiogenesis limits our ability to detect small, subclinical changes that may be occurring in response to growth-factor delivery. Despite these limitations, angiogenesis is a critical process that occurs in all humans and, if appropriately modulated, can provide therapeutic benefit to the large population of patients with ischemic coronary artery disease.

Processes involved in Blood Vessel Formation

An understanding of the biology of growth factors and their therapeutic potential requires the understanding of the processes involved in new blood vessel formation and has been previously reviewed (21, 22). Vasculogenesis, angiogenesis, and arteriogenesis are three processes that may contribute to the growth of blood vessels (57).

Vasculogenesis is the formation of new vessels from pluripotent stem cells, as seen in embryonic development. Increasing evidence suggests that vasculogenesis may also occur in the adult, as seen in the mobilization of endothelial progenitor cells from bone marrow and the incorporation of these cells into foci of neovascularization.

Angiogenesis refers to the growth of capillaries from enlarged venules that sprout capillary buds, become divided by periendothelial cells (intussusception), or are separated by transendothelial cell bridges (bridging) to form capillaries. The process starts with vasodilation and increased permeability to allow extravasation of proteins that modify the extracellular matrix. This is followed by endothelial cell proliferation and migration and tube formation with endothelial cell differentiation in response to the local tissue environment. Angiogenesis is the manner by which capillaries proliferate in healing wounds, along the border of myocardial infarctions, as well as in neoplasms. Whether these vessels are capable of producing physiologically relevant increases in tissue perfusion is debated.

Arteriogenesis is the process that results in the appearance of arteries possessing a fully developed tunica media by proliferation of preexisting arterioles into true collateral arteries. Smooth muscle cells may differentiate from various cell types, including endothelial cells and bone marrow precursors. Arteriogenesis involves smooth muscle cell growth and proliferation, migration, and differentiation to a contractile phenotype (23). An example of arteriogenesis is the development of angiographically visible collaterals in patients with advanced obstructive coronary or peripheral vascular disease.

Angiogenic Signaling

The formation of new blood vessels involves a complex molecular signaling cascade. A significant number of cytokines involved in this process have been identified, including members of the fibroblast growth factor (FGF) family, the vascular endothelial growth factor (VEGF) family, the platelet-derived growth factor (PDGF) family, and angiopoietins (127). VEGFs and FGFs are the most widely studied and the only ones used for clinical studies.

Vascular endothelial growth factors are a family of heparin-binding glycoproteins shown to act as mitogens for vascular endothelial cells as well as to stimulate endothelial progenitor cell mobilization from the bone marrow (5). The family of VEGF molecules includes VEGF [A-D] as well as placental growth factor (PIGF). These ligands interact with a number of different tyrosine kinase receptors (flt-1, flk-1, and flt-4) (15).

VEGFs are expressed in cardiac myocytes and vascular smooth muscle cells, with increased expression in the setting of vascular injury, acute and chronic ischemia, and hypoxia (109). VEGFs bind to their tyrosine kinase receptor, which activates PI3 kinase, leading to the phosphorylation of Akt (protein kinase B). Phosphorylation of Akt has numerous downstream effects, among which is the phosphorylation and activation of endothelial nitric oxide synthase (eNOS), eventually leading to the production and release of nitric oxide (NO) (110), Downstream effects of VEGFs include vascular permeability, increased endothelial cell growth and survival, and formation of tubular structures (127). The VEGF family of growth factors has been demonstrated to be a crucial component of redox cell signaling that occurs in response to ischemia and reperfusion and provides the stimulus for neovascularization (64, 65).

The FGF family consists of 23 proteins that are classified by their expression pattern, receptor-binding preference, and protein sequence (29, 34). FGF is present in the normal myocardium (24). Its expression is stimulated by hypoxia (9) and hemodynamic stress (89). FGF-2 is a pluripotent molecule and modulates numerous cellular functions in multiple cell types. In the context of angiogenesis, it induces endothelial cell proliferation, survival, and differentiation, and also is involved in the migration of endothelial cells, smooth muscle cells, macrophages, and fibroblasts (29). These effects are mediated through its interaction with the tyrosine kinase receptor FGFR1 (29, 131), leading to the activation of protein kinase C (α and ε isoforms) and also involves syndecan-4 as a downstream mediator. Although FGF signaling also involves NO release (100), in contrast to VEGF, a lesser number of studies have tied the angiogenic effects of FGF-2 directly to NO. Additionally, FGF-2 stimulates endothelial cells to produce a variety of proteases, including plasminogen activator and matrix metalloproteinases (27, 120), promoting chemotaxis.

Role of Substrate in Determining Effects of Therapeutic Angiogenesis

As discussed earlier, one of the major reasons for the discordant results between successful animals models and less efficacious clinical studies is the presence of important pathophysiologic changes in patients with end-stage coronary artery disease in whom angiogenic therapy has been attempted. Despite important advances in risk factor management and medical therapy, patients with advanced coronary artery disease have a number of influences that can impair their response to therapeutic angiogenic therapy. Cardiovascular risk factors, such as hypertension, hypercholesterolemia, diabetes, metabolic syndrome, and smoking all have independent effects on vascular function. However, in addition to these independent effects, a common pathway in which this vascular impairment is manifest is the presence of endothelial dysfunction. Increasing awareness of these antiangiogenic influences in patients with coronary disease has led to the emergence of the notion that the substrate, or the molecular, cellular, and microvascular environment, on which the therapeutic angiogenic agent acts is as important, if not more important, than the agent itself. In the following sections, we discuss the link between endothelial dysfunction, hypercholesterolemia, and diabetes, and the response to therapeutic angiogenesis.

The endothelium, nitric oxide, and angiogenesis

The endothelium is a critical component in the maintenance of normal vascular function and the response to injury. Although many different aspects of endothelial dysfunction exist, in the context of vascular physiology and angiogenesis, it is generally defined as a reduction in the release of nitric oxide in response to a stimulatory agent. This reduction in stimulated NO release can be evaluated by using a number of *in vivo* or *ex vivo* experimental methods. Most commonly, *ex vivo* assessments of arteries or arterioles are performed by using tension or size-based assessment of vascular reactivity.

A strong relation exists between the release of NO and the regulation of blood-vessel growth and development, especially that mediated by the actions of VEGF. Arnal *et al*. (4) showed that proliferating endothelial cells express about sixfold as much eNOS mRNA as do confluent cells. Substance P and VEGF, which both stimulate the release of NO (93, 134), induce new vessel formation *in vivo* in addition to increasing the permeability, migration, and proliferation of postcapillary endothelial cells in tissue culture (70). Bouloumie *et al.* (132–134) demonstrated that VEGF enhances the expression of eNOS in native and cultured endothelial cells, an effect that may be important in the process of VEGF-induced angiogenesis. Inhibitors of NOS suppress angiogenesis, and the proliferative effect of VEGF is decreased in the presence of NOS inhibitors. Uhlmann *et al.* (112) measured the proliferation and migration of choroidal endothelial cells after VEGF stimulation in the presence or absence of N^{ω}-nitro-arginine methyl ester (L-NAME), a NO inhibitor, and found that pretreatment with L-NAME attenuated the VEGF-induced angiogenic response, in direct correlation with a reduction in basal NO release. NO may also play a crucial role in the VEGF-mediated angiogenic response of vascular smooth muscle cells (VSMCs). Jozowicz *et al.* (51) recently examined the effect of exogenous and endogenous NO on the synthesis of VEGF by rat and human VSMCs by exposing cells to exogenous NO donors, or to the genetic augmentation of eNOS or iNOS. NO donors potentiated by twofold the generation of VEGF protein by rat or human VSMCs. Similarly, rat or human VSMCs transiently transfected with plasmid cDNA encoding eNOS or iNOS synthesized up to threefold more VEGF than did those transfected with control plasmid cDNA, an effect that was reversed after treatment with L-NAME, an eNOS inhibitor.

In comparison to VEGF, a lesser number of studies have tied the angiogenic effects of FGF-2 to local NO availability. Still, NO likely acts as an important signal in the angiogenic response to FGF-2 as well, presumably by terminating its proliferative actions and promoting the differentiation of endothelial cells into vascular tubes (6). This role is supported by the work of Muhohara *et al.* (74), who showed that the inhibition of endothelial NOS by L-NAME attenuated endothelial cell migration but not proliferation *in vitro*. These authors also demonstrated that endogenous endothelium-derived NO maintains the functional expression of integrin $\alpha_v\beta_3$, a mediator for endothelial migration, survival, and angiogenesis, suggesting that endothelium-derived NO plays a crucial role in mediating angiogenesis by supporting endothelial cell migration, at least partly *via* an integrin-dependent mechanism. Recently, Sieber *et al.* (96) studied the role of NO in the effects of FGF-2 in a rat model of portal hypertension

secondary to portal vein ligation. These authors used two Teflon rings, filled with collagen I, that were fixed in the mesenteric cavity, with one supplemented with 100 ng of FGF-2. The role of NO was tested in a subset of animals by adding the NO-formation antagonist N^{ω}-nitro-L-arginine (NNA) to drinking water. After 16 days, the rings were explanted and embedded, and the vessels were morphometrically counted. FGF-2 significantly stimulated vessel formation per implant in control rats, but not in rats with portal hypertension, suggesting that endothelial dysfunction and diminished NO availability may have played an inhibitory role on the effects of FGF-2. Interestingly, the numbers of ingrown vessels without FGF-2 stimulation were higher in rats that had portal vein ligation compared with controls. NNA substantially inhibited angiogenesis in both groups, and FGF-2 did not reverse angiogenesis prevented by NNA.

It also has been demonstrated that tube development by growing endothelial cells in three-dimensional gels in response to transforming growth factor-β is dependent on NO and inhibited by antagonists of NOS (81). Moreover, the stimulated synthesis and release of endothelium-derived NO by VEGF and FGF-2 has been shown to be largely regulated by tyrosine kinases (93), further implicating the role of NO in blood vessel formation mediated by these two proteins. Interestingly, activity of the tyrosine kinase Src was also found to protect endothelial cells from apoptosis during VEGF-mediated angiogenesis in chick embryos and mice (31).

Indirect evidence also supports for a crucial role of NO in the angiogenic process. In a rat gastric ulcer model, Ma *et al.* (61) showed that angiogenesis changed in parallel with eNOS expression, and that L-NAME administration significantly reduced both, suggesting that eNOS plays a significant role in gastric ulcer healing. Dewilt *et al.* (28), in a model of renal subcapsular adenocarcinoma in rats, reported that an additional antitumor effect was demonstrated when L-NAME was added to the synergistic combination of melphalan and tumor necrosis factor (tumor sizes decreased from 70 to 100%), suggesting an antiangiogenic role of L-NAME for the treatment of solid tumors in a systemic or regional setting.

Important *in vivo* evidence suggesting that endothelial factors play a major role in mediating the angiogenic response is found in the murine studies of Jang (48) and Duan (30), whose apoE- hypercholesterolemic mice exhibited attenuated collateral vessel formation in response to a FGF-2 disk angiogenesis system and hindlimb ischemia, respectively. This inhibition was, in both studies, fully reversed by the oral administration of L-arginine, which is the substrate for endothelial NO production. Overall, the bulk of evidence therefore suggests that NO production and perhaps other yet unidentified endothelial factors play a significant role in mediating the endogenous as well as the exogenous angiogenic responses, and likely accounts for the attenuated effects of angiogenic therapy observed in humans with end-stage, inoperable CAD who display significant endothelial dysfunction (33, 36, 54, 130).

Effects of hypercholesterolemia

Hypercholesterolemia in patients may occur for a variety of reasons, including dietary intake or abnormalities in lipid and cholesterol metabolism. The effects of diet-induced hypercholesterolemia on endothelial function have been repeatedly

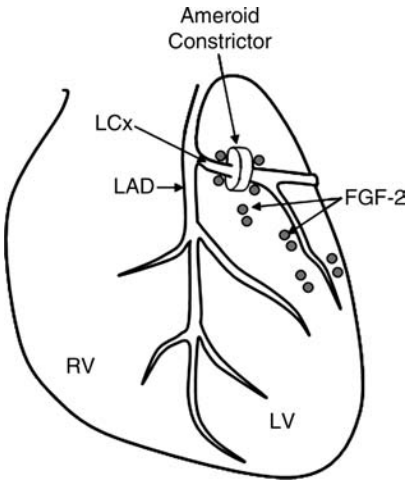

FIG. 1. Schematic showing the commonly used model of circumflex coronary artery ameroid constrictor for the creation of chronic myocardial ischemia as well as perivascular implantation of sustained release FGF-2 heparin alginate beads.

demonstrated in a number of animal models as well as in clinical studies. Cohen *et al.* (25) demonstrated that pigs fed a high-cholesterol diet for as little as 9 weeks had attenuated endothelium-dependent relaxation to serotonin in coronary ring segments placed in an organ chamber, despite the absence of intimal proliferative changes on light or electron microscopy. Similarly, Hasdai *et al.* (40) showed that pigs fed a hypercholesterolemic diet for 10 weeks had reduced vasorelaxation to bradykinin, abnormal responses to the endothelin B–receptor agonist sarafotoxin 6c (41), and impaired arteriolar relaxation to insulin-like growth factor (42). Impairments in smooth muscle function in the setting of hypercholesterolemia have also been demonstrated by Shishido and colleagues (95) in hypercholesterolemic rabbit aortae, in addition to endothelial dysfunction.

To examine the effects of hypercholesterolemia and endothelial dysfunction on angiogenesis, swine fed a diet rich in fat and cholesterol for 13 weeks were subjected to chronic myocardial ischemia by using a circumflex coronary artery ameroid constrictor (Fig. 1). In this model, the endogenous response to chronic myocardial ischemia as well as the exogenous angiogenic response to sustained-release perivascular administration of VEGF and FGF-2 were evaluated (13, 87, 118). All hypercholesterolemic animals demonstrated impaired coronary microvascular relaxation responses to adenosine diphosphate (ADP) and VEGF, suggesting reduced NO availability and endothelial dysfunction. In addition, hypercholesterolemic animals also exhibited impaired relaxation response to the NO donor, sodium nitroprusside, suggesting abnormalities in smooth muscle relaxation. The endogenous response to myocardial ischemia was impaired in the hypercholesterolemic animals, as evidenced by reduced perfusion of the collateral-dependent territory, as well as reduced endothelial cell density in the ischemic circumflex region (13) (Fig. 2). Furthermore, the response to growth factors, VEGF (118) and FGF-2 (87), was impaired compared with that of normocholesterolemic controls. In addition to the

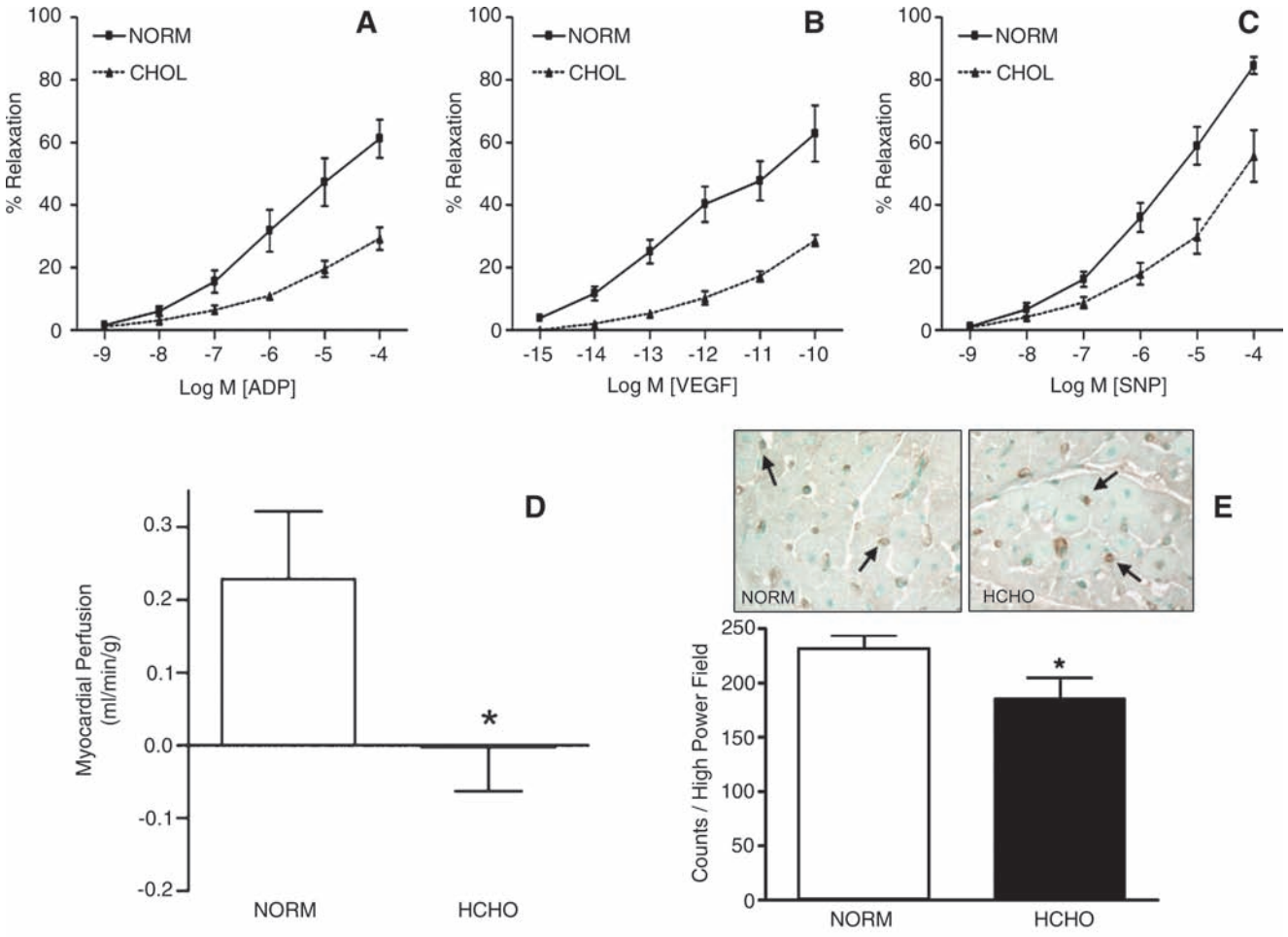

FIG. 2. Summary of functional changes observed in a swine model of hypercholesterolemia and chronic myocardial ischemia. Hypercholesterolemic (HCHO) swine had impaired microvessel relaxation to (**A**) adenosine diphosphate (ADP), (**B**) vascular endothelial growth factor (VEGF), and (**C**) sodium nitroprusside (SNP) compared with normocholesterolemic controls (NORM). HCHO animals had reduced collateral-dependent perfusion (**D**) and reduced microvessel density (**E**), indicating impaired angiogenesis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

functional effects, a number of abnormalities at the molecular level were observed in this model.

When examined in a prolonged, stable state 7 weeks after ameroid constrictor placement, the animals demonstrated no significant differences in the protein expression of angiogenic growth factors, their receptors, or any of the downstream mediators, including eNOS. An interesting observation, however, was an increase in oxidation levels of structural proteins in hypercholesterolemic animals, the most prominent of those proteins being the structural protein, actin (51 kDa). This suggests increased oxidative burden in hypercholesterolemic animals (Fig. 3). Reactive oxygen species (ROS) can be generated from numerous sources within the cell, including mitochondria, nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase, xanthine oxidase, and eNOS uncoupling (19). In the context of angiogenesis, they are released in response to angiogenic stimuli (for example, ischemia) and play an important role in angiogenic signaling (66). However, ROS also rapidly combine with NO, forming peroxynitrite, and can, therefore, reduce the amount of bioavailable NO (101). Prolonged, excessive production of ROS can also cause irreversible oxidation of cellular proteins, leading sometimes to altered function (59). The increased burden of oxidative stress in the hypercholesterolemic animal group, as demonstrated by higher levels of oxidized proteins, combined with equivalent levels of eNOS expression in both groups, suggests that the reduced NO bioavailability and resulting endothelial dysfunction in these animals may be due to increased NO degradation, in the presence of ROS, rather than impaired NO synthesis.

Another interesting observation was that the hypercholesterolemic animals demonstrated a significantly higher expression of the antiangiogenic protein, endostatin. Endostatin was originally identified by O'Reilly *et al*. (77) from conditioned medium of a hemangioendothelioma cell line as a highly active and endothelial specific angiogenic inhibitor. It is an endogenous 20-kDa protein that is a C-terminal fragment of collagen XVIII produced by proteolytic cleavage, by a variety of matrix metalloproteinases (MMPs). Endostatin not only may inhibit angiogenesis but also may block migration and proliferation of endothelial cells and increase apoptosis (77). Conversely, one of the proposed physiological effects of

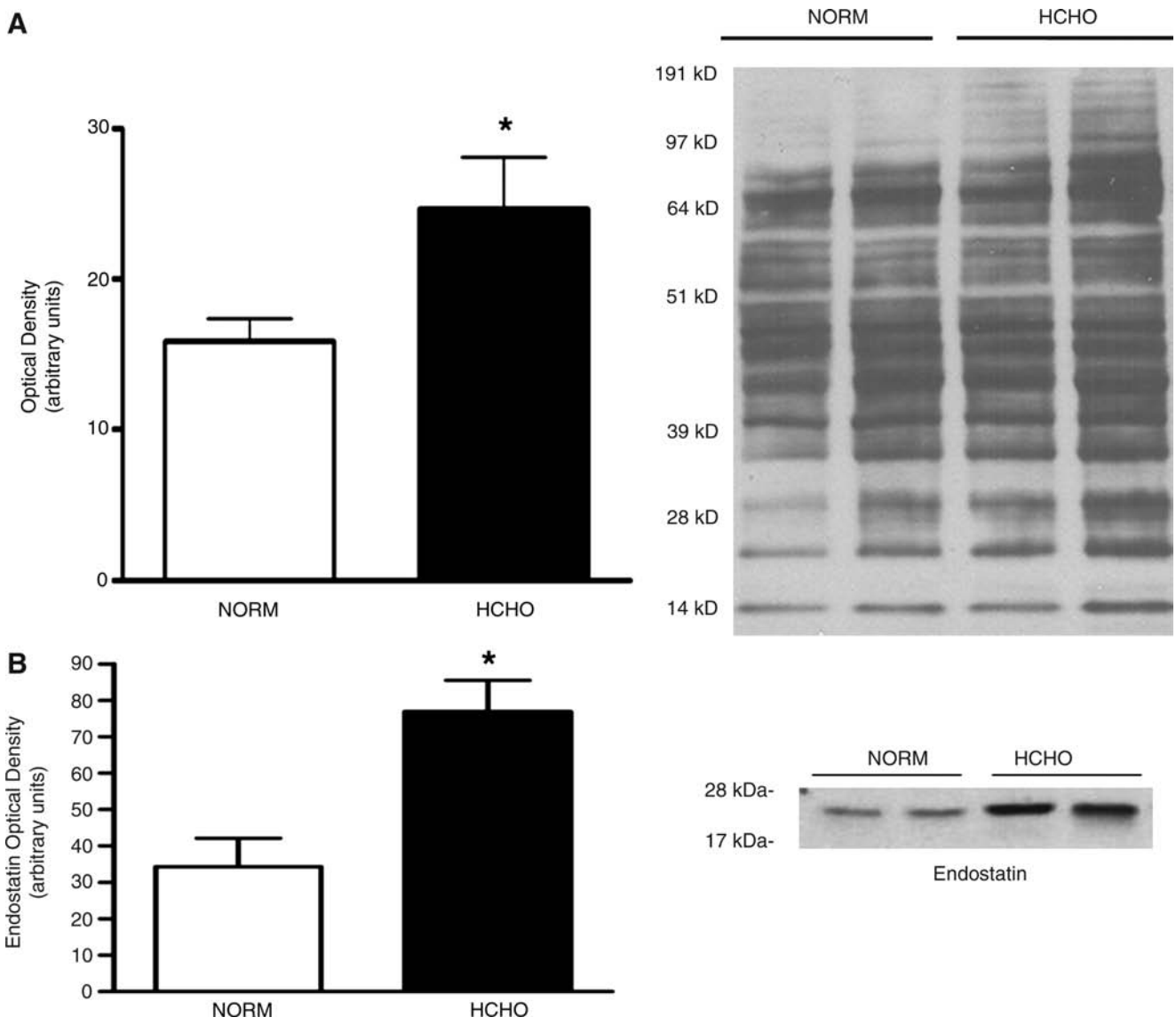

FIG. 3. Molecular findings in the setting of hypercholesterolemia. These included (**A**) increased protein oxidation, and (**B**) increased expression of endostatin in hypercholesterolemic (HCHO) *versus* normocholesterolemic (NORM) animals.

endostatin is antiatherosclerosis. In 1999, Moulton *et al*. (71) investigated endostatin as well as TNP-470, another substance known to inhibit the growth of capillaries, in apolipoprotein E–deficient mice fed a high-cholesterol diet for 16 weeks. They found that endostatin significantly reduced intimal neovascularization and plaque growth. More recently, Moulton *et al*. (72) indicated that loss of collagen XVIII, the source of endostatin, enhanced neovascularization of aorta in the collagen XVIII–knockout mouse. Hence, it is possible that endostatin is an important endogenous protective factor against atherosclerosis in hypercholesterolemia. Nevertheless, endostatin is very likely responsible, at least in part, for the blunted endogenous and exogenous angiogenic response in the setting of hypercholesterolemia.

Last, when the relative efficacies of therapeutic doses of VEGF and FGF-2 were compared in a hypercholesterolemic model, FGF-2 resulted in greater perfusion of the collateral-dependent territory compared with VEGF (17). Interestingly, when evaluated in healthy, normocholesterolemic swine, the effects of intramyocardially delivered VEGF and FGF-2 are not significantly different (47). However, in a model of hypercholesterolemic endothelial dysfunction, FGF-2 appears to be a more effective agent for therapeutic angiogenesis. Possible explanation for this finding may be that VEGF-induced angiogenesis may be more NO dependent and that reduced NO bioavailability, due to increased ROS in this model, makes VEGF less effective. In addition, endostatin has significant inhibitory effects that are specific to VEGF signaling, and its increased expression in this model may be responsible for the reduced angiogenic effect of VEGF.

Diabetes and myocardial angiogenesis

More than 35 million people in the United States are affected by diabetes and glucose intolerance. These individuals carry up to 8 times the risk of cardiovascular events compared

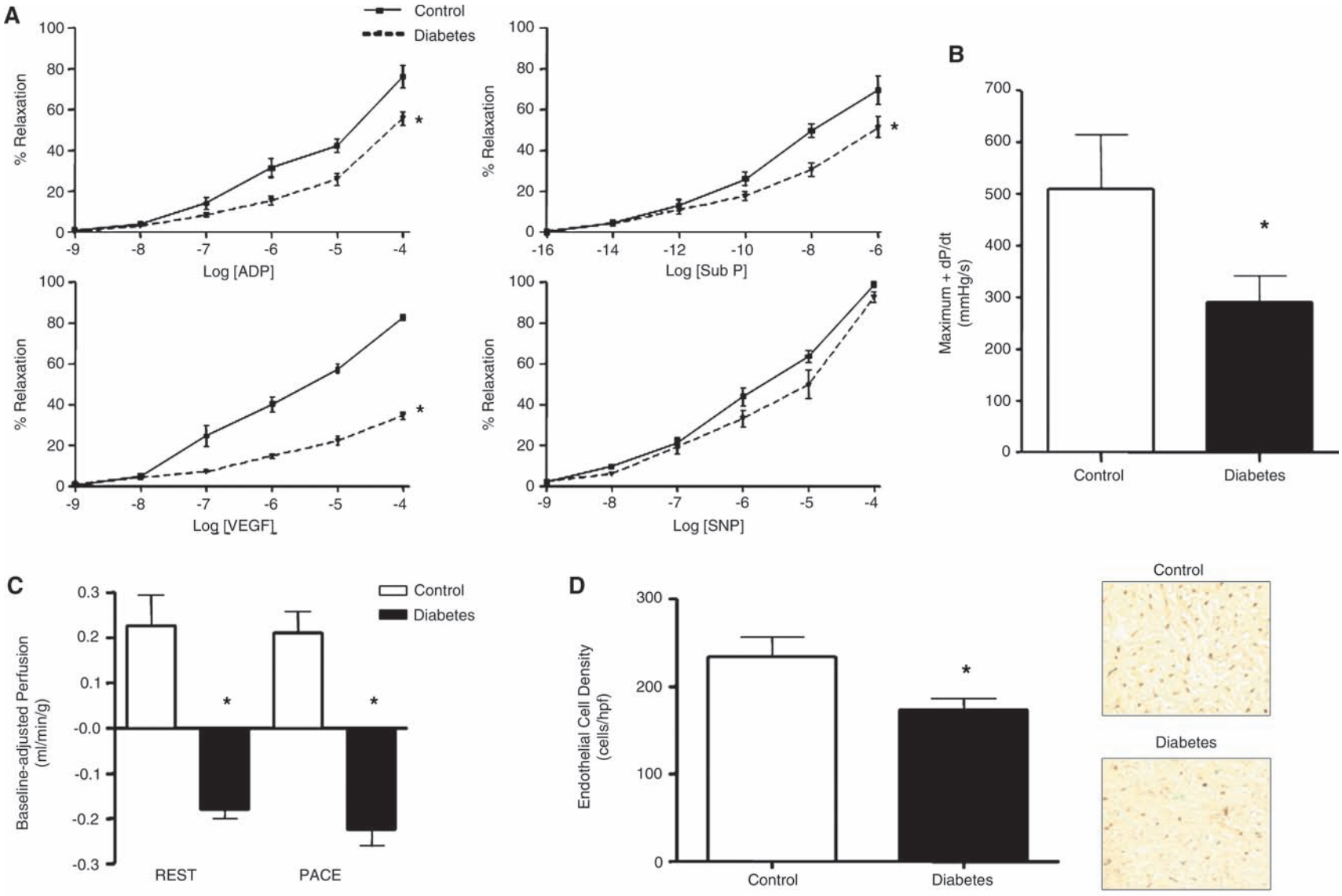

FIG. 4. Summary of functional changes in a swine model of alloxan-induced diabetes and chronic myocardial ischemia. Diabetic animals (DM) exhibited (**A**) endothelial dysfunction, (**B**) impaired left ventricular systolic function, (**C**) reduced collateral-dependent perfusion during rest and pacing, and (**D**) reduced microvessel density in the ischemic territory compared with nondiabetic controls. ADP, adenosine diphosphate; Sub P, substance P; VEGF, vascular endothelial growth factor; SNP, sodium nitroprusside. *$p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

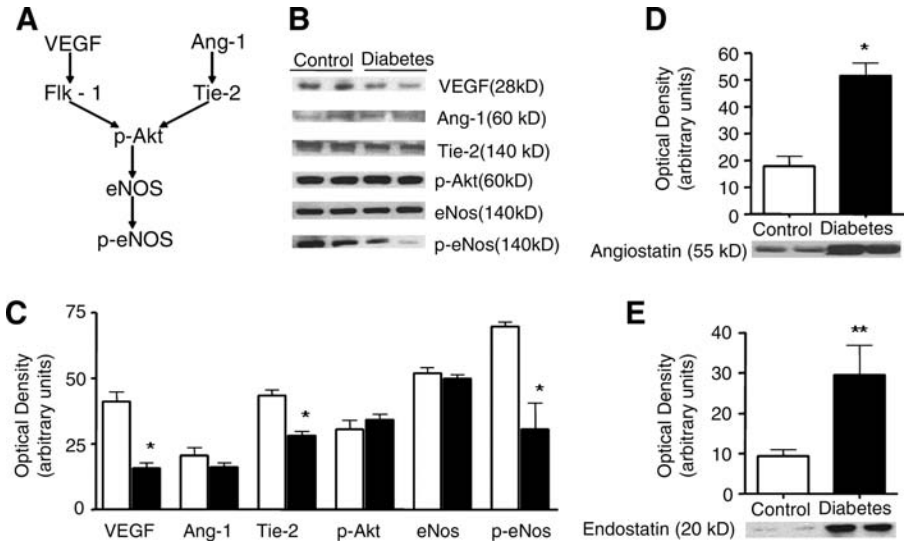

FIG. 5. (A) Schematic of proangiogenic signaling pathway, and (B) expression, and (C) quantification of proangiogenic mediators. Diabetic swine demonstrated reduced VEGF, Tie-2, and phospho-eNOS expression. Expression of antiangiogenic proteins, angiostatin (**D**), and endostatin (**E**) was significantly elevated in diabetic swine. VEGF, vascular endothelial growth factor; Ang-1, angiopoietin-1; eNOS, endothelial nitric oxide synthase.

with individuals without diabetes, making cardiovascular disease the largest cause of mortality in this population (39). Diabetic patients have accelerated atherosclerosis and also exhibit a diminished angiogenic response to myocardial ischemia, as shown angiographically (3) and in autopsy studies (128). Numerous abnormalities are found at the functional, microvascular, and molecular levels that contribute to this impaired angiogenic response.

Endothelial dysfunction in the presence of diabetes has been demonstrated in various murine (79, 103), rodent (84, 85, 104) and swine models (7) of streptozotocin (STZ)-induced diabetes as well as in coronary and noncoronary vasculature of humans with type I (20, 49, 73) and type II diabetes (68, 80, 91, 116, 121, 126). Although various mechanisms for endothelial dysfunction in diabetes have been proposed, the most commonly invoked mechanism is that of increased oxidative stress. Despite the mildly increased NOS expression and activity demonstrated in *in vitro* and *ex vivo* studies of diabetic animals, inactivation of NO by rapid reaction with reactive oxygen species appears to account for decreased NO bioavailability and impaired endothelium-dependent relaxation (19, 52, 84, 85, 102). Rosen *et al.* (84, 85) demonstrated that this increased oxidative stress in diabetic rat hearts coincides with increased levels of urine nitrites. Endothelial dysfunction seen in these studies was reversed by the administration of antioxidants like superoxide dismutase and tocopherol. Consistent with this hypothesis, increased superoxide production and increased NADPH activity has been demonstrated in coronary arteries of diabetic swine (129). Furthermore, in a diabetic rat model of hindlimb ischemia, Hirata *et al.* (46) directly correlated a diminished angiogenic response to bone marrow cell implantation to reduced plasma levels of bioavailable NO. The formation of reactive oxygen and nitrogen species, like peroxynitrite, with subsequent increase in nitrotyrosine activates poly(ADP-ribose) polymerase (PARP), a DNA-repair enzyme, which initiates an energy-consuming cycle resulting in cellular dysfunction (79, 103). Activation of PARP also induces transcription factor NF-κB, leading to the activation of a number of pro-inflammatory cytokines such as ICAM-1 and TNF-α, which have also been implicated in diabetic endothelial dysfunction. In addition to the decreased NO bioavailability due to increased oxidative stress, other proposed mechanisms for diabetic endothelial dysfunction include the uncoupling of the homodimeric eNOS (116), leading to reduced NO production, a reduced synthesis of vasodilatory prostacyclin (97), and endothelial cell dysfunction due to the chronic inflammatory state, characterized by elevated levels of inflammatory cytokines.

The dynamic involvement of the extracellular matrix (ECM) in the angiogenic process was previously described (111). Myocardial ischemia, which is a potent stimulus for collateral vessel formation, is associated with an increased breakdown of the various components of the ECM through an increased expression of elastase and matrix metalloproteinases (MMPs) and downregulation of tissue inhibitors of metalloproteinases (TIMPs). *In vitro* studies have demonstrated a diminished vascular tube formation in response to growth factors in a glycated collagen matrix (56). Nonenzymatic glycation of the ECM proteins also has been shown to reduce the angiogenic response *in vivo*. Tamarat *et al.* (105) demonstrated reduced endogenous angiogenesis in a model of hindlimb ischemia in STZ-induced diabetic mice, which was reversed by the administration of aminoguanidine, an inhibitor of advanced glycation end product (AGE) formation. Weirauch *et al.* (123) studied collateral vessel formation in permanently instrumented dogs under hyperglycemic conditions and demonstrated reduced collateral-dependent perfusion associated with increased MMP-9 activity and increased expression of the antiangiogenic protein, angiostatin.

Altered expression of angiogenic growth factors and related mediators also is known to occur with diabetes. Diminished activation of HIF-1α, a transcription factor that triggers the angiogenic response, in an acute ischemia model (62, 78, 106), as well as alterations in the expression of angiopoietins and the tie-2 receptor, have been shown in diabetic rats (78). Sasso *et al.* (88) studied the expression of VEGF and its downstream mediators in myocardial biopsies of patients with or without type II diabetes and found that whereas VEGF expression was increased, VEGF-receptor activation and downstream signaling were reduced.

The effects of diabetes on the angiogenic response was studied in a large-animal model of a 15-week period of alloxan-induced diabetes in Yucatan miniswine with subsequent creation of chronic myocardial ischemia by using a circum-

flex coronary artery ameroid constrictor (16). In this model, diabetic animals successfully achieved blood glucose levels between 250 and 400 mg/dl and demonstrated significant impairments in coronary microvascular relaxation to ADP and substance P. Furthermore, microvascular relaxation in response to VEGF was even further reduced, suggesting an impairment in VEGF signaling beyond the reduction in bioavailable NO. Collateral-dependent perfusion in the circumflex territory was profoundly reduced and was also associated with reduced left ventricular function. Consistent with the finding of reduced perfusion and function, diabetic animals had reduced vascular density compared with normoglycemic controls in the ischemic territory (Fig. 4). Together, these findings demonstrate an impaired endogenous angiogenic response to chronic myocardial ischemia.

At the molecular level, the myocardium of these animals exhibited reduced expression of VEGF and of the angiopoietin receptor, Tie-2, as well as reduced phosphorylation and activation of eNOS. Expression of antiangiogenic proteins, endostatin (about a threefold increase) and particularly, angiostatin (about a 4.5-fold increase) was also significantly elevated in diabetic animals *versus* controls (Fig. 5). Overall, this large-animal model successfully characterized the functional, microvascular, and molecular abnormalities that have been observed in human diabetes and offers an opportunity to evaluate novel therapeutic strategies to enhance the angiogenic response in the setting of diabetes.

Role of Substrate Modification

In response to limited therapeutic efficacy of growth factor–based angiogenic therapy in clinical trials, researchers have investigated a number of strategies to enhance the angiogenic response. These include the search for a more-potent angiogenic agent or combination of agents, improved and sustained delivery strategies that may involve sustained-release devices or gene-based or cell-based delivery, as well as more-targeted delivery strategies (*i.e.*, intramyocardial and perivascular delivery compared with intravenous or intracoronary routes). In addition, some investigators explored substances that can modify the substrate to improve the response to the therapeutic angiogenic agent. Substrate-modifying strategies are briefly discussed below.

L-Arginine

Numerous studies have clearly demonstrated that the presence of endothelial dysfunction is associated with impaired endogenous and exogenous angiogenic responses. This endothelial dysfunction is secondary to a reduction in bioavailable NO, which may occur because of reduced NO production or increased consumption. L-Arginine, the substrate for endothelial nitric oxide synthase, can increase the amount of bioavailable NO by increasing its production. Evidence for its pro-endothelial effects comes from animal studies as well as demonstration of improved coronary vasorelaxation in response to acetylcholine in patients with coronary disease (83). In the context of angiogenesis, studies by Jang (48) and Duan (30) in a hypercholesterolemic mouse model of hindlimb ischemia were the first to demonstrate amelioration of a blunted angiogenic response to FGF-2 administration *in vivo* through dietary supplementation with L-arginine. These concepts have since been validated in a large-animal model of hypercholesterolemic swine. In these animals, oral supplementation with L-arginine reversed endothelial dysfunction and improved collateral-dependent perfusion in response to chronic myocardial ischemia (75), as well as with VEGF (117) and FGF-2 (119) supplementation. These findings have been further translated to the clinical setting in a trial in which patients undergoing coronary artery bypass surgery with at least one non-revascularizable territory were randomly assign to receive placebo, VEGF alone, L-arginine alone, or VEGF and L-arginine in a factorial trial design (86). Although small in size, this study demonstrated trends toward smaller perfusion defects and trends toward improved angina scores in the combination-therapy group. Contrary to these findings, the VINTAGE MI clinical trial (90), which randomized patients to L-arginine *versus* placebo after acute myocardial infarction, had increased mortality in the L-arginine group. It is important to recognize in this context that patients with acute myocardial infarction are a very different patient population compared with patients with chronic stable coronary disease and that L-arginine supplementation may still hold promise as an adjunctive therapy for therapeutic angiogenesis. Finally, it should be noted that L-arginine is also a substrate for arginase and leads to the formation of polyamines, and the role of this pathway in the setting of angiogenesis has not been well elucidated.

Statins

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are commonly used in patients with coronary disease. In addition to inhibiting the rate-limiting step in cholesterol biosynthesis, statins appear to have a number of off-target or pleiotropic effects. Statins have been shown to improve peripheral and coronary endothelial dysfunction in coronary artery disease patients (8). They can increase NO bioavailability by activating Akt, which subsequently leads to endothelial nitric oxide synthase (eNOS) activation (94), as well as through its antioxidant effects (63). Interestingly, *in vitro* and murine studies have suggested a biphasic and dose-dependent effect of statins on angiogenesis (114, 124). In a swine model of hypercholesterolemia, oral supplementation with high-dose atorvastatin reversed the hypercholesterolemia-induced endothelial dysfunction, as demonstrated by normalization of microvessel relaxation to ADP and VEGF. However, improvements in endothelial function did not lead to improved endogenous angiogenic response to chronic myocardial ischemia (14) or to exogenous VEGF protein administration (12). In this model, atorvastatin-treated animals had significantly and persistently elevated levels of Akt phosphorylation, a known effect of statins, as well as increased expression of the antiangiogenic protein, endostatin. These abnormalities were also replicated in normocholesterolemic swine (11). These studies communicate that, in addition to endothelial function, other determinants of the angiogenic response can affect the response to substrate-modifying therapies.

Insulin

Glycemic control remains the mainstay of treatment in diabetes and has been shown to improve both macrovascular and microvascular complications of diabetes (1, 2). Insulin treatment, with or without oral hypoglycemic agents, is the

most common method used clinically to achieve glycemic control. Insulin has multifaceted effects on the myocardium, which mainly involve the regulation of fuel consumption, glucose transport, glycogen synthesis, and glycolysis (18). More relevant to vascular function, however, is the demonstrated ability of insulin to increase endothelial nitric oxide (NO) availability in the vasculature (125). Furthermore, the insulin receptor, which is present in the myocardium, is a tyrosine kinase receptor that shares many of the downstream mediators common to angiogenic growth factors and their receptors [*e.g.*, PI3 kinase and MAP kinases (125)]. In addition to its direct effects on the myocardium and coronary vasculature, insulin exerts indirect effects through the reduction in systemic blood glucose levels. By reducing blood glucose levels, insulin can avoid the adverse effects of chronic hyperglycemia, which include increased oxidative stress, chronic inflammation, and the nonenzymatic glycation of proteins, particularly in the extracellular matrix (122, 129). In the large-animal model of alloxan-induced diabetes and chronic circumflex territory myocardial ischemia described earlier, parenteral insulin treatment was successfully used to achieve glycemic control (fasting blood glucose, <150 mg/dl) (15). Insulin treatment resulted in significant improvements in, but not normalization of, diabetic endothelial dysfunction. Significant improvements also were observed in collateral-dependent perfusion, as well as systolic and diastolic left ventricular function and capillary density in the ischemic territory. These functional findings of an improved endogenous angiogenic response were accompanied by increased expression of proangiogenic growth factors VEGF and angiopoietin-1, as well as its receptor, Tie-2. Last, the expression of the antiangiogenic proteins, angiostatin and endostatin, which were significantly elevated in the setting of diabetes, was reduced with insulin therapy. Thus, insulin treatment holds significant potential as a substrate-modulating agent for therapeutic angiogenesis in the setting of diabetes.

Other agents

A number of agents hold some potential as substrate-modifying agents for therapeutic angiogenesis because of either their pro-endothelial or antioxidant properties. Ascorbic acid and α-tocopherol (vitamins C and E) have demonstrated antioxidant properties that may be useful in improving endothelial function in certain disease states. Preliminary evidence from rodent models of hindlimb ischemia suggests that supplementation with vitamins C and E, along with L-arginine, may enhance the angiogenic effect of bone marrow cell infusion (76). Resveratrol, an ingredient found in red wine, has been shown to have antioxidant and cardioprotective effects in a variety of disease states and may have a beneficial effect on angiogenesis (26, 107). Other agents that have pro-endothelial properties and may play a role in enhancing therapeutic angiogenesis include angiotensin-converting enzyme (ACE) inhibitors, PPAR-γ agonists, and other oral hypoglycemic agents. These substances must be studied in large-animal models, to better elucidate their effects on the coronary vasculature and the angiogenic response.

Conclusions

For more than a decade, cardiovascular researchers and clinicians have explored therapeutic angiogenesis by using growth factors or cell-based therapies as treatment options for patients with advanced coronary artery disease. A number of therapeutic agents have undergone extensive preclinical evaluation followed by phase I, II, and III clinical trials. Despite encouraging results in animal models, clinical trials have showed minimal measurable benefits in patients. These investigations, however, have given us a new level of insight into the complexity of the angiogenic process and determinants of therapeutic success. For angiogenic therapy to become a clinically viable therapeutic option, it will have to involve modulation of multiple potent growth factors and be delivered in a targeted manner to the desired territory, with a sustained effect and without major adverse effects. Additionally, the therapeutic strategy will have to address the antiangiogenic influences present in the host tissue. Improved understanding of factors influencing the substrate with approaches for substrate modification will likely be an important part of this therapeutic strategy.

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Abbreviations

ACE, Angiotensin-converting enzyme; ADP, adenosine diphosphate; AGE, advanced glycation end product; CAD, coronary artery disease; CABG, coronary artery bypass graft; DNA, deoxyribonucleic acid; ECM, extracellular matrix; FGF, fibroblast growth factor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ICAM, intracellular adhesion molecule; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MMP, matrix metalloproteinase; mRNA, messenger ribonucleic acid; NADPH, nicotinamide adenine dinucleotide phosphate hydrogen; NO, nitric oxide; NOS, nitric oxide synthase; NNA, N^{ω}-nitro-L-arginine; PARP, poly(ADP-ribose) polymerase; PCI, percutaneous coronary intervention; PDGF, platelet-derived growth factor; PIGF, placental growth factor; ROS, reactive oxygen species; STZ, streptozotocin; TIMP, tissue inhibitors of metalloproteinases; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor; VSMC, vascular smooth muscle cell.

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