

ADVANCED MOLECULAR AND CELL BASED THERAPIES FOR ATHEROSCLEROSIS

MAHAMMAD RAHMATHULLA SB^{1*}, LAKSHMI DEVI K²¹Department of Medical Biochemistry, College of Medicine and Health Sciences, Adigrat University, Adigrat, Ethiopia. ^{1,2}Department of Biochemistry, Sri Krishnadevaraya University, Anantapuram, Andhra Pradesh, India. E-mail: mdrtl.biochem@gmail.com

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ABSTRACT

Cardiovascular disease (CVD) is the most frequent cause of premature death in modern industrialized countries, accounting for 4.35 million deaths each year in Europe and 35% of deaths in the United Kingdom. Atherosclerosis is a pathological process characterized by formation of subintimal plaques that can reduce or obstruct blood flow through the vessel. It is a result of normal blood vessels aging, with initial lesion developing before age 30 (so-called fatty streaks). Risk factors that significantly accelerate this process are serum lipid impairment, hypertension, cigarette smoking, diabetes mellitus, obesity, physical inactivity and hereditary factors, i.e., positive family history of cardiovascular disorders. Compelling experimental and clinical evidence has demonstrated that both systemic and local inflammation might play a prominent role in the pathogenesis of atherosclerosis and its clinical complications. Since inflammatory processes accompany all stages of atherosclerosis, measurement of plasma/serum concentrations of circulating inflammatory biomarkers might aid in identifying individuals at high risk for CVD. Currently, employed treatment strategies include risk factor modification, conventional pharmacotherapy, and percutaneous or surgical revascularization. Yet despite these interventions disease progression often results in recurrent symptoms, with many patients exhausting all therapeutic options.

Keywords: Atherosclerosis, Risk factors, Pathophysiology, Inflammation. Biomarkers for Atherosclerosis, Polyphenols, Molecular and cell-based therapies.

INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death for both men and women in the United States and much of the western world and is predicted to be the leading global killer by 2020 [1]. CVD, most strokes, and limb ischemia are all caused by atherosclerosis. CVD are the number one killer of modern humankind. According to the World Health Organization (WHO) about 17.5 million people die every year from CVD and it is estimated this number will increase up to 20 million by 2015. Atherosclerosis comes from the Greek words; "athero" meaning gruel or paste and "sclerosis" meaning hardness. Atherosclerosis begins with damage to the artery caused by elevated levels of cholesterol and triglycerides in the blood, as well as high blood pressure. Several other factors are also associated with the onset and progression of atherosclerosis, i.e. hyperglycemia, hyperhomocysteinemia, disruption of the immune system, glycation end products, and infectious agents.

Atherosclerosis and its complications are the major cause of morbidity and mortality in both developed and developing nations. There is an urgent need to understand the pathogenesis and progression of atherosclerosis, and to develop a strategy to prevent an epidemic episode. Crucial advances in our understanding of the pathogenesis of atherosclerosis and its complications have been achieved in recent years.

Atherosclerosis and its thrombotic complications are responsible for nearly all cases of CVD [2]. Atherothrombosis is a diffuse immunoinflammatory process characterized by the deposition of lipid and other blood-borne material within the arterial wall of almost all vascular territories [3]. Atherosclerosis is a diffuse disease that progresses silently until it is clinically manifested. Epidemiological evidence has identified acute thrombus anchored on a ruptured atherosclerotic lesion, in 70-80% of cardiovascular deaths. The major characteristic of atherosclerosis is the deposition of cholesterol in the subendothelial space, leading to the narrowing of the arterial lumen. Lipid accumulation is the result of an imbalance between cholesterol influx and efflux [4,5]. The magnitude of the thrombotic process, triggered by plaque rupture, is modulated by different elements that determine plaque and blood thrombogenicity. Tissue factor

exposure, thrombin formation, fibrin deposition, platelets aggregation, circulating procoagulant microparticles, and soluble tissue factor are key players in thrombus formation and propagation [6]. The original term "risk factor" was coined by Framingham Heart Study investigators in one of their initial manuscripts published in 1961 [7]. Several actions of alcohol could explain why precisely recent heavy drinking increases the risk of ischemic stroke. Trauma to the neck, which frequently occurs during alcoholic intoxication, is certainly one reason. Another is alcohol-induced cardiac arrhythmias, which predispose to cardiogenic brain embolism. Risk factors for CVD are listed in Table 1.

The application of gene transfer, antisense oligodeoxynucleotide (ODN) technology, bone marrow-derived cell transplantation, or immunomodulation techniques to the treatment of atherosclerosis has resulted in therapeutic alternatives to restore vessel wall function and interrupt the progression of vascular disease.

PATHOPHYSIOLOGY OF ATHEROSCLEROSIS

Atherosclerosis is a chronic immunoinflammatory disease characterized by lipid and matrix deposition, neoangiogenesis, inflammation and immune activation, vessel wall remodeling, and abnormal vasomotor regulation. Inflammatory/immune gene

Table 1: Risk factors for CVD

Major risk factors
Cigarette smoking
Elevated blood pressure elevated serum total (and LDL) cholesterol
Low serum HDL cholesterol
Diabetes mellitus
Advancing age
Other risk factors
Obesity
Dietary habits
Physical inactivity
Family history of premature CHD

CVD: Cardiovascular disease, CHD: Coronary heart disease, LDL: Low density lipoprotein, HDL: High density lipoprotein

activation appears to be a common pathophysiologic underpinning in the evolution and progression of atherosclerosis. The normal artery wall is composed of two organized layers: Intima and media. The intima is made up of a single layer of endothelial cells (EC) that are seated on the basement membrane and then the internal elastic lamina (IEL). Beneath the IEL is the medial layer, comprising vascular smooth muscle cells (VSMCs) surrounded by basement membrane and embedded in interstitial extracellular matrix. The boundary of the media is marked by the external elastic lamina. All infants have focal thickening of the coronary artery intima due to VSMC proliferation [8]. Although focal thickening is an important hallmark of the developing atherosclerotic plaque, this is considered to be an adaptive response to turbulent blood flow rather than pathological. Endothelial dysfunction initiated by the risk factors already described permits the entry of lipids and inflammatory cells into the artery wall. Once in the artery, monocytes differentiate into macrophages which take up the lipid and become foam cell macrophages. This results in the formation of lesions termed "fatty streaks," recognized as the onset of atherosclerosis.

Fatty streaks are small, slightly raised lesions caused by focal collections of foam cell macrophages in the intima. They may be precursors of larger atherosclerotic plaques but may also regress. Progression of the fatty streak to a more complex lesion occurs due to the formation of a necrotic core and fibrous cap. Foam cell macrophages, engorged with lipid, begin to die and release their contents, which contributes to the formation of a necrotic core. The release of the cytoplasmic contents of the foam cells leads to the accumulation of extracellular lipids and growth factors which induce inflammation. The occurrence of VSMC migration and proliferation results in the formation of a fibrous cap. VSMCs migrate into the intima where they proliferate and deposit extracellular matrix. The increase in cell number and presence of matrix causes augmentation of the bulk of the plaque, which now protrudes into the lumen. This is termed as stable advanced plaque. The size and composition of the plaque determine its outcome.

Classification schemes have been devised to categorize the various plaque types [9-12]. A plaque with a large necrotic core, high content of inflammatory cells, and thin fibrous cap is termed an "unstable plaque," and is more prone to rupture than a plaque with a smaller necrotic core, lower content of inflammatory cells, and thick fibrous cap, termed a "stable plaque." Rupture of the plaque leads to thrombus formation, which can occlude the lumen and cause the symptoms of myocardial infarction (MI) or stroke. However, plaque rupture does not always lead to occlusion of the artery, and the plaque may restabilize and heal over. This is at a cost since the "healed plaque" is larger [13] and repeated episodes of plaque rupture and healing is associated with a greater incidence of a fatal event [14,15].

PATHOPHYSIOLOGICAL FACTORS FOR ATHEROSCLEROSIS

Atherosclerosis is multifactorial and polygenic in origin and develops decades earlier than its clinical manifestation [16]. A strong positive relation between alcohol consumption and the risk of mortality from stroke is apparent [17]. In the Scandinavian countries, binge drinking has been observed to associate with both an increased risk for ischemic stroke mortality [18] and the progression of atherosclerosis [19].

Intravascular inflammation in atherosclerosis

Inflammation is itself an adaptive, homeostatic mechanism that has evolved as a protective response to cellular injury. Tissue injury - For example infarction, trauma, or infection-initiates a cascade of interactions that constitute the process of inflammation.

Central to this mechanism is the circulating leukocytes of the immune system, including monocytes, polymorphonuclear neutrophils, and B and T lymphocytes. These cells interact with one another and with vascular EC via a vast array of surface receptors and released signaling molecules (inflammatory cytokines). The ultimate purpose of the

inflammatory response is to sequester and remove the offending substrate and, allow normal, healthy tissue to replace it. Chronic inflammation specifically involves circulating monocytes (which differentiate into tissue macrophages) and T lymphocytes that are recruited to the site of injury. It is this model of chronic vascular inflammation that has been applied to atherosclerosis and which now shapes our understanding of this disease. Atherosclerosis has long been associated with elevated cholesterol levels, and histologic evaluation of atheromata supported the notion that central to this disease is the accumulation of lipid within the vessel wall [20].

The lesions of atherosclerosis are considered to have important roles in driving the atheromatous process from initial endothelial injury to final plaque disruption and disease manifestations such as stroke and MI [21,22].

Potential serologic biomarkers of inflammation

C-reactive protein	Leukocyte-derived enzymes
Soluble CD40-ligand	Myeloperoxidase
Soluble cell adhesion molecules	Lipoprotein-associated phospholipase A2
ICAM-1	Secretory phospholipase A2 Type II
VCAM-1	Matrix metalloproteinases
E-selectin	Endothelial progenitor cells

ICAM-1: Intracellular adhesion molecule-1, VCAM-1 = Vascular cell adhesion molecule-1

BIOMARKERS FOR ATHEROSCLEROSIS

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of a physiological, as well as a pathological process or pharmacological response to a therapeutic intervention. Classical biomarkers are measurable alterations in blood pressure, blood lactate levels following exercise and blood glucose in diabetes mellitus. Any specific molecular alteration of a cell on DNA, RNA, metabolite, or protein level can be referred to as a molecular biomarker. Adipocyte enhancer-binding protein 1 has been identified as a transcriptional repressor that impedes macrophage cholesterol efflux, promoting foam cell formation, via peroxisome proliferator-activated receptor γ -1 and liver-X-receptor a down regulation [22].

Ghrelin, a peptide hormone from the stomach, stimulates food intake and decreases fat utilization. Ghrelin binds to growth hormone secretagogue receptor (GHSR). GHSR density has been shown to be upregulated in atherosclerotic lesions. Ghrelin concentrations and carotid artery atherosclerosis are positively associated in males even after adjustment for the commonly recognized risk factors of atherosclerosis [23]. List of biomarkers for atherosclerosis is shown in Table 2.

POLYPHENOLS FOR CARDIO-PROTECTIVE EFFECT

Plant-based foods have a wide range of aromas, colors, and tastes. These qualities make them distinctive and attractive for food consumption. These qualities are thought to be due to the presence of compounds called phytochemicals. Many foods and herbs contain phytochemicals, which are possibly involved in optimizing health and preventing and/or treating CVD [24]. Phytochemicals are biologically active compounds present in small quantities in plants for instance polyphenols.

Table 2: Biomarkers for atherosclerosis

Adipocyte enhancer-binding protein 1 asymmetric dimethyl arginine	Lipoprotein-associated phospholipase A2 macrophages chemoattraction
Cathepsin D	Nitric oxide: Impairment of production
E-selectin	Oxidative stress
Ghrelin	Serum amyloid A
Ma HSP-27 (low levels)	T-cell chemokine activity

HSP: Heat-shock protein

Number of studies has demonstrated that consumption of polyphenols limits the incidence of coronary heart diseases [25-28]. Atherosclerotic lesions may be present and clinically silent for decades before becoming active and producing pathological conditions such as acute MI, unstable angina or sudden cardiac death [29]. Tea catechins have been shown to inhibit the invasion and proliferation of the smooth muscle cells in the arterial wall, a mechanism that may contribute to slow down the formation of the atheromatous lesion [30]. Association between polyphenols intake or the consumption of polyphenol-rich foods and incident of CVD were also examined in several epidemiological studies and it was found that consumption of polyphenol-rich diet have been associated to a lower risk of MI in both case-control and cohort studies [31]. The studied plant extract contains antioxidants and hepatoprotective activity through a regulatory action on cellular permeability, stability and suppressing oxidative stress. A number of scientific reports indicated that certain flavonoids, triterpenoids and steroids have protective effects on the liver [32-34].

MOLECULAR AND CELL BASED THERAPIES

Novel cell-based therapeutic strategies are being developed in response to the short-comings of available treatments. Potential repair by cell grafting or mobilizing endogenous cells holds particular attraction in heart disease, where the meager capacity for cardiomyocyte proliferation likely contributes to the irreversibility of heart failure.

Antisense ODN

An alternative approach to the modulate atherosclerosis utilizes a methodology to decrease the expression of proteins integral to atheroma formation by targeting transcription and translation. Several different technologies have been employed, with varying degrees of success owing to technical limitations associated with specificity and/or long-term activity. One approach is the use of antisense ODN that are complementary to the messenger RNA (mRNA) of interest and which bind stoichiometrically to mRNA sequences. The second method utilizes ribozymes, a unique class of RNA molecules that catalytically cleave specific target RNA species, leading to their degradation. Transfection of cis-element double-stranded decoy ODN attenuates cis-trans interaction, leading to the removal of transactors from the endogenous cis elements, with subsequent modulation of gene expression [35].

Antisense ODN suppress gene expression at the RNA level, are generally nucleic acids in length and are designed to have a sequence that is complementary to a segment of the target gene mRNA. These compounds form a heteroduplex with target RNA to block translation, either by sterically inhibiting ribosome movement along the mRNA or by mediating its destruction by activation of RNase H. Although antisense ODN decrease target protein expression, their utility as therapeutic agents is limited by their non-specific biological effects. Oligomers that contain stretches of G nucleotides form a structure known as a G-quartet that can interact in a sequence-independent manner with growth factors, signaling molecules, enzymes, and components of the extracellular matrix [36-41].

Antisense ODN to regulators of cell growth, including c-Myb, c-Myc, NFκB, p65, CDK2, and CDC2 kinase/proliferating-cell nuclear antigen, have been used to decrease neointima formation in experimental vascular injury and atherosclerosis models [42]. Other studies have utilized antisense ODN to modulate atherosclerotic plaque composition [43]. For example, rabbits were injected with an antisense ODN to cholesteryl ester transfer protein, an enzyme that facilitates the transfer of cholesteryl ester from high-density lipoprotein (HDL) to apoB-containing lipoproteins, and fed a high-cholesterol diet for 16 weeks. After this time, total cholesterol was decreased, and HDL cholesterol was increased compared to control or sense ODN-treated rabbits. Furthermore, aortas harvested from antisense ODN-treated rabbits demonstrated a decreased atheroma burden and fewer lipid-laden plaques compared with control animals [44].

Antisense ODN have also been utilized for genetic engineering of bypass grafts to render them resistant to atherosclerosis. In early studies, investigators targeted the cell cycle regulatory proteins CDC2 kinase/proliferating-cell nuclear antigen to limit VSMC proliferation. Interestingly, this strategy reduced neointimal hyperplasia and increased medial hypertrophy in rabbit jugular veins that were grafted into the carotid arteries, suggesting redirection of vein graft biology toward that observed for normal arterial conduits [45]. Similarly, transfection of bypass grafts with antisense ODNs directed against CDC2 kinase or the antiapoptotic mediator Bcl-xL has been shown to limit neointima formation and the graft failure associated with cardiac transplantation in a murine heterotopic cardiac allograft model [46,47].

Bone marrow-derived progenitor cell transplantation

The complexities associated with bone marrow-derived cell transplantation are, in part, due to the diverse composition of bone marrow. Owing to the presence of hematopoietic stem cells and other early precursor cell lines, the efficacy of experimental protocols often lies in the sorting procedures applied prior to transplantation. Some studies have transplanted unsorted bone marrow cells, whereas others have isolated specific cell populations, such as pluripotent mesenchymal stem cells or the mononuclear fraction. Furthermore, depending on the isolation technique (e.g. simple isolation versus growth and differentiation in culture), the cell fractions may differ significantly in their composition and phenotype. It is, therefore, difficult at times to compare results between studies and, to date, unclear which cell line offers the greatest therapeutic efficacy.

Initial studies in small animal models suggested that the direct injection of unsorted bone marrow cells expanded *in vitro* improved left ventricular salvage in MI models. This finding has been attributed to the observation that these cells are capable of differentiating into cardiomyogenic cells *in vivo* to regenerate infarcted myocardium [48]. Bone marrow-derived cells were pretreated with 5-azacytidine prior to implantation in a rat MI model to induce cardiomyogenic differentiation, and infused into the coronary circulation. After 8-9 weeks, bone marrow cells were identified in the myocardial scar and peri scar tissue, and these cells were found to express the cardiomyocyte-specific protein troponin I. Bone marrow-derived cells were also found to form gap junctions with adjacent host cardio myocytes. Incorporation of bone marrow cells resulted in a significant improvement in fractional shortening and end-diastolic and end-systolic diameter of the left ventricle. This study suggested that bone marrow cells could be delivered via a percutaneous approach, and that, once infused, cells were capable of targeted migration and differentiation to improve cardiac function [49]. Although these studies differ with respect to animal model and method of delivery, the studies performed in sheep suggested that unsorted bone marrow-derived cells were not enriched for a cell population that was efficacious in improving the contractile function of post-infarction scar tissue [50].

Endothelial progenitor cells (EPC)

Recent attention has focused on bone marrow-derived EPC to effect repair of atherosclerotic vascular lesions. The reservoir of EPC has been shown to contribute to neovascularization following vessel wall injury, implying that methodologies to modulate the function of these cells may represent a novel therapeutic intervention.

Bone marrow-derived and peripheral-blood EPC express a number of endothelial-specific markers, including vascular endothelial growth factor receptor-2 (VEGFR-2), Tie-2, vascular endothelial cadherin, CD34, CD146, and E-selectin. These markers have been exploited to isolate cells from the bone marrow and peripheral blood. EPC also express the stem cell marker CD133, and CD133+VEGFR-2+ cells may be induced to differentiate to mature CD133-VEGFR-2 - ECs that participates in neovascularization. At any given time these cells represent only 0.01% of the total cells in the circulation. Following trauma or injury, however, this number may increase up to 12% [51,52].

Numerous studies have been performed to evaluate the contribution of circulating EPC in atherosclerotic vascular disease and vessel wall repair in an effort to determine the therapeutic value of EPC transplantation. EPC have been targeted specifically as a deficit in the number of circulating EPC has been associated with an increased risk of CVD [53]. Furthermore, in patients with established cardiac disease, risk factors for coronary artery disease have been shown to influence EPC number and function. In these patients, tobacco use was found to be the major independent predictor of decreased EPC levels, whereas impaired progenitor cell migration was influenced predominantly by hypertension [54]. It has also been speculated that EPC senescence may contribute to the increased risk of atherosclerosis associated with aging [55]. Preliminary clinical studies, therefore, suggest that bone marrow-derived mononuclear cells may have therapeutic utility and minimal adverse side-effects [56]. Macrophages from these mice expressed levels of apoE that were comparable to those from wild-type mice, and vector-driven expression of apoE in macrophages was sufficient to reverse both hypercholesterolemia and atheroma development in this model [57].

Immunomodulation

Atherosclerosis is recognized as an inflammatory disorder, and immunohistochemical analysis has confirmed the presence of polyclonal memory T lymphocytes in atherosclerotic vascular lesions. These CD4+T-cells were found to be reactive against oxidized low density lipoprotein (ox-LDL), thereby identifying a major target of the cellular immune response in the plaque. These T-cells are of the T helper-1 subset and secrete the proinflammatory cytokines interferon- γ (IFN- γ), interleukin-2 (IL-2), and tumor necrosis factor- α to activate macrophages and promote inflammation. Activated T-cells also express CD40 ligand which binds to its receptor CD40 on macrophages, B-cells, EC, and VSMC [58]. B-cells are also believed to play a role in the immune response to atherosclerosis as elevated levels of circulating antibodies to ox-LDL have been demonstrated in patients with advanced atherosclerosis [59]. These findings suggest that atherosclerosis, as an inflammatory disorder of the vessel wall, lends itself to immunomodulation as a therapeutic intervention.

Autoantigens

Several putative target antigens to initiate an immune response have been identified and studied in both animal models and humans. There is abundant evidence to demonstrate that ox-LDL plays a significant role in the observed immune response in atherosclerosis. In apoE knockout mice with aortic plaques, CD4+ and CD8+ T-cells have been found in the lesions and the mice have elevated circulating levels of antibodies against modified LDL. Furthermore, T-cells specific for epitopes present on ox-LDL have been demonstrated in human atherosclerotic lesions, and scavenger receptors can modulate the uptake of ox-LDL for presentation to antigen-specific T-cells [58].

A second group of candidate autoantigens is the heat-shock protein (HSP) family. These proteins are secreted by injured cells to act as chaperones and limit the denaturation of other cellular proteins. HSP has also been shown to serve as targets for an autoimmune response in inflammatory disorders. In fact, HSP70 has been located in human plaques, and experimental studies with rabbits have shown that immunization with mycobacterial HSP65 yields atherosclerotic-type lesions in the absence of a hypercholesterolemic diet. Anti-HSP60 antibodies have been detected in the blood of atherosclerotic animal models and immunization with HSP60 augments disease in both mice and rabbits. HSP60 may also activate toll-like receptor-4 in a manner similar to endotoxin, suggesting that HSP60 may activate innate immunity as well as T and B-cells [60].

It has been suggested that atherosclerosis is mediated by viral infection, and this hypothesis is supported by the discovery of viral genomes, including herpes simplex, cytomegalovirus, and *Chlamydia pneumoniae*, in atherosclerotic plaques. Interestingly, chlamydia

HSP60 resembles human HSP60 and may elicit similar inflammatory responses. Yet despite these findings, studies performed to establish a link between infectious agents and atherosclerosis have yielded conflicting results [59,61].

Immunomodulation therapies

Therapeutic efforts to reduce atherosclerotic lesion burden by immunomodulation have investigated immunosuppressive agents, targeted therapies to reduce inflammatory cytokines, and immunization strategies. Cyclosporin A, an immunosuppressive drug utilized in solid organ transplantation, has been associated with accelerated atherosclerosis; however, the role of cyclosporin A in the development of atherosclerosis remains controversial, owing to conflicting experimental observations. For example, in hyperlipidemic C57BL/6 mice the administration of cyclosporin A resulted in advanced atherosclerotic lesions, suggesting that a T-cell-mediated response was atheroprotective. In contrast, using a rat cardiac transplant model, other investigators have shown that cyclosporin A did not promote atherosclerosis, and studies in CD4+CD8+T-cell depleted mice revealed a reduction in fatty streak formation, implicating T-cells in the pathogenesis of atherosclerosis [62]. The net effect is manifested as an acute inflammatory response in the media of the vessel wall, with subsequent neointimal formation [63]. The IL-1 receptor antagonist, an endogenous inhibitor of IL-1, has been shown to decrease fatty streak formation in apoE knockout mice, and IL-1 receptor antagonist expression has been demonstrated in human coronary artery EC [64,65]. Interestingly, intravenous immunoglobulin preparations were also shown to contain anti-ox-LDL antibodies, suggesting another mechanism by which this preparation may inhibit atherosclerosis. Therapies to reduce levels of inflammatory cytokines have also proved successful in decreasing atherosclerotic plaque formation. The IL-1 receptor antagonist, an endogenous inhibitor of IL-1, has been shown to decrease fatty streak formation in apoE knockout mice, and IL-1 receptor antagonist expression has been demonstrated in human coronary artery EC [66].

CONCLUSION

Recent advances in our understanding of the vascular biology of atherosclerosis have defined this disease process as a complex disorder of the vessel wall characterized by ineffective repair mechanisms and a heightened inflammatory response.

Atherosclerosis is now widely viewed as a chronic inflammatory disease that may someday respond to targeted therapy. Advancements in our basic understanding of the molecular mechanisms of vascular wall inflammation are now yielding novel targets for pharmacotherapy and gene therapy that may affect the course of disease. Measurable indices of inflammation predict risk for atherothrombotic events, even in individuals with previously unrecognized atherosclerosis, and may be used to follow response to therapy. Through the prism of inflammation, lines of previously independent investigation now appear to converge, revealing an ever more complex interdependency. By targeting the molecular and cellular mechanisms that are dysregulated in atherosclerosis, gene transfer, antisense technology, EPC transplantation, and immunomodulation have each demonstrated efficacy in decreasing atheroma formation. Future efforts to combine these strategies may offer enhanced therapeutic benefit in the treatment of atherosclerotic vascular disease.

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