Cardiovascular polygenic disorders

Circulating cAMPft + solute and water reabsorption (kidney)

Network of pathways and genes postulated to be associated with blood pressure regulation.

ACE, angiotensin-converting enzyme; ADH, antidiuretlc hormone (vasopressin); ANP, atrial natriuretic peptide; AT1R, angiotensin II type 1 receptor; AV, atrioventricular; ECE, endothelin-converting enzyme ELAM, endothelial leukocyte adhesion molecule 1 (E-selectin); ET-1, endothelin-1; IP3, inositol tris-phosphate; NO, nitric oxide; NOS, nitric oxide synthase; SAH, SA hypertension-associated homolog (rat); 7-TMD, seven-transmembrane domain. Reprinted from Marteau J-B, Zaiou M, Siest G, Visvikis-Siest S. Genetic determinants of blood pressure regulation. *J Hypertens* 2005; 23: 2127-2143 with permission from Lippincott Williams and Wilkins.

CHAPTER 6

Atherosclerosis

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Introduction

For hundreds of years observers have been interested in the unusual lesions of atherosclerosis. Renaissance artist Leonardo da Vinci complained of the “waxy fat” that made some arteries difficult to draw and provided some early descriptions of arteriosclerosis in elderly men. The first complete and accurate description of intimal atheromatous lesions is that of the eighteenth century Italian anatomist Antonio Scarpa who wrote graphically of the “slow, morbid ulcerated, steatomatous, fungus,

squamous degeneration of the internal coat of the artery." The word "atheroma" derives from the Greek for gruel or porridge and was first used with reference to human arteries by Swiss physiologist Albrecht von Haller in 1755; the term "atherosclerosis" as we use it today to describe disease of the coronary artery intima was coined in the first years of the twentieth century by Leipzig pathologist, Felix Marchand [1,2].

Data from previous clinical and epidemiologic studies have shown that several risk factors, including age, male sex, family history of myocardial infarction (MI), increased serum total and low density lipoprotein cholesterol (LDL-C), decreased serum high density lipoprotein cholesterol (HDL- c), smoking and diabetes mellitus, predict the risk for atherogenesis [3-11]. More recently, inflammation linked with disadvantageous plasma lipoprotein profile and chronic infections were suggested as risk factors for coronary artery disease (CAD) [12-14]. It has become evident that atherosclerosis is a complex multifactorial phenomenon. Furthermore, risk factors appear to cluster and interact in individuals and families, making it challenging to determine the level of risk. Evaluation of risk is further hampered by the largely unknown relationship and interactions among the underlying genetic and environmental factors. Currently, atherosclerosis is considered to be a highly complex heterogeneous disease involving the actions of more than 400 genes [15] and an ever increasing number of genetic, environmental and endogenous risk factors continue to be identified as acting singly and in combination to modify gene expression to contribute to or to protect against the development of CAD. Today atherosclerosis, presenting as CAD, stroke and peripheral artery disease, is the most common cause of morbidity and mortality in western and westernizing societies. About half of all people in the USA die from atherosclerosis-related complications, and cardiovascular disease world-wide is expected to increase significantly over the next 20 years [16].

Up until about 20 years ago, the lesions of atherosclerosis were mainly regarded as degenerative by-products of the atherosclerotic process;

atherosclerosis itself was considered to be a build up of bland degenerative lipid products, and angina and thrombosis were thought to be the consequence of narrowing of the artery to occlusion as a result of lipid accumulation. However, this view has drastically changed. For the past 20 years researchers have been dissecting out the intricate cellular and molecular signaling and communication pathways, the genetic, molecular and cellular activity that drives atherosclerotic lesion formation. Thanks in large part to this work, atherosclerosis today is regarded as a complex, ongoing inflammatory process. 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 [17].

In this chapter we explore first the cellular and tissue changes that initiate the process of lesion formation in atherosclerosis and, second, the molecular and gene level changes as the disease advances from benign to increasingly more dangerous. Studies in the molecular biology of atherosclerosis over the past two decades have provided numerous clues to novel diagnostic, prognostic and therapeutic approaches to atherosclerosis and in the final section we review how molecular biologic insights can be translated into clinical applications for the future.

Endothelial dysfunction and lesion formation: A general view

Theories of the pathogenesis of atherosclerosis have undergone considerable changes in the past few decades. Earlier conceptualizations of atherosclerosis as for the most part a disorder of lipid storage have given way to the “response to injury” hypothesis originally described by Ross and Glomset [18]. In this model, atherosclerosis is considered to be essentially an inflammatory and immune response process, triggered and maintained as a response to ongoing systemic biochemical injury (reviewed in [19,20]). This hypothesis focuses on the cardiovascular system not as a set of passive mechanical structures but at the molecular

biologic level as active players in atherosclerotic events. Gimbrone and Topper [21] have well described the blood vessel as a “community of cells.” It is by exploring the microcomponents of this community and their inter – and intracellular interactions and signals as they work together to maintain homeostasis in health and become maladaptive and ultimately self-destructive in disease that we can best understand atherosclerosis.

The lesions of atherosclerosis start with vascular endothelial dysfunction. The vascular endothelium is the 700 m2, single-cell-thick luminal lining of the vascular system. The healthy functioning of the vascular endothelium is the first line of defense against atherosclerosis and this highly interesting tissue has lately received a great deal of research attention [21-23].

The endothelium is most obviously a tissue of structural importance. It is the innermost layer of the artery and acts as a barrier between the blood flowing in the intravascular space and the wall of the artery itself. The endothelium is also a complex and active tissue, even a cardiovascular organ in its own right, with paracrine, endocrine and autocrine functions [21]. The endothelial cells synthesize and release vasoactive substances and have a number of important functional properties. First, healthy endothelial cells regulate the vascular tone of the cardiovascular system; second, they are antithrombotic, inhibiting platelet aggregation and coagulation so that blood circulates through the arterial vessels without clotting; and third, the endothelium is nonadhesive. Endothelial cells are able to sense changes in their microclimate; to signal these changes to other cells and to respond to alterations in order to maintain vascular homeostasis.

It is thus in specific areas of the vascular system where the endothelium is functioning less well that the first changes leading to atherosclerosis become evident. In areas of endothelial dysfunction the endothelial cells lose some protective function, becoming pro – rather than anti-

atherogenic. In particular, in areas of altered function the endothelium becomes more permeable to plasma lipoproteins, shows increased monocyte adhesion, altered vasoreactivity and other signs of inflammatory changes begin to occur in the vessel wall.

It is at these areas of endothelial dysfunction that the first lesions associated with atherosclerosis develop, the so-called fatty streaks. Fatty streaks are yellowish inflammatory lesions that develop in the intima within the artery wall. Varying from the size of a pinhead to covering large areas, fatty streaks are to be found in the arteries of very young children [24] and even in premature fetuses [25], and by the age of 10-14 years some 50% of childhood autopsy specimens show evidence of fatty streak lesions [26]. In humans, fatty streaks can be found in the aorta in the first few years of life; the coronary arteries in the teens; and cerebral arteries in third to fourth decades of life [16].

Analysis of characteristic fatty streak lesions finds them to contain inflammatory monocytes and T lymphocyte cells as well as oxidized lipids such as low density lipoprotein (LDL). Intra – lesional monocytes become macrophages, able to digest large amounts of the surrounding oxidated lipoprotein particles. Such lipid laden macrophages, or foam cells, together with T cells form the bulk of fatty streaks. It is these lipid filled foam cells that account for the gruel – or cereal-like yellowish substance that is observed in atheroma.

The next stage in development of atherosclerosis is the formation of “intermediate or fibrofatty lesions.” Similar in appearance to the fatty streaks, intermediate lesions are more complex in composition. Ongoing inflammation stimulates smooth muscle cells to migrate to the area of injury. Contractile smooth muscle cells undergo phenotypic changes, becoming noncontractile and then fibrous.

Neither the fatty streaks nor the more complex intermediate lesions are immediately harmful. Such lesions can in fact be viewed as protective

responses to insult, as is wound healing in general in the body. If the injury were a one time or occasional event, then these changes would be benign and reversible. However, as Ross [26] observes, in the atherogenic milieu the biochemical injury tends to be constant and chronic, such as smoking, diabetes or hypercholesterolemia. Such ongoing insult prevents the inflammatory process from ceasing, becoming maladaptive and harmful as the lesions continue to develop.

The next stage of atherogenesis is the formation of arterial plaques. These are well-defined lesions containing a lipid core, collagen, elastic fibres and proteoglycans and covered with cap of fibrous tissue, composed of smooth muscle cells and connective tissue. Patients with early atherosclerotic plaques often do not exhibit symptoms of chronic ischemia as the arterial lumen can be remarkably normal at this stage, because of the phenomenon of positive remodeling. However, the absence of symptoms does not mean that these patients are not at risk of acute atherothrombosis, as even the smallest of plaques is liable to rupture. As the plaque grows, the lesions may obtrude into the artery lumen and it was long thought that it was this gradual and insidious narrowing of the lumen over time that was the causal event in angina and coronary thrombosis. However, it is now believed that although occlusion may have a role in some cases, it is the physical disruption of plaques that is the cause of acute coronary events. That is, the lesions become unstable and either stimulate *In situ* thrombosis or, more rarely, break off to form distal emboli. Either of these events can cause obstruction of the coronary arterial tree, which ultimately results in the manifestation of acute cardiac ischemia.

Steps in lesion formation: Molecular and gene levels

The challenge for the molecular biologist is to identify the genes and gene expression changes that drive the pathologic process of lesion formation from dysfunction of the normal healthy endothelium to fatty intimal

deposits, plaque formation, rupture and thrombosis [23].

The initiating events in endothelial dysfunction have long been a subject of investigation. If endothelial dysfunction were solely a consequence of biochemical risk factors such as smoking or high cholesterol or homocysteine levels then we would expect that blood vessels would be uniformly prone to disease: that atheroma would be found throughout the length of the arteries. However, it has long been known that atherosclerotic lesions do not occur uniformly or randomly throughout the vascular system. On the contrary, atheroma is more likely to be found in specific locations in the vasculature: in arterial branches and bifurcations; straight vessels, by contrast, are less likely to develop atherogenic lesions [27].

The most relevant geographical differences between vascular regions where atheromatous lesions occur compared with where they do not occur is that blood flow patterns are significantly altered in arterial branches compared to straight arterial regions. Hemodynamic shear stress forces such as turbulence tends to affect curvatures in the arterial landscape; straight vessels experience uniform, relatively constant laminar shear stress. Thus, shear stress may act as a “local” risk factor, contributing to endothelial dysfunction.

Are there genes whose expression may be altered under conditions of altered shear stresses? One such molecule is the nitric oxide synthase 3 *(NOS3)* gene encoding the enzyme endothelial cell nitric oxide synthase (eNOS). The *NOS3* gene is a key factor linking shear stress and endothelial dysfunction.

The biologically ubiquitous gas nitric oxide was named molecule of the year by *Science* magazine in 1992 [28]. Nitric oxide was identified as the original

Endothelium derived relaxant factor [29] and is of crucial importance in

maintaining healthy endothelium [30]. In vascular tissue, nitric oxide is atheroprotective: it regulates vascular tone and vasomotor function, it counteracts leukocyte adhesion to the endothelium, opposes vascular smooth muscle proliferation and inhibits platelet aggregation. Nitric oxide is thus a major component of defense against vascular injury, inflammation and thrombosis [22].

Nitric oxide is synthesized in endothelial tissue by eNOS encoded by *NOS3*. The *NOS3* gene expression is influenced by biomechanical fluid shear stresses generated by local conditions of blood flow. Cultured human endothelial cells undergo gene expression changes under conditions of altered shear stress. For example, Topper *Et al.* [27] showed that steady laminar blood flow, mimicking conditions occurring as blood flows through straight vessels, upregulates NOS3, as well as manganese superoxide dismutase and other atheroprotective genes. By upregulating genes that are anti-oxidant, antithrombotic and anti-adhesive, laminar blood flow creates conditions within the artery that are athero – protective. Turbulent blood flow, by contrast, which might occur at artery branches, does not appear to upregulate *NOS3*. Thus, specific areas within the artery may have decreased local nitric oxide production, leading to endothelial dysfunction at these vulnerable areas.

Areas of altered endothelial function are also characterized by increasing "stickiness" of the endothelial cells to circulating monocytes. Normally, mononuclear leukocytes circulate freely through the blood and do not adhere to the vascular endothelium. In conditions conducive to atherosclerosis such as hypercholesterolemia, however, large numbers of mononuclear cells can be found attaching to the endothelium in the specific areas prone to atheroma [31,32].

Abnormal localized stickiness is a result of endothelial cell activation or overexpression of specific leukocyte adhesion molecules. Leukocyte adhesion molecules are proteins which, when activated on the surface of

the endothelial cells, increase adherence of monocytes and T cells to the endothelial surface [33]. Adhesion molecule expression occurs at the specific focal sites that are prone to develop atherosclerosis. Normal endothelium shows little to no such expression of adhesion molecules.

One of the first endothelial adhesion molecules to be identified was vascular cell adhesion molecule 1 (VCAM-1). VCAM-1 is specialized to recruit circulating leukocytes, specifically monocytes and T lymphocytes. Early experiments showed that in response to cholesterol feeding in rabbits, endothelial cells express mononuclear leukocyte selective VCAM-1 in localized areas of the aorta close to developing atheroma [34]. VCAM-1 expression is increased in early stage disease [35] and is also expressed in advanced atherogenic plaques [36]. Mice genetically engineered with defective VCAM – 1 expression showed reduced foam cell lesion development [37]. Other adhesion molecules upregulated in atherosclerosis include P selectin, E selectin and intercellular adhesion molecule 1 (ICAM-1) [33].

Once monocytes and T cells attach to activated endothelial cells via adhesion molecules, these cells are able to effect passage through the single cell layer of endothelium, between the endothelial cells and into the coronary vessel intima. Little is known about the process of transmigration. Specific proinflammatory chemokines are expressed in atheroma and have been identified, including monocyte chemoattractant protein 1 (MCP-1) [38] and various T-cell chemoattractants [39].

Endothelial dysfunction is also characterized by increased permeability to lipoproteins [21]. It is now firmly established that cholesterol levels are important contributors to atherogenesis, in particular levels of LDL. In endothelial dysfunction, circulating LDL attaches itself to the wall of the artery in the areas that endothelial cells have become altered because of shear stress. Via transcellular or pericellular mechanisms, LDL transmigrates through the normally impermeable endothelial barrier into

the intima.

Although LDL circulating in plasma is nontoxic, once trapped in the subendothelial matrix, LDL seems to become more susceptible to enzymatic induced oxidative changes, becoming oxidized LDL (oxLDL), a proinflammatory substance [20]. For example, oxLDL contains bioactive lysophos – phatidyl choline and other phospholipids, which act to upregulate several genes including the adhesion molecules VCAM-1 and ICAM-1 and growth factors such as platelet derived growth factor (PDGF) and heparin epidermal binding growth factor-like protein (HB-EGF) [40,41]. Growth factors are important mediators of smooth muscle cell and fibroblast migration and proliferation. The exact role of oxLDL and the relevance of this modified lipoprotein to atherosclerosis continue to be under investigation.

The combination of monocytes and oxLDL within the arterial intima initiates a number of gene changes driving the activation of monocytes. This transformation involves the activation of molecular scavenger receptors on mononuclear cells. Scavenger receptors are proteins structured to be able to recognize and rapidly to accumulate oxLDL [42]. Several molecules of this class have been identified including scavenger receptors of the SRA series and CD36 (reviewed in [20]). Genetically engineered ApoE deficient mice lacking scavenger receptor expression are less likely than control mice to develop atherosclerosis [20].

Macrophages contribute to host cell defense by acting as phagocytes, recognizing and removing foreign or noxious substances, and it is thought that initial macrophage removal of cytotoxic and inflammatory oxLDL is a protective process [20,43]. Macrophages also act as signaling cells in the inflammatory cascade and release cytokines such as tumor necrosis factor α (TNF-α) [44,45]. Macrophage derived TNF-α has important proinflam – matory autocrine and paracrine effects. Cell stimulation by TNF-α leads to the downstream activation of the proinflammatory

transcription factor nuclear factor κB (NF-κB) [46]. Activation of NF-κB leads to the upregulation of various adhesion molecules (e. g. VCAM-1, E selectin), the production and release of proinflammatory cytokines (e. g. interleukin-1β [IL-1β]) and the induction of molecules that favor a prothrombotic state (e. g. tissue factor, plasminogen activator inhibitor 1) [47]. Therefore, TNF-α has a net effect of propagating endothelial dysfunction as well as promoting the influx of more inflammatory cells.

TNF-α increases expression of macrophage- produced iNOS. We have earlier spoken of NO as atheroprotective when produced in small amounts by endothelial cells. When produced in high levels by macrophages, inducible NO (iNOS) is antimicrobial. However, antimicrobial NO is also atherogenic. The high levels of NO produced by iNOS damages proteins and DNA [48].

Macrophages can also secrete IL-1β which is another potent proinflammatory cytokine [49]. IL – 1β has similar effects to TNF-α as it also induces NF-κB activation [47]. Indeed, the importance of IL-1β in atherogenesis was illustrated by a study that showed that the blockade of IL-1β in an ApoE -/- mouse model of atherosclerosis attenuated the formation of atherosclerotic plaques [50].

Macrophages that have taken up great quantities of lipids are called foam cells, and lesions full of foam cells are called fatty streaks. The initial pathologic changes in the fatty streak are fully reversible. However, with persistence of the atherogenic stimuli, the fatty streak may progress to become an atherosclerotic plaque. The conversion of the fatty streak to the atherosclerotic plaque occurs as a result of the death of foam cells and the influx of vascular smooth muscle (SMC) cells to form the characteristic lipid core and fibrous cap, respectively. The origin of the SMCs in atherosclerotic plaques is currently a matter of debate. Classically, plaque SMCs are believed to arise from the preexisting medial SMCs [51].

More recently, it has been proposed that at least some of the plaque SMCs are derived from stem cells [52]. Whatever the origin of the SMC in the atherosclerotic plaque, their phenotypic characteristics are different from those of SMCs found in healthy blood vessels. Normally, SMCs are quiescent, contractile and nonproliferative and act to control artery tonus. Within the atherosclerotic environment SMCs become noncontractile and proliferative. SMCs become altered under the influence of cytokines and growth factors from leukocytes, platelets and endothelial cells. γ-Interferon (IFN-γ), which is secreted by CD4+ lymphocytes of the Th1 phenotype, has been shown to promote vascular SMC proliferation [53,54]. Indeed, this effect has been suggested to be caused by the induction of the PDGF receptor in SMCs [54]. Another molecule, macrophage inhibitory factor (MIF), a potent proinflammatory cytokine secreted by SMCs, endothelium and macrophages, has been shown to promote SMC proliferation [55,56]. Indeed, MIF immunoreactivity has been demonstrated to co-localize to areas of atherosclerosis [55]. In addition, MIF -/- mice are more resistant to atherosclerosis than their MIF +/+ littermates [53]. Analysis of atherosclerotic lesions from MIF -/- mice has shown decreased lipid deposition and reduced smooth muscle proliferation and intimal thickening.

Conversely, some cytokines have been demonstrated to suppress SMC proliferation. The antiinflammatory cytokine, IL-10, has been observed to inhibit the proliferation of SMC after vascular injury [57]. In addition, IL-10 hyperexpression in a murine model of atherosclerosis has been shown to reduce plaque formation [58].

Many of the cytokine-induced SMC changes are mediated by modification of gene expression. Indeed, *In vitro* stimulation of SMCs by TNF-α induced the upregulation of more than 50 genes and the downregulation of around 20 others [59]. The genes whose expression are affected by TNF-α treatment are diverse, and they range from those involved in the immune response through those encoding structural proteins to those

involved in cellular metabolism [59].

The next stage in atherosclerosis is the formation of plaque [60]. It is now thought that it is the rupture of the plaque leading to thrombosis rather than stenosis leading to occlusion that is the precipitating factor in the majority of acute atherosclerotic events [61,62]. Plaque rupture is now suspected to be the cause of approximately 70% of fatal MI and sudden coronary deaths [63].

The atherosclerotic lesion is composed of a mass of fatty material and inflammatory cells overlaid with a fibrous cap. The major question in considering plaque lesions is: What causes some lesions to suddenly rupture and cause life-threatening thrombosis? [64]. Gene expression changes and other factors that contribute either to plaque stability or to plaque instability are subjects of increasing interest in this regard (reviewed in [60,65]).

Stable and unstable plaques show striking dissimilarities in architecture and histology. The unstable vulnerable plaque lesion has a large lipid core, which can comprise some 40% and upwards of the lesion, and a thin fibrous cap [66]. Stable plaque comprises about 14% lipid and the stable cap, which can occupy more than 70% of the lesion, acts to protect against rupture [61]. The unstable lesion also contains large numbers of inflammatory macrophages and T cells [22]. By contrast, the stable cap is relatively nonactivated and noninflammatory [60].

The stability of the fibrous cap is largely determined by its collagen content. Collagen and other cap proteins such as elastin and glycosaminoglycans are in turn synthesized by the SMCs. In the unstable inflammatory lesion, fibrous cap thinning is caused, on the one hand, by increased collagen breakdown by proteases and, on the other, by decreased collagen synthesis by SMCs.

The formation of the calcified plaques is also dependent on gene

expression. Genes that are active in bone formation are also activated during plaque formation [67,68]. This may also contribute to plaque stability.

It is now recognized that collagen and elastin breakdown are affected by activated macrophages that overproduce matrix metalloproteinases, in particular collagenases and gelatinases, enzymes that have a key role in breaking down collagen and elastin [16]. Advanced plaque is also made up of about 20% T cells, which produce the lymphokine IFN-γ that inhibits the production of collagen matrix by SMCs. Macrophage content in unstable plaque is high and, in turn, macrophage expression of these proteins is strongly induced by inflammatory cytokines such as TNF-α, PDGF and IL-1 [69-71].

The other important process occurring in plaque destabilization is a reduction in SMCs. Cytokines such as TNF-α and IFN-γ induce apoptosis of SMCs. As a consequence, SMC synthesized collagen, elastin and glycosaminoglycans decrease and large areas of necrotic and apoptotic SMCs begin to accumulate in unstable plaques [72-74]. Increased apoptosis of SMCs brings into play the Fas death pathways and other cell death pathways. The lipid cores of unstable plaque lesions have been described as a cemetery of cells and cell types [74]. Extracellular protein tenascin C, which regulates cell adhesion, both induces metalloproteinase expression and causes SMC apoptosis. It is not present in normal vessel but is expressed in unstable plaque [75,76].

Plaque rupture usually occurs specifically at the edges of the lesion. It is here that inflammatory activity is the most intense, T lymphocytes and lipid-filled macrophages predominate and SMCs are less common. When the cap ruptures the contents of the lesion plaque lipids, tissue factor, collagen and other materials come into contact with blood components, initiating platelet activation, coagulation and thrombosis [64].

In addition to being prone to rupture, the inflamed plaque has a

prothrombotic effect. This finding is not surprising, given that the thrombotic and inflammatory pathways are intimately linked [77]. Indeed, it is now believed that thrombosis can play a vital part in the initiation of atherosclerosis. The adhesion of platelets to seemingly normal endothelium has been shown to promote the formation of the atherosclerotic plaque in the apoE -/- mouse [78]. The initial adhesion of the platelet to the vessel wall has been shown to be mediated by a member of the integrin family, GPIb/IX/V [78]. Indeed, this adhesion molecule may represent a potential pharmacologic target as its inhibition can attenuate the formation of the atherosclerotic plaque in the mouse model of atherosclerosis [78]. GPIb/IX/V can bind von Willebrand factor (vWF) adherent to the damaged vessel wall, thus allowing the platelet to roll on the endoluminal surface of the vasculature [79]. This interaction slows the passage of the platelet in the blood stream, thus allowing for other platelet adhesion molecules, such as GPIIb/IIIa, to establish more permanent links to the vessel wall. GPIIb/IIIa is another member of the integrin family, whose main physiologic ligands are fibrinogen and vWF [80]. The presence of multiple GPIIb/IIIa binding sites on fibrinogen allows this molecule to act as a cross-link between different platelets, thus facilitating platelet aggregation [77]. GPIIb/IIIa binding to fibrinogen also mediates various physiologic changes within the platelet leading to shape change and platelet granular release.

Thrombosis also has a vital role in the progression of atherosclerosis. Exposure of the subintima to blood, through plaque rupture, disruption of plaque microvessels or superficial endothelial disruption, leads to activation of the coagulation cascade as well as circulating platelets [17]. The resulting thrombus can be integrated into the atherosclerotic plaque, thus contributing to its size. In addition, thrombus constituents, such as erythrocyte membrane cholesterol, may provide additional antigenic stimuli for plaque growth [81]. Platelets are also a rich source of proinflammatory cytokines, such as RANTES, CD40L and IL-1β, which further promote inflammation [77]. CD40L has an important role in plaque

destabilization by inducing the production of various metalloproteinases (MMPs) responsible for the breakdown of the extracellular matrix in the plaque [82]. Indeed, CD40L levels are associated with the presence of lipid-rich plaques in humans [83]. It is therefore not difficult to visualize a vicious cycle whereby the inflamed plaque initiates local thrombosis, which then promotes further inflammation and plaque destabilization.

Genetic background of atherosclerosis: Atherosclerosis is a complex trait

Common DNA sequence variants which each may have a small to moderate phenotypic effect have been suggested to determine genetic susceptibility to common complex traits such as atherosclerosis [84-86]. Currently, however, the DNA sequence variants, whether rare or common, conferring the susceptibility to atherosclerosis in the general population are still largely unknown.

Atherosclerosis and other complex traits do not follow a simple Mendelian mode of inheritance. Instead, relatives of an affected individual are likely to have disease-predisposing alleles, making the disease more common among the first degree relatives of the proband and less common in less closely related relatives, resulting in a familial aggregation of the complex trait. However, the observed familial aggregation does not necessarily mean a strong genetic contribution. It may be by chance alone because atherosclerosis is common at the population levels or to great extent explained by shared environmental factors but previous studies have shown that family history of coronary heart disease (CHD) significantly increases the risk for CHD [3,87,88]. Heritability is also used to estimate the degree of genetic involvement. Heritability is the fraction of the total phenotypic variance of a trait caused by genes. It is worth noting that heritability does not reveal how many genes are involved or how the different genes interact. For CHD the her- itability has been estimated to be 56-63% [89].

Currently, a gene for a monogenic disease can be mapped and identified,

providing that there are enough informative families available for analysis.

However, the success in identifying genes for complex diseases including atherosclerosis has been relatively modest (reviewed in [90]), and most progress has been made with rare familial (Mendelian) forms of these complex traits [91]. Table 6.1 shows some of the factors hampering gene identification in atherosclerosis.

To increase the impact of genetic involvement and thus the possibilities of identifying contributing genes for complex diseases, investigation of cases with a likely familial component; families with multiple affected individuals; subjects with an early onset disease; and extreme phenotypes can be utilized [92]. Importantly, study samples originating from genetically isolated populations such as Sardinians and Finns, where genetic and environmental heterogeneity are reduced, have been very successfully used to identify genes for rare monogenic diseases (reviewed in [93,94]). These populations may provide some advantages into gene identification of complex traits as well, although there are likely to be multiple predisposing alleles for complex traits even in these population isolates. An important advantage of population isolates may turn out to be the relatively low environmental and lifestyle variability that can be expected among these populations. These are important factors because they present less confounding factors into the statistical analyses.

Alleles contributing to complex traits are also suggested to have only a minor to moderate effect on the phenotype [84-86]. Furthermore, in complex traits such as atherosclerosis the underlying DNA sequence variants may differ from the ones identified for typical monogenic diseases [94], and they may mostly even represent noncoding variants, residing in conserved regulatory regions. For associated single nucleotide polymorphisms (SNPs) in noncoding regions, the functional analyses are challenging, and currently they focus on investigation of cross-species

conservation and/or identification of regulatory elements such as transcription factor, RNA splicing factor and microRNA binding sites using approaches of bioinformatics [95].

Two main projects facilitating gene identification in atherosclerosis: the Human Genome Project and the International HapMap Project

The Human Genome Project (HGP) was started in 1988 first to map and then to sequence the human genes. The initial emphasis was in building both physical and genetic linkage maps of 22 human autosomal chromosomes in order to provide dense maps of microsatellites, expressed sequence tags and sequence-tagged sites for mapping purposes. The ultimate goal was to sequence the human genome. The first version was available in 2001 [96,97] and, in 2003, the HGP announced the completion of the DNA reference sequence of *Homo sapiens*. The HGP provided the essential tools for gene identification of complex traits, including atherosclerosis. As a result of the successful HGP, human genetics is expected to be one of the key players in providing new insights and better understanding of diseases, not only of the rare monogenic disorders but also of common complex diseases such as CAD, and other atherosclerosis – related disorders including stroke, diabetes and hypertension.

Meaningful analysis of the enormous amount of data that the HGP produced is crucial for successful genetic analysis of atherosclerosis and other complex traits [90]. To tackle the millions of SNPs available for association tests, approaches identifying the causal variants among those in an associated haplotype were recently developed [98-101]. The important message of these studies was that most of the human genome may consist of blocks of variable length over which only a few common haplotypes are detected. The mean size of blocks was initially estimated to be 22 kb and ~80% of the genome in blocks >10 kb in populations of European ancestry [102]. In African-Americans, the mean size of blocks

was initially estimated to be 11 kb and ~60% of the genome in blocks >10 kb [102]. These observations led to the establishment of the International HapMap Project which is currently determining the linkage disequilibrium (LD) patterns across the human genome in blood samples taken from people in Japan, Nigeria and China as well as from people of northern and western European ancestry in the USA in order to allow the efficient selection of SNPs for regional and genome-wide association studies [103]. The overall aim is to provide a restricted number of tag SNPs for genotyping to cover most of the common variation in the human genome without genotyping redundant SNPs. The ultimate success of this project is to great extent dependent on the hypothesis of common variants underlying common disorders [84,86]. Using these data generated by the HapMap project, the tag SNPs capturing most of the genetic variation can be selected for regional and genomewide association analyses, hopefully providing a powerful shortcut to gene identification of atherosclerosis among other complex traits. For example, currently, the genotypes of millions of SNPs for 30 trios from Centre d'Etude du Polymorphisme Humain (CEPH) subjects of north European ancestry are available online (Http://www. hapmap. org/).

Previous data show that some genomic regions fit better to the block theory than others [104]. Thus, the actual practical usefulness of the haplotype method will depend on the specific patterns of LD in the region of interest [104] as well as on the underlying LD structure of the study population. Furthermore, the haplotype-block strategy is agnostic about types and location of functional SNPs. However, in most Mendelian diseases the identified causative mutations have turned out to be coding variants (reviewed in [94]). Thus, an alternative strategy, focusing on identification and testing for association of SNPs in coding and regulative regions, has been proposed for association testing of complex traits [94]. In this sequence based approach, about 10 times smaller number of SNPs need to be genotyped than when using the haplotype-block strategy [94]. In addition, low frequency disease alleles could also be detected. This

may be of importance because rare DNA sequence variants may have a role in individual families, and because both rare and common variants seem to confer for instance the susceptibility to low plasma levels of HDL-C in the general population [105].

Candidate genes contributing to the development of atherosclerosis

Genetic components of atherosclerosis can be investigated using several strategies, including genome-wide scans for novel genes (Table 6.2) and candidate gene approaches (reviewed in [90]). Candidate gene studies of known genes, mainly using case-control study samples have been the method of choice for several decades. In these studies, alleles of unrelated affected subjects are compared with alleles of unrelated unaffected subjects. When an association is detected, however, it may be difficult to demonstrate the direct causality. Differences in age, sex or ethnicity between case and control groups can contribute to the observed association and cause stratification bias resulting in false positive associations [106]. Therefore, careful selection of a control group is crucial for a meaningful case-control study. Multiple testing can also lead to false positive results. These difficulties partly explain the small number of findings replicated in several study samples and populations. Replication in independent study samples is of utmost

Table 6.2 Genome-wide screens for myocardial infarction (MI), coronary artery disease (CAD) or coronary artery calcification using linkage or association analysis [90]. After Lusis *Etal.* 2004 [90].

LTA indicates lymphotoxin-alpha and ALOX5AP arachidonate 5-lipoxygenase-activating protein gene.

importance when evaluating the significance of association results.

Genes suggested to contribute to CAD risk include apolipoprotein E,

apolipoprotein (a), methyltetrahydrofolate reductase, angiotensin-converting enzyme and *NOS3* genes [107-111]. The size and nature of these and other CAD susceptibility genes are largely unknown. An autosomal dominant form of CAD was recently shown to be caused by a mutation in the myocyte enhancer factor-2 (MEF2A) transcription factor gene [91], implicating the MEF2A signaling pathway in the pathogenesis of myocardial infarction (MI). However, subsequent studies showed that mutations in this gene are not a common cause of CAD at the population level [112,113]. Recently, the gene encoding 5-lipoxyge – nase activating protein was also shown to confer risk of MI and stroke in subjects from Iceland and UK [114], and the finding was replicated in Japanese [115]. Table 6.3 shows the currently known common DNA variations contributing to CAD and its risk factors (reviewed in [90]).

Example of a candidate gene for atherosclerosis: *NOS3*

Identifying polymorphisms that may indicate increased susceptibility to atherosclerosis and heart disease is an area of active research interest. A major contender for prognostic polymorphisms is the *NOS3* Gene. *NOS3* (or the endothelial isoform of nitric oxide synthase) is expressed primarily in vascular endothelium [116]. The nitric oxide synthesized by this enzyme has antiplatelet effects as well as promoting smooth muscle relaxation. Indeed, nitric oxide is the predominant vasodilator found in the healthy vasculature. Therefore, loss of nitric oxide activity would promote vasoconstriction and platelet activation. Impairment of nitric oxide activity has been described in many conditions that predispose to atherosclerosis, including hypertension, hypercholesterolemia and diabetes [117-119].

Theoretically, genetically controlled subtle disturbances in nitric oxide synthesis caused by perturbations in the *NOS3* gene might predispose

Table 6.3 Common DNA sequence variations contributing to coronary

heart disease (CHD) and its risk factors. After Lusis *Et al.* 2004 [90]. Only genes showing evidence of linkage or association in multiple studies are cited.

Trait	*Gene*	*Variation*	*Reference*
LDL/VLDL	LDL receptor PCSK9 ApoE ApoAI-CIII-AIV-AV cluster	Many mutations Many mutations Three common missense alleles explain ~5% of variance of cholesterol Multiple polymorphisms	Goldstein *Et al.* 1995 [192] Abifadel *Et al.* 2003 [193] Sing *Et al.* 1985 [194] Talmud *Et al.* 2002 [195]
HDL levels	Hepatic lipase ABCA1	Promoter polymorphism Many polymorphisms	Shohet *Et al.* 2002 [196] Frikke-Schmidt *Et al.* 2004 [197]
FCHL	Upstream transcription factor 1	Intronic and 3′UTR polymorphims	Pajukanta *Et al.* 2004 [167]
Lp(a)	Apo(a)	Many alleles of apo(a) explain >90% variance	Boerwinkle *Et al.* 1992 [198]
Homocysteine	Methylene tetrahydrofolate reductase	Missense polymorphism	Kang *Et al.* 1993 [199] Ma *Et al.* 1996 [200]

Coagulation	Fibrinogen B Plasminogen activator inhibitor type 1 Factor VIII	Promoter polymorphism Promoter polymorphism Common missense	Hamsten *Et al*. 1993 [201] Hamsten *Et al*. 1993 [201] Hamsten *Et al*. 1993 [201] Thomas *Et al*. 1995 [202]
Blood pressure	Angiotensinogen ***β***2-Adrenergic receptor Alpha-adducin	Missense and promoter polymorphisms Missense polymorphism Missense polymorphism; support from studies in rats	Caulfield *Et al*. 1995 [203] Lusis *Et al*. 2002 [204] Lusis *Et al*. 2002 [204]
CAD	Angiotensin converting enzyme Serum paraoxonase Toll-like receptor 4 Arachidonate 5-lipoxygenase – activating protein	Insertion-deletion polymorphism Missense polymorphism affecting enzymatic activity; animal studies support Missense polymorphism Haplotype of 4 SNPs	Staessen *Et al*. 1997 [205] Shih *Et al*. 2001 [206] Tward *Et al*. 2002 [207] Kiechl *Et al*. 2002 [208] Helgadottir *Et al*. 2004 [114]
			Gretarsdottir

Stroke	Phosphodiesterase 4D	Regulatory polymorphism	*Et al.* 2003 [209]
Diabetes, obesity and insulin resistance	PPAR***γ*** Calpain 10 Hepatocyte nuclear factor-4***α*** Transcription factor 7-like 2	Missense polymorphism Promoter polymorphism Intronic	Altshuler *Et al.* 2000 [210] Horikawa *Et al.* 2000 [211] Silander *Et al.* 2004 [212] Grant *Et al*. 2006 [214]

CAD, coronary artery disease; FCHL, familial combined hyperlipidemia; HDL, high density lipoprotein; LDL, low density lipoprotein; PPAR, peroxisome proliferator activated receptor; SNP, single nucleotide polymorphism; VLDL, very low density lipoprotein.

Individuals to develop atherosclerosis, given some environmental or endogenous upset to uncover the effects of the gene alteration [111]. There is some evidence that certain polymorphisms in this gene may affect atherosclerosis development. Among several polymorphisms found in the *NOS3* gene (reviewed in [111]), the Glu298Asp polymorphism is of especial interest. This polymorphism, which is located in a coding region of the gene, exon 7, could alter mature protein activity, thus affecting enzyme activity and reducing local nitric oxide synthesis. Hingorani *Etal.* [120] investigated Glu298Asp in an English study sample. They found a strong association between the Glu298Asp polymorphism and the risk for coronary heart disease. Studies have also linked Glu298Asp to essential hypertension, resistance to therapy [121] and to coronary spasms [122]. However, the relevance of the data remains uncertain, as other investigators have not been able to replicate these findings [123].

Other polymorphisms have also been found in the *NOS3* gene.

Polymorphisms in the promoter may influence mRNA transcription; intronic polymorphisms are less likely to have functional roles. In Japanese, the promoter polymorphism T – 786C was shown to be linked to vasospasm, which in turn is linked to low endothelial NO [124]. T-786C is also associated with MI [125] and diabetes [126]. Wang *Et al.* [127] reported that risk of CAD was increased in smokers with a 27 base pair repeat polymorphism in intron 4 of *NOS3*.

The evidence linking *NOS3* polymorphisms to clinical states is still inconsistent [30]. With increasing availability of large scale genotyping techniques such as microarray technology, the search for interesting diagnostic or prognostic polymorphisms will be made easier, as thousands of gene variants can be searched simultaneously.

Polymorphisms related to identifying individuals at risk of unstable plaque and plaque rupture are also under investigation. For example, MMP gene expression is regulated at the transcriptional level and responds to variety of factors such as TNF-α. Genetic variations in the MMP promoter regions may act to affect extracellular matrix degradation, thereby increasing susceptibility to atherosclerosis [74,128]. A polymorphism in MMP – 3 promoter was also linked to MI independently of other risk factors, suggesting a susceptibility to plaque rupture (reviewed in [128]). Several SNPs in thrombospondin genes, deficiencies of which are associated with increases in MMP-2, have been associated with premature familial MI [74].

Genomic approaches to identify genes for atherosclerosis

During the last decade, a genome-wide scan has become a popular approach to identify novel genes for complex traits, and several scans have been performed for MI, CAD [129-133] as well as for coronary artery calcification [134], mainly using linkage analysis of families or affected sib-pairs (Table 6.2) (reviewed in [90]). For this approach no a priori knowledge of disease pathophysiology is required, thus enabling identification of novel genes and pathways for atherosclerosis. In 1996 the

idea of genome-wide association studies was introduced [85]. Association analysis has been shown to be more powerful than linkage analysis for detecting alleles of complex traits with only modest effects [85]. However, searching the whole genome using an association approach requires genotyping of hundreds of thousands of SNPs [94]. So far, only a few such studies have been completed for complex traits, because of the technical demands related to genotyping of such a large number of SNPs [135,136]. In one such study, analyses of 92,788 gene-based SNPs showed that functional SNPs in the lymphotoxin-alpha gene are associated with susceptibility to MI [135]. In another study, over 100,000 SNPs were genotyped and the human complement factor H gene was identified to be associated with age-related macular degeneration [136]. Advances in genotyping technologies have recently made this approach both more affordable and feasible. However, genome-wide association analyses are still facing a number of challenges, including problems related to multiple testing, suitable study design, SNP selection and interactions between polymorphisms [137,138]. Selecting the tag SNPs, produced by the HapMap Project, instead of random, evenly spaced SNPs may turn out to be the most effective way to cover most of the genetic variation in the human genome. However, it is difficult to detect rare causative SNPs using this approach, suggesting that substantial resequencing of genes is warranted to identify rare causative variants.

DNA microarrays provide a practical and economic tool for studying gene expression in atherosclerosis on a genomic scale [90]. Complementing classic linkage and association studies, expression arrays can relate changes in gene expression to atherosclerosis and have great potential to identify novel genes, their pathways and networks associated with atherosclerosis. Recently, when atherosclerotic plaques of patients with stable and unstable angina were investigated for gene expression differences, several genes previously linked to hemostasis, such as the protein S (PROS1) gene, the cyclo-oxygenase

1 (COX-1) gene, the IL-7 gene, and the MCP-1 and MCP-2 genes, were shown to be expressed at significantly lower levels in samples from unstable angina patients [139]. These findings suggest that these genes may have a role in plaque rupture.

The limitations of expression arrays are often related to the relevant tissues and small sample sizes available for investigation in humans. Small sample sizes typically available for microarrays as well as increased genetic heterogeneity can make distinguishing statistically significant differential expression between cases and controls especially challenging. Furthermore, atherosclerosis is likely to result from small quantitative differences in multiple genes, rather than major expression changes in a few genes. This phenomenon is exemplified by a recent study of type 2 diabetic males [140], where analysis of co-regulated sets of genes rather than individual genes identified metabolic pathways that are altered in diabetic individuals. In more detail, Mootha *Et al.* [140] used the Gene Set Enrichment Analysis (GSEA) to identify a set of PGC-1α responsive genes involved in oxidative phosphorylation that were coordinately downregu – lated by approximately 20% in the muscle of diabetic individuals, with no single gene showing significant differential expression between diagnostic categories.

As a result of the successful HGP, recent progress of the HapMap Project and advancing genotyping and microarray technologies, it is finally possible to identify the DNA sequence variants conferring susceptibility to atherosclerosis using whole-genome approaches where information obtained from linkage, association, gene expression and functional analyses are combined to verify the signals.

Genes, lipids and atherosclerosis

Stein *Et al.* [141] point out that some people are unlikely to develop atherosclerosis and CHD even in the face of high dietary cholesterol intake or frank hypercholesterolemia. Individuals show a wide variety of

responses to dietary cholesterol, about 9% of populations studied are hyper-responders and 9% are hypo-responders. For example of the latter, a case was reported of one man who ate about 25 eggs a day (about 6 g cholesterol) and yet remained nor – mocholesterolemic at 88 years of age [142]. It has been known for some time that genetic variation can have dramatic effects in causing inter-individual variation in cholesterol levels. Some of the genes implicated in lipid metabolism are discussed below.

Familial hypercholesterolemia is one of the most common inborn errors of metabolism and is most often a result of mutations of the low density lipo-protein receptor (LDL-R) gene [143]. Although countless loss of function mutations have been described in the LDL-R gene (these can be viewed at Http://www. ucl. ac. uk/fh), these can generally be classified into one of five categories:

1 Those that do not produce a detectable LDL-R (e. g. 'null alleles');

2 Those that code for a protein that cannot be transported from the endoplasmic reticulum to the Golgi body and hence to the cell surface;

3 LDL-R that cannot bind the corresponding ligand;

4 LDL-R that binds LDL normally but cannot be internalized; and

5 LDL-R that cannot be recycled to the cell surface after transporting cholesterol into the cell [143].

Individuals who are heterozygous for a mutant allele have a two – to threefold increase in LDL-C and develop premature CHD after the age of 35. Homozygotes exhibit 6-8 times above normal levels of cholesterol and develop ischemic heart disease in their teenage years. Therefore, it is important to identify individuals who suffer from familial hypercholesterolemia in order to institute early lipid lowering treatment.

Apolipoprotein E (ApoE), found in various classes of lipoproteins, binds to

LDL-R and mediates the uptake of the lipoprotein by the cell [144]. ApoE is believed to have a protective effect against the development of atherosclerosis. Mice that are -/- for the ApoE gene are severely hypercholes – terolemic and are prone to early onset atherosclerosis [145]. Numerous polymorphisms of the gene coding for ApoE have been described [144]. Of the three common alleles, ε2, ε3 and ε4 at a single locus, ε3 and ε2 are the most and least common alleles, respectively [144,146]. ε2 homozygosity can give rise to type III hyperlipoproteinemia [146], a condition that is associated with premature atherosclerosis brought about by the defective cellular uptake of lipoprotein remnants [147,148]. Based on the finding that the majority of cases of type III hyperlipoproteinemia are homozygous for ε2, it is perhaps surprising to find that ε2 heterozygosity is not associated with CHD [149]. However, ε3⁄4 heterozygosity is associated with an increased risk of ischemic heart disease when compared with ε3⁄3 homozygotes, with an odds ratio of 1.30 (95% confidence interval [CI], 1.18-1.44) [149]. The mechanism by which ε4 heterozygosity affects atherogenesis is unclear at present.

Lipoprotein lipase is important in the regulation of triglyceride-rich lipoproteins such as VLDL and chylomicrons. Impairment of lipoprotein lipase activity may delay the clearance of these lipoproteins from the circulation [150]. An Asp9Asn mutation in the lipoprotein lipase gene may be associated with disease progression while some other variations are believed have a protective effect against MI [150,151].

Lipoprotein (a) levels appear to be a marker of the risk of disease progression in atherosclerosis and cardiovascular risk, although not all studies have shown consistent results [152-154]. Genetic variation seems to have an effect on plasma lipoprotein (a) levels, although the significance of this remains to be determined [155].

Cholesteryl ester transfer protein (CETP) mediates the transfer of cholesteryl esters from HDL to LDL and VLDL, therefore promoting the

transport of cholesterol to the hepatocyte [156]. Inhibition of CETP has been shown to protect against atheroma formation in rabbits [157]. A recent meta-analysis of over 13,000 patients has shown that the so-called Taq1B polymorphism of the CETP gene can be related to cardiovascular risk [158].

Sitosterolemia is an autosomal recessive condition in which there is excessive intestinal uptake but decreased biliary secretion of plant sterols and cholesterol [159]. Patients with this condition usually have hypercholesterolemia and are at risk of developing premature atherosclerosis. Sitostero – lemia has been found to be caused by mutations in the genes coding for ABCG5 and ABCG8 [160]. These genes belong to the ATP-binding cassette (ABC) superfamily of transmembrane transporters that are responsible for the movement of a diverse range of substances [161]. ABCG5 and ABCG8 are expressed in the liver and intestines and have been shown to be responsible for the secretion of cholesterol into the bile [162].

Another member of the ABC superfamily, ABCA1, controls the efflux of intracellular cholesterol to lipid-poor ApoA-I, the major apolipoprotein of HDL [161]. Impairment of ABCA1 activity leads to an autosomal recessive condition known as Tangier disease [163]. Patients with this condition have accumulation of cholesterol in various tissues, leading to a multisystem disorder featuring premature atherosclerosis, hepatosplenomegaly, polyneuropathy and epidermal lesions. In a mouse model of atherosclerosis, overexpression of the ABCA1 gene attenuated atherogenesis and increased HDL levels [164]. These findings suggest that HDL levels may reflect ABCA1 activity [165]. Indeed, certain mutations in the ABCA1 genes have been shown to be associated with reduced HDL levels and an increased risk of developing CHD [165,166].

Upstream transcription factor 1 (USF1), residing on chromosome 1q21, regulates several genes of lipid and glucose metabolism. Variants of USF1

were recently associated with a common familial dyslipidemia, familial combined hyperlipidemia (FCHL) in Finnish [167], Mexican [168] and Caucasian families [169]. As FCHL predisposes the affected individuals to CAD, it is of importance that a recent study implicated USF1 variants in CAD [170] and that the 1q21 region has also been linked to MI [133]. Taken together these studies suggest that USF1 should be further investigated as a potential candidate gene conferring the susceptibility to CAD.

Genetic polymorphisms, inflammation and atherosclerosis

There is great interest in elucidating the role played by polymorphisms of the genes regulating inflammation. Early studies on a rodent model have shown that a genetic predisposition to inflammation co-segregates with the tendency to form atherosclerotic plaques [171].

5-Lipoxygenase is an enzyme involved in the production of the leukotrienes, and thus has a major role in promoting inflammation. A recent study showed that 6% of an American population sample possess two minor variant alleles in the promoter polymorphism of the 5-lipoxygenase gene [172]. It was found that individuals with the variant alleles have higher intima-media thickness measurements and a greater degree of systemic inflammation (as measured by high-sensitivity C-reactive protein [CRP]). Indeed, the 5-lipoxygenase gene has been shown to be associated with adverse cardiovascular events in both Icelandic and Scottish subjects [114,173]. In addition, LDLR -/- mice that had one copy of their 5-lipoxygenase gene removed showed a dramatic reduction in plaque formation when compared with “normal” LDLR -/- controls [174]. However, this finding has not been replicated in LDL -/- 5-LO -/- and ApoE -/- 5-LO -/- mice [175].

A genome-wide association study of Japanese subjects has found a polymorphism in the lympho – toxin-α (also known as TNF-β) gene to be associated with susceptibility to MI [135]. However, the result of this study was not replicated in another publication from another Japanese group

[176]. A study performed in a German population similarly failed to reveal an association between the lympho – toxin-α polymorphism and CHD [177]. Similarly, studies on polymorphisms of TNF-α gene provided conflicting results [177,178].

Genes, coagulation, fibrinolysis and atherosclerosis

Variations in genes controlling the coagulation pathway may also play a part in influencing the natural history of atherosclerosis. A high plasma level of fibrinogen has been associated with an increased risk of developing adverse cardiovascular events [179]. Fibrinogen is the precursor of fibrin. In addition, its numerous binding sites for the platelet GPIIb/IIIa receptor enables it to act as a cross-link during platelet aggregation [77]. A recent study has suggested that genetic variation may play a part in determining plasma fibrinogen levels and that the genes responsible for this are located on chromosomes 2 and 10 [180]. A -455G/A polymorphism in the β-fibrinogen gene has also been shown to affect plasma fibrinogen levels [181]. However, at present, it is unclear if this polymorphism may influence the risk of atherothrombosis, as some studies have reported a positive association between the two while others have not [181,182]. At present, there is no strong evidence to suggest that genetic polymorphisms in genes coding for the other components of the coagulation pathway would influence atherothrombosis.

Plasminogen activator inhibitor-1 (PAI-1) is the major circulating inhibitor of tissue-type plasminogen activator (t-PA). PAI-1 acts to regulate thrombolytic activity by preventing excessive systemic fibrinolysis. Therefore, PAI-1 can be considered to be a procoagulant molecule. Indeed, plasma levels of PAI-1 can be related to the risk of developing adverse cardiovascular events [183]. However, it is unclear if elevated PAI-1 levels are a cause or effect of atherosclerosis. In addition, the fact that the elevations of PAI-1 levels are accompanied by rises in t-PA further complicates matters. Most studies on the PAI-1 gene have focused on the

4G/5G insertion/deletion polymorphism at position -675 in the promoter region of the gene [184]. The 4G (deletion) allele is associated with higher PAI-1 levels and it is conceivable that patients with this allele may be more prone to thrombosis. Because studies on the 4G/5G polymorphism have yielded conflicting results, a meta-analysis was performed which showed a weak association between the 4G allele and the risk of atherothrombosis [185].

Other genes

An autosomal dominant form of CHD has been linked to a 21-bp deletion in the transcription factor MEF2A [91]. MEF2A is known to be involved in vasculogenesis but the pathophysiologic pathway by which the MEF2A polymorphism may influence the atherogenesis is uncertain at present. Some of the other candidate genes that may be related to the development of atherosclerotic disease are summarized in Table 6.3.

The future in atherosclerosis

Atherosclerosis is no longer thought of as a lipid accumulation or passive degeneration but as an active process of cells signaling and actively participating in atherosclerotic remodeling. Hundreds of genes are likely to be involved in this process as well as alterations in cell-cell communication [186]. Increased understanding of the molecular and cell biologic events underlying the atherosclerotic process has led to new concepts to develop the necessary prognostic indicators, diagnostic tests and targeted therapies for atherosclerosis and CHD. Plaque stabilization and identification of vulnerable patients is also emerging as an important goal to prevent complications of atherosclerosis [63].

The concept of atherosclerosis as inflammation has led to several new insights into novel, clinically applicable, risk factor markers. For example, measurement of inflammatory markers such as plasma CRP or serum amyloid A could provide a noninvasive method for assessing

cardiovascular risk. Large increases in levels of these proteins can be discerned in plasma following inflammatory stimuli. CRP, produced in the liver and possibly in cells in atheromatous plaque in response to upstream proinflammatory cytokines, is being developed as a potentially important risk factor with predictive power for cardiovascular events [187].

In addition, because early endothelial changes may herald the development of later disease development, tests to assess the health of endothelium may serve as markers for disease and as targets for therapy [21,33]. For example, methods to detect marker “activation antigens” such as the endothelial leukocyte adhesion molecules, E selectin, ICAM-1 and VCAM-1, could be developed as biomarkers in tissues or circulating blood to identify inflammatory endothelial cell changes for early diagnosis [21]. Because VCAM-1 is an important early step in atherosclerotic processes, targeting this protein might be of therapeutic value, and several animal studies have suggested that blocking VCAM-1 acts to reduce neointimal hyperplasia [33].

Novel therapies for suppressing endothelial activation could provide new means of preventing atherosclerotic changes. For example, the peroxisome proliferator activated receptor (PPAR) subfamily appears to have anti-inflammatory properties and can reduce VCAM-1 and tissue factor gene expression by cells in atheroma [19].

The atherosclerosis as inflammation hypothesis has also led to new understanding of the mechanisms by which several existing pharmaceuticals are effective in reducing the complications of CAD. Angiotensin-converting enzyme (ACE) inhibitors promote bradykinin a nitric oxide stimulus, and decreased angiotensin increases nitric oxide bioavailability [22]. Thus, these agents appear to have effects on improving endothelial function beyond blood pressure decreases. The important role of the unstable plaque and plaque rupture in thrombosis has also increased interest in mechanisms that may effect plaque stabilization, by

lipid lowering, ACE inhibition and antibiotics (reviewed in [60,188]).

Gene therapy

Gene therapeutic technologies might be used to remedy eNOS deficits (reviewed in [189]). Rabbits that are fed a high cholesterol diet are prone to develop endothelial dysfunction and atherosclerosis [189]. Impairment of nitric oxide activity has been shown to be a key factor behind this observation. Enhancement of NOS activity in these rabbits by the use of an adenovirus-mediated NOS gene transfer restored endothelial derived relaxing factor activity [190]. The use of the adenovirus-mediated NOS gene therapy was also shown to reduce macrophage infiltration of the carotid arteries of cholesterol-fed animals [191]. In addition, the expression of adhesion molecules such as ICAM-1 and VCAM-1 are significantly downregulated in the carotid arteries of the treated rabbit [191]. Therefore, it is possible that *NOS3* gene therapy may be useful in the treatment of human atherosclerosis.

Other gene therapy targets include MMP inhibitors (reviewed in [128]). Tissue inhibitors of MMPs might be increased at the local tissue level by administration of exogenous recombinant tissue inhibitors or by stimulating increased endogenous expression via gene therapy. Synthetic MMP inhibitors have also been investigated, including antibiotics such as doxycycline [128]. In addition, the apoptosis of fibroblasts on the surface of plaques would increase the likelihood of plaque rupture, and blocking apoptosis would be another approach to preventing complications of atherosclerosis [15]. It might also be possible to interfere with important transcription factors that regulate groups of genes involved in atherosclerosis. For example, NF κB, a key inflammation regulator in the vessel wall, PPARs and Sp/XKLF family of zinc finger genes may all prove to be important targets (reviewed in [186]).

Conclusions

Many highly intriguing genes have been discovered to be active in the progress of atherosclerosis. Furthermore, identification of genes contributing to susceptibility to atherosclerosis and other complex traits is expected to accelerate rapidly because the Human Genome Project and the HapMap Project have made the sequence of the human genome and human genome sequence variation data publicly available. Even so, we need more detailed knowledge about the numerous genes and gene targets involved in various stages of development of complex atherosclerotic lesions. New genomic technologies such as microarray chip technologies are beginning to be used to identify novel genes and pathways as well as to understand gene-gene and gene-environment interactions. For example, recent studies comparing gene expression in stable and ruptured plaque, as well as various responses of macrophages to oxLDL are pioneering the way to the fuller utilization of genomic technology in exploring the complexities of atherosclerosis.

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CHAPTER 7