

Genetic determinants: is there an “atherosclerosis gene”?

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(Received December 2, 2003; accepted December 9, 2003)

Genetische Determinanten der Atherosklerose: Gibt es das Atherosklerose-Gen?

Zusammenfassung. Atherosklerose ist, wie man heute weiß, eine chronisch entzündliche Erkrankung, die durch eine Reihe von Ereignissen ausgelöst wird. Prädispositionsstellen dafür sind Gefäßabschnitte mit turbulenter Strömung, wie sie physiologischer Weise in Koronararterien und Gefäßbifurkationen zu finden sind, aber auch überall dort wo Gefäßkrankungen keinen laminaren Blutfluss zulassen. Laminarer Blutfluss führt nämlich durch die Endothel-NO-Synthase (eNOS) zur Generierung von Gefäß-schützendem Stickoxid (NO). Bei turbulenter Strömung fällt dieser Schutzmechanismus weg, Endothelzellen werden aktiviert und es kommt zur Lipideinlagerung in den Extrazellularraum. Makrophagen oxidieren daraufhin die eingelagerten Lipoproteine und Phospholipide. Es hat sich gezeigt, dass die Höhe des LDL-Cholesterinspiegels im Blut – und damit die mögliche Oxidation im Extrazellularraum – entscheidend ist diese Erkrankung über Endothelzellaktivierung und Entzündungsreaktionen voranzutreiben. Zusätzlich können virale oder bakterielle Infektionen wie durch Chlamydia pneumoniae den Entzündungsprozess unterstützen. Die daraus resultierende chronische Entzündung wird besonders von Entzündungszellen und Entzündungsmediatoren getragen, wodurch es zu einer signifikanten Erhöhung von Entzündungsmarkern wie CRP oder PAI-1 im Blut kommt.

Schlüsselwörter: Atherosklerose, Entzündung, Plaque-Stabilität, Cholesterin.

Summary. It is now clear that atherosclerotic disease is a chronic inflammatory disease triggered by a sequence of events initiated at sites with turbulent flow under normal conditions such as in the coronary arteries or at bifurcations or where normal laminar flow is replaced by turbulent flow because of vessel pathologies. Normally, laminar flow is protected by generation of NO by endothelial

NO synthase (eNOS), which becomes activated via stretch activated channels. When the flow turns turbulent, such protective NO generation ceases, leading to endothelial cell activation and lipid deposition into the extra-cellular space. There, lipoproteins and specifically phospholipids become oxidized by cells of the monocytic-macrophage lineage. Only when the LDL-cholesterol level is high enough lipid peroxidation products are generated in sufficient amounts to perpetuate the disease by generating a feed forward loop of endothelial cell activation leading to an inflammatory response. That inflammatory response might also be added by bacterial or viral infections such as Chlamydia pneumoniae or viruses. The disease then progresses to a chronic inflammatory state, whereby the immune system seems to contribute significantly and markers of chronic inflammation such as fibrinogen, leukocytes, PAI-1 and CRP are found increased.

Key words: Atherosclerosis, inflammation, plaque stability, cholesterol.

Introduction

Our current understanding of the vascular biology of atherogenesis and its clinical manifestations suggests a pathophysiology that is much more complex than mere lipid accumulation. Recent advances support the current view of atherosclerosis as an inflammatory process that initiates and promotes lesion development to the point of acute thrombotic complications and clinical events. These clinical events, which represent the most common causes of death in Western societies, are the consequences of a disease which starts already in childhood [5]. It is now clear that the atherosclerotic disease is a chronic inflammatory disease [45–47, 58, 59] resulting from the interaction of modified lipoproteins, macrophages, T cells and physiological components of vessel walls like endothelial cells and smooth muscle cells. A couple of genetic and environmental risk factors of atherosclerosis have been identified by epidemiological studies, among them elevated levels of serum cholesterol, which eventually suffice to promote the development of atherosclerosis. This led to the development of serum cholesterol-lowering drugs called “statins”, which have been shown

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to reduce the risk of cardiovascular mortality in patients with a broad range of cholesterol levels significantly [1]. Furthermore, there is additional evidence that plasma levels of cholesterol do not necessarily have to correlate with the progression of atherogenesis. This was most strikingly illustrated by two individuals having the same founder gene defect leading to homozygous familial hypercholesterolemia with comparable amounts of plasma cholesterol levels. While the first one died at the age of 3 of coronary artery disease, the second one, despite developing symptoms of cardiovascular disease, died at the age of 33 of unrelated causes [34]. Therefore, it is evident that a basic understanding of this multifactorial disease is intended for novel therapeutic approaches. In this review, the current view of the pathomechanism of atherosclerosis is given, whereby special targets of interest are pointed out possibly leading to novel therapeutic approaches.

Blood flow and mechanical shear stress

The localization of atherosclerotic lesions to arterial sites associated with disturbed flow patterns suggests an important role for local hemodynamic forces in atherogenesis [24, 25]. Sites with turbulent flow are found under normal conditions in the coronary arteries or at bifurcations and under pathological conditions wherever vessel pathologies prevent normal laminar flow. Indeed, laminar flow is normally protected by the generation of NO by endothelial NO synthase (eNOS), which becomes activated via stretch activated channels [20]. Deletion of the eNOS gene against the background of apo E deficiency results in hypertension and increased atherosclerosis [39]. When the flow turns turbulent, such protective NO generation ceases [53], leading to endothelial cell activation and lipid deposition into the extra-cellular space. There is increasing evidence that the vascular endothelium, which is directly exposed to various fluid mechanical forces generated by pulsatile blood flow, can discriminate among these stimuli and transduce them into genetic regulatory events. At the level of individual genes, this regulation is accomplished via the binding of certain transcription factors, such as NF κ B and Egr-1, to shear-stress response elements (SSREs) that are present in the promoters of biomechanically inducible genes. Indeed, recent data indicate that shear stress increases eNOS transcription by NF κ B activation [17].

On the level of NOS activity, asymmetric dimethylarginine (ADMA) has been shown to be an endogenous inhibitor, which itself is metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). It could be shown that DDAH I overexpression increases NOS activity *in vitro* and *in vivo* [18]. Furthermore it was shown that all-trans-Retinoic acid increases the expression of DDAH II, thereby facilitating NO synthesis [2].

Lipoproteins

Low density lipoprotein (LDL) particles, which carry the majority of serum cholesterol, have an essential role as a vehicle for the delivery of cholesterol to the peripheral tissue. LDL particles contain apolipoprotein B-100 (apo B-100), whose N-terminal domain is recognized by LDL

receptors to take up LDL into the cells. Increased LDL cholesterol levels are associated with increased risk of cardiovascular disease. The circulation of LDL is determined to a large extent by its rate of uptake through the hepatic LDL receptor pathway. If this pathway is disturbed by depletion of functional LDL receptors as seen in patients with homozygous familial hypercholesterolemia, massive accumulation of LDL is seen [26]. Targeted disruption of genes of the LDL receptor or apo E receptor, an alternate receptor for the uptake of different lipoproteins, as well as overexpression of the apo B gene in mice, results in remarkable increases in serum cholesterol levels, especially when such animals are fed a high cholesterol diet. This results in extensive atherosclerotic disease, whereby crossing these animals with animals that have been engineered to overexpress or lack genes of interest led to estimating the role of other proteins contributing to this multifactorial disease. It has to be mentioned that in all animal models of atherosclerosis used, extreme levels of lipids cause extensive lesions over a short period of time, whereas in man, development of atherosclerosis takes decades and lesions are histologically characterized by a lower cell count. Although, as mentioned above, serum cholesterol levels in man do not necessarily have to correlate with the progression of atherosclerosis, elevated serum levels of LDL and VLDL cholesterol it is a generally accepted risk factor for the development of atherosclerosis.

Fatty streaks underlying the endothelium are the first morphological changes in atherosclerosis [61]. LDL is thought to become susceptible to enzymatic and non-enzymatic modifications when retained by extracellular matrix. Thereby oxidative modification is thought to promote further progression of atherosclerotic lesions: depending on the extent of oxidation there, one can distinguish minimal modified LDL (mmLDL), which is still recognized by the LDL receptor, from extensive modified lipoproteins, in which apo B components are fragmented and cannot be recognized by LDL receptors anymore. These lipoproteins are taken up by several so-called scavenger receptors expressed on macrophages and smooth muscle cells. Many lines of evidence suggest that oxidative modifications of LDL promote the initial formation of fatty streaks as well as inflammatory reactions at all stages of the disease process. Only when the LDL-cholesterol level is high enough [51, 60] lipid peroxidation products are generated in sufficient amounts to perpetuate the disease. It has turned out that among mmLDL, especially oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC) is capable of stimulating endothelial cells to secrete monocyte chemoattractant protein (MCP-1) [52], which can directly attract monocytes, and IL-8 [6]. *Bochkov* et al. [9] could show that oxidized phospholipids increase the synthesis of early response protein-1 (EGR-1), a transcription factor that rapidly induced transcriptional upregulation of many genes that mediate thrombosis and inflammation. So tissue factor is upregulated by oxPAPC via EGR-1 in an NF κ B independent manner, while the anticoagulant glycoprotein thrombomodulin is downregulated by oxLDL [37]. In addition, several studies have demonstrated that mmLDL and oxidized phospholipids are able to inhibit lipopoly-

saccharide-induced upregulation of cell adhesion molecules in endothelial cells [44, 56], thereby preventing acute inflammatory response. This was most strikingly illustrated by an animal model, in which injection of oxidized phospholipids inhibited inflammation and protected mice from lethal endotoxin shock [8]. Also hemoxygenase-1 (HO-1), which has antioxidant and anti-inflammatory properties [43, 54, 55], is upregulated by oxPAPC [40]. Oxidized phospholipids may thus switch transcriptional mechanism of inflammation and promote a shift from acute to chronic inflammatory processes.

Therefore a number of potential oxidant-generating systems have been investigated that could directly or indirectly target LDL lipids, including myeloperoxidase, inducible nitric oxide synthase (iNOS) and 15-lipoxygenase (15-LO) [15, 32]. Especially 15-LO has turned out to play an essential role in atherogenesis, because overexpression of 15-LO in vascular endothelium of LDL receptor-deficient mice led to accelerated early atherosclerosis, while disruption of the homologous leukocyte 12/15-LO gene diminishes atherosclerosis in apo E-deficient mice.

Although oxidative modifications in the lipid and apo B components of LDL are shown to play a central role in the pathogenesis of atherosclerosis, in prospective clinical trials, antioxidant treatment with various agents like vitamin E or beta carotene of patients with preexisting atherosclerosis have been disappointing [74].

Monocyte/macrophage recruitment

Monocytes attach to endothelial cells that have been induced to express cell adhesion molecules by mmLDL and inflammatory cytokines. Adherent monocytes migrate to the subendothelial space and differentiate into macrophages. Beside VCAM-1 [14], other adhesion molecules like E-selectin and P-selectin are expressed on endothelial cell surfaces over lesion-prone areas to play quantitative roles in monocytes recruitment [21]. Migration of monocytes into the artery wall is stimulated by oxLDL, which can on the one hand directly attract monocytes [67] and on the other hand, as mentioned above, indirectly attract monocytes via upregulation of chemotactic molecules from endothelial cells like MCP-1. Knock-out animals deficient in MCP-1 or in its receptor CCR2 markedly reduce the development of atherosclerosis in apoE^{-/-} or apoB-overexpressing genetic background [11, 27, 29]. In addition, indirect evidence exists that IL-8, which is also upregulated by oxPAPC and has been shown to be present in human atherosclerotic lesions, may also play a role in monocyte/macrophage trafficking [10]. Interference with the MCP-1/CCR2-induced or with IL-8 induced monocyte chemotaxis may be a novel therapeutic approach.

After monocytes are attracted and differentiated to macrophages, so called “foam cells” are formed, which are characterized by massive amounts of cholesterol esters. Cholesterol esters are taken up as modified LDL via scavenger receptors. Among them CD36 and scavenger receptor-A (SR-A) are thought to be most important [22, 69].

As a consequence, simple fatty streaks are transformed into more complex lesions. This transition is promoted by the immigration of vascular smooth muscle cells from the media into the intima of the artery wall. There, smooth muscle cells tend to proliferate, taking up modified lipoproteins, and synthesize extracellular matrix proteins, which contribute to the formation of a fibrous cap.

Immunologic responses

At this phase of lesion development, interaction of monocytes/macrophages with T-cells takes place. Activated macrophages express class II histocompatibility antigens such as HLA-DR that allow them to present antigens to T lymphocytes. T-cells are activated when they bind antigen processed and presented by macrophages, which results in secretion of cytokines. Among them, in-

- **Initial events** are thought to be activation of the vessel wall by a change in shear stress from linear to turbulent
 - less NO generation
 - endothelial cell activation
 - lipid influx
 - monocyte recruitment and lipid peroxidation in the extracellular space
- **Perpetuated by**
 - infectious agents (viruses or Chlamydia pneumoniae)
 - oxidized phospholipids or oxidized cholesterol esters
- **Progression:**
 - immune responses
 - Complement recruitment
- **Chronic Inflammatory State**
 - increased levels of markers of inflammation
 - fibrinogen, leukocyte count, inflammatory cytokines, C-reactive protein (CRP), PAI-1
- **Late events**
 - plaque stability
 - stable plaque, collagen rich cap
 - lipid rich plaque (TF, PAI-1)
- **Restenosis**
 - response to injury
 - inflammation
 - proliferation and migration of SMCs

Fig. 1. Patho-mechanisms in atherosclerotic vascular diseases

interferon- γ is secreted by Th1 cells, which has been shown to inhibit smooth muscle cell proliferation, decreases collagen synthesis and leads to a reduction of scavenger receptor expression in monocytes. In contrast, interferon- γ also induces upregulation of inflammatory cytokines in macrophages and increases expression of MHC class II molecules. When interferon- γ receptor is absent in apoE-deficient mice, significantly less atherosclerosis was seen, indicating a pro-atherogenic role for interferon- γ [30]. Smooth-muscle cells from the lesions also have class II HLA molecules on their surfaces, presumably induced by interferon- and can also present antigens to T cells [31]. One possible antigen may be oxidized LDL [68], which leads to the generation of a variety of auto-antibodies to oxidatively modified lipids that occurs with progressive atherosclerosis. The autoantibody titers to epitops of oxLDL strongly correlate with the extent of atherosclerosis [36]. Many of these antibodies, mostly IgM, block binding and degradation of oxLDL by macrophages. It has been shown that these antibodies share complete genetic and structural identity with T15 natural antibodies against phosphorylcholine, which provide optimal protection to mice against lethal infection with *S. pneumoniae* [62]. Immunization of LDL receptor-deficient mice with *Streptococcus pneumoniae* resulted in a decreased extent of atherosclerosis [7].

Also Th-2 derived cytokines have been identified as modulating the atherosclerotic process. Among them IL-4 has an antiatherogenic effect in decreasing interferon- γ activity in macrophages and inhibition of Th-1 cell functions. In contrast, it has also pro-atherogenic effects by upregulating 15-LO, which promotes LDL oxidation. Heat-shock protein 60 may also contribute to autoimmunity. This and other heat-shock proteins have several functions, including the assembly, intracellular transport and breakdown of proteins and the prevention of protein denaturation. These proteins may be elevated on endothelial cells and participate in immune responses [72].

These observations suggest that immunization or other immune-based intervention might be beneficial in preventing atherosclerotic progression.

Instability and rupture of plaque

In most patients, myocardial infarctions occur as a result of erosion or uneven thinning and rupture of the fibrous cap, often at the shoulders of the lesion where macrophages enter, accumulate and are activated and where apoptosis may occur [42]. When a plaque ruptures, plaque lipids, matrix and tissue factor are exposed to hemostatic components of the blood leading to the initiation of the coagulation cascade.

Apoptosis of macrophages and smooth muscle cells is promoted by local cytokines, modified LDL and cell-cell interactions leading to the formation of a necrotic core [42].

Several classes of proteolytic enzymes have been found in regions of plaque rupture, among them matrix-metalloproteinases (MMP) such as collagenases, elastases and stromelysins, which are secreted mainly by T-cell stimulated macrophages thereby degrading the extracellular matrix [12, 23]. These changes may also be accom-

panied by the production of tissue-factor procoagulant and other hemostatic factors [49], further increasing the possibility of thrombosis. Similarly, lack of plasminogen activator inhibitor-1 promotes growth and abnormal matrix remodeling of advanced atherosclerotic plaques in apolipoprotein E-deficient mice [48].

Although neovascularization in human atherosclerotic lesions is associated with remodeling and protease activation, suggesting that neovascularization could contribute to plaque instability and rupture, high levels of circulating endothelial progenitor cells correlate with a reduced cardiovascular risk [33].

Nuclear targets

From all these findings we can conclude that inhibition of a single protein, which contributes to this multifactorial disease would only diminish, but most likely not block the progression of atherosclerosis. Transcription factors represent a more complex target for therapeutic intervention, because they usually regulate large numbers of genes. For instance, members of the NF κ B family coordinately regulate gene clusters that control inflammatory responses and have been implicated in the development of atherosclerosis [70]. In fact, activation of the pro-inflammatory nuclear factor kappa B (NF- κ B) [13, 41] and up-regulation of NF- κ B dependent genes [41] by balloon injury has been shown in animal experiments [25].

The transcription factor NF- κ B is a common factor promoting the inflammatory response of cells. In resting cells, NF- κ B is retained in the cytosol by complex formation with its natural inhibitor I κ B [3, 4]. Upon stimulation by inflammatory cytokines, I κ B is phosphorylated, ubiquitinated and degraded by the proteasome. In turn, NF- κ B is liberated and enters the nucleus to initiate transcription of certain adhesion molecules, cytokines and other inflammatory mediators [19]. The NF κ B signaling process is complex and several negative (e.g. upregulation of the NF κ B inhibitor I κ B [73]) and positive (e.g. upregulation of XIAP that activates NF κ B [35]) feedback loops participate. Among the genes regulated by NF κ B are also the family of inhibitors of apoptosis proteins (IAPs) [66] that are responsible for survival of cells especially endothelial cells under inflammatory conditions. Therefore, blocking the NF κ B pathway would not only inhibit the inflammatory response including recruitment of mononuclear cells, but would also favor cell death by lower overall contribution of anti-apoptotic mechanism.

During the inflammatory process, however, not only NF κ B becomes activated, but also other signal transduction pathways are affected. These pathways include activation of the MAP kinase ERK1/2 and the downstream transcription factor EGR-1 [50]; in addition inflammation also upregulates the orphan nuclear receptor Nur77 (NAK-1) [28]. Therefore, whenever the NF κ B pathway is inhibited, only part of the inflammatory response is affected. These partially NF κ B independent pathways, however, might also participate in feedback loops. In addition to leaving some inflammatory pathways unaffected, shutting off the NF κ B system might result in sometimes unexpected effects.

In conclusion, inflammation and specifically the NF κ B pathway can participate at several stages in vascular remodeling: NF κ B directly contributes to an inflammatory loop via adhesion molecules and chemokines recruiting monocytes that in turn release growth factors and other cytokines that augment smooth muscle cell proliferation. This proliferative effect is counterbalanced to some extent by EGR-1 induced upregulation of Nur77/NAK-1 that has pro-apoptotic properties. On the other hand, NF κ B induces anti-apoptotic proteins that act synergistically with the proliferative EGR-1 pathway and antagonistic to the pro-apoptotic Nur77/NAK-1.

Another family of transcription factors is the peroxisome proliferators-activated receptor subfamily (PPAR α , γ and δ). While PPAR α stimulates expression of apoA-I and raises human plasma HDL levels and PPAR α activators inhibit the inflammatory response of human aortic smooth-muscle cells [65], PPAR γ exerts both pro- and anti-atherogenic effects on macrophage gene expression. On the one hand, activators of PPAR γ upregulate the expression of scavenger receptor CD36 in macrophages, but on the other hand decrease MMP-9 and TNF- α expression indicating an anti-inflammatory role in atherosclerosis [38, 57, 71]. These findings have important clinical implications, because PPAR γ is the molecular target of the thiazolidinedione (TZD) class of insulin sensitizer used for treatment of type II diabetes mellitus patients [64].

Searching for new genes linked with the atherosclerotic disease

Different genetic backgrounds in mice determine the extent of atherosclerosis [16]. Comparable observations were made in humans, knowing that a family history of heart disease represents an important risk factor. Therefore, the association of common single nucleotide polymorphism (SNPs) will possibly lead to the identification of new susceptible genes for atherosclerosis.

Recent development of gene-expression-profiling technologies has enabled the large-scale analysis of gene expression changes during disease progression. This technology allows to be investigated the complex interactions of multiple cell types in atherosclerotic lesions, which leads to a better understanding of the pathology of cardiovascular diseases and the potential identification of underlying genetic defects in a number of animal and tissue-culture models [63].

The combination of proteomic and genomic technologies, animal models and human and murine genome sequencing efforts for a better understanding of the complex pathogenesis of atherosclerosis may allow the development of new powerful anti-atherosclerotic drugs.

Is there an “atherosclerosis gene”? From the evidence shown above, it is clear that several systems and pathways contribute at different levels and during different disease stages to the initiation, progression and late events of the atherosclerotic disease. The question whether an “atherosclerotic gene” exists can clearly be denied. This disease is a classical multifactorial disease, where disease susceptibility is given by a different pat-

tern of genetic variations together with a strong environmental component.

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