Institutionen för Medicin,
Enheten för Akutmedicin
Karolinska Universitetssjukhuset, Huddinge,
Karolinska Institutet, Stockholm, Sweden

Natural antibodies against phosphorylcholine as potential protective factors in atherosclerosis, cardiovascular disease and systemic lupus erythematosus

Jun Su

蘇峻

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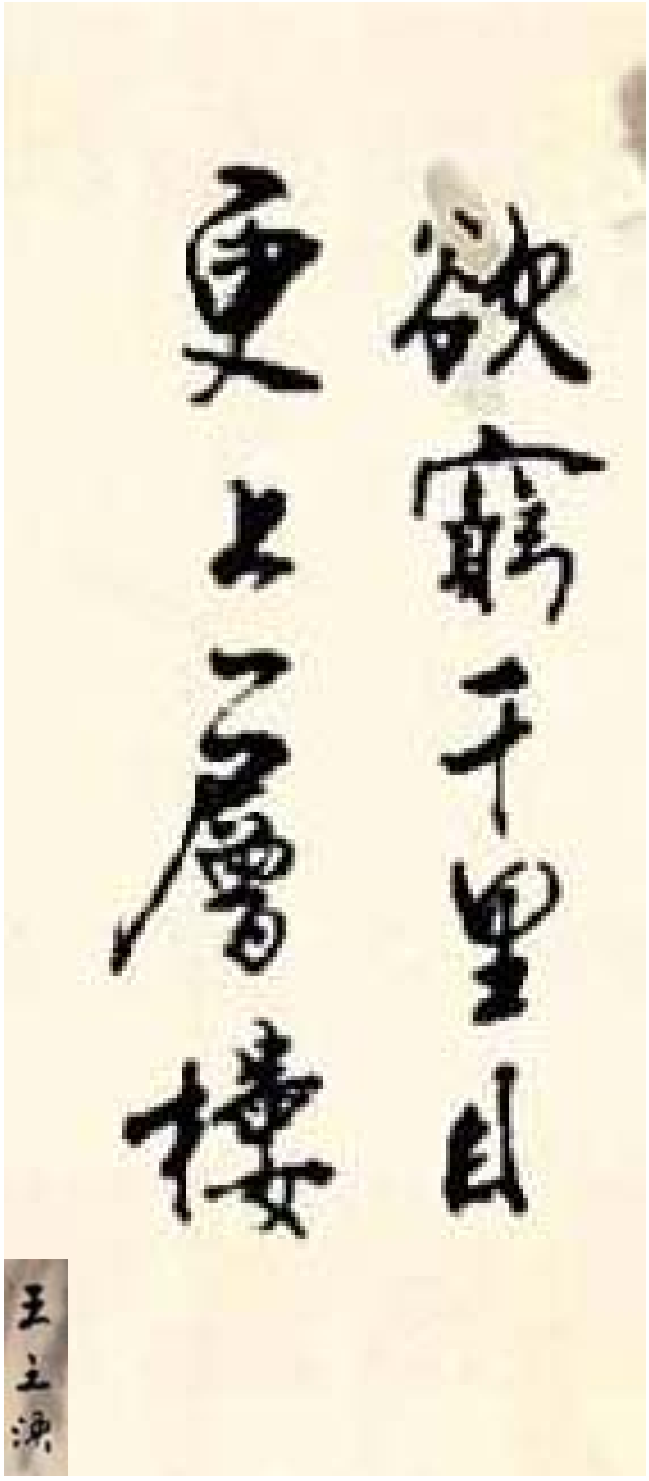

You can enjoy a grander sight by ascending to a greater height

Wang Zhihuan
Translated by Jun Su

献给翔, 梓源, 贝嘉和泽睿

To Xiang, Nicole, Rebecca and Alex

Abstract

Atherosclerosis is the most important contributor to cardiovascular disease (CVD). Although it was initially considered as a bland lipid storage disease, processes of inflammation and autoimmunity are now considered to play a major role in atherosclerosis.
Oxidation of low density lipoprotein (OxLDL) has been implicated in atherogenesis and much of the lipid content in the atherosclerotic lesions consists of OxLDL.
OxLDL binds to and is taken up by macrophages through scavenger receptors (SRs) and subsequently these macrophages develop into foam cells, which marks the initiation of atherosclerosis.
The important role of autoimmunity and inflammation in atherosclerosis and CVD is illustrated by Systemic lupus erythematosus (SLE), an autoimmune disease, where the risk of CVD is very high and the prevalence of atherosclerotic plaques is increased. This association is only partially linked to the traditional risk factors for CVD. In addition to these, systemic inflammation and autoantibodies against phospholipids among others can be considered SLE-related risk factors to CVD. CVD in SLE is not only an important clinical problem, but may also shed light on the role of immunity in atherosclerosis.
The purpose of this thesis was 1) to develop an assay for anti-phosphorylcholine (anti-PC) antibodies and define the correlation between anti-PC levels and atherosclerosis, CVD and SLE, 2) to determine whether PC-epitopes on OxLDL are involved in the uptake of OxLDL by human macrophages, 3) to investigate if anti-PC antibodies have any anti-inflammatory role in CVD.
An ELISA-based method was developed to measure anti-PC antibodies levels in clinical cohorts. We used sepharose columns to purify anti-PC antibodies from human IgM and IgG. Next, we determined the affinity and specificity of such purified anti-PC antibodies by competition experiments. We established a method to study different properties of anti-PC, including macrophage uptake of OxLDL and effects of anti-PC on inflammatory reactions relevant in atherosclerosis, by use of flow cytometry and confocal microscopy.
In the first study, we developed an assay for and determined anti-PC levels in hypertensive subjects. We found that high levels of IgM anti-PC antibodies and IgM anti-OxLDL antibodies predict a favorable outcome in the development of carotid atherosclerosis.
In the second study on SLE and SLE related CVD, we determined, surprisingly, that low levels of IgG and IgM anti-PC are more prevalent in SLE and SLE-related CVD and that levels of IgG anti-PC are decreased in these groups. Further, we found that anti-PC antibodies have anti-inflammatory effects on adhesion molecule expression stimulated by an inflammatory phospholipid.
In the third, prospective study, we found that low levels of IgM anti-PC antibodies predict CVD in 60-year old men, and determined that human IgM anti-PC antibodies block the uptake of OxLDL in macrophages which may be one explanation as to why anti-PC may protect against atherosclerosis development in humans.
In the fourth paper, which is a follow-up study of the 60-year old cohort, we determined, that in contrast to IgM anti-PC, IgG anti-PC did not predict risk for CVD. Specific IgG anti-PC antibodies extracted from total IgG only had minimal effects in preventing uptake of OxLDL. Howerer, upon pre-incubation with antibodies against FC-receptors, IgG anti-PC were able to induce a reduction in uptake of OxLDL, indicating that FC-receptors may contribute to OxLDL uptake in macrophages.
In conclusion, we report for the first time that high levels of IgM anti-PC antibodies is a novel protective factor for atherosclerosis, and low IgM anti-PC antibodies is an independent risk marker for development of CVD in humans. Further, we demonstrate that anti-PC can inhibit the uptake of OxLDL in macrophages and exert anti-inflammatory effects relevant in the atherosclerotic lesions. It is thus conceivable that raising anti-PC levels through active immunization or passive transfer of anti-PC could be a novel therapeutic possibility.

LIST OF PUBLICATIONS

I. **Jun Su**, Anastasia Georgiades, Ruihua Wu, Thomas Thulin, Ulf de Faire, Johan Frostegård. Antibodies of IgM subclass to phosphorylcholine and oxidized LDL are protective factors for atherosclerosis in patients with hypertension. Atherosclerosis 188 (2006) 160–166

II. **Jun Su***, Xiang Hua*, Hernan Concha, Elisabet Svenungsson, Anna Cederholm and Johan Frostegård. Natural antibodies against phosphorylcholine as potential protective factors in SLE. Rheumatology 2008 47(8):1144-1150. (*Contributed equally)

III. Ulf de Faire, **Jun Su**, Xiang Hua, Anna Frostegård, Mats Halldin, Mai-Lis Hellenius, Max Wikström, Ingrid Dahlbom, Hans Grönlund, Johan Frostegård. Low levels of IgM antibodies to phosphorylcholine predict cardiovascular disease in 60-year old men: effects on uptake of oxidized LDL in macrophages as a potential mechanism. (Submitted)

IV. **Jun Su**, Xiang Hua, Anna Frostegård, Ingrid Dahlbom, Max Vikström, Mai-Lis Hellenius, Ulf de Faire, Johan Frostegård. Antibodies against phosphorylcholine of IgG and IgM subclass in cardiovascular disease: clinical and functional differences. (Manuscript)

LIST OF ABBREVIATIONS

AII	angiotensin II
ABCA1	ATP-binding cassette transporter A1
ACAT	acyl coenzyme A:acylcholesterol transferase
aCL	anticardiolipin antibody
AGE	advanced glycation end products
anti-MDA-LDL	autoantibodies against malondialdehyde-LDL
anti-OxLDL	antibodies against oxidized LDL
APC	antigen presenting cell
anti-PC	anti-phosphorylcholine
aPL	anti-phospholipid antibodies
apoA1	apolipoprotein A-1
apo B	apolipoprotein B
apoE KO	apoE knock out
APS	antiphospholipid syndrome
CD40L	CD40 ligand
CE	cholesteryl ester
CETP	cholesterol ester transfer protein
C-PS	cell-wall polysaccharide
CRP	C-reactive protein
CVD	cardiovascular disease
DCs	dendritic cells
dsDNA	double-stranded DNA
ECs	endothelial cells
ECA	endothelial cell activation
ELISA	enzyme-linked immunosorbent assay
FC	free cholesterol
FcRs	Fc receptors
HDL	high density lipoprotein
hsCRP	high sensitive CRP
HSP-60	heat-shock protein 60
ICAM	intercellular adhesion molecule-1
IFNγ	interferonγ
IL	interleukin
IMT	intima-media thickness
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoprotein
LDLRs	low density lipoprotein receptors
LDLR KO	LDL receptor knock out
LPC	lysophosphatidylcholine
MCP-1	monocyte chemoattractant protein-1
Lp-PLA2	Lipoprotein-associated phospholipase
M-CSF	monocyte colony-stimulating factor
MDA	malondialdehyde
MDA-LDL	malondialdehyde-modified LDL

MHC class II	major histocompatibility complex class II
MI	myocardial infarction
mmLDL	minimal modified LDL
MMPs	matrix metalloproteinases
NAbs	natural antibodies
NECH	neutral cholesterol ester hydrolase
NO	nitric oxide
NPPC	p-nitrophenyl PC
OxLDL	oxidized low density lipoprotein
OxPLs	oxidized phospholipids
PAF	platelet-activation factor
PAF-AH	PAF-acetylhydrolase
PAI-1	plasminogen activator inhibitor type-1
PAMPs	pathogen-associated molecular patterns
PC-KLH	PC conjugated -keyhole limpet hemocyanin
PGI2	prostacyclin
PC	phosphorylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PNPP	alkaline phosphatase substrate
PON	paraoxonase
POVPC	1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero- 3-phosphorylcholine
PPARγ	peroxisome proliferator-activated receptor γ
PRRs	pattern-recognition receptors
PS	phosphatidylserine
PtC	phosphatidylcholine
RE	reticuloendothelial
ROS	reactive oxygen specials
SLE	systemic lupus erythematosus
SM	sphingomyelin
SMCs	smooth muscle cells
SRs	scavenger receptors
TD	T cell–dependent
TF	tissue factor
TG	triglycerides
TGF β	transforming growth factor β
Th1	T helper 1
Th2	T helper 2
TI-2	T cell–independent type 2
TLRs	Toll-like receptors
TNF	tumor necrosis factor
t-PA	tissue-type plasminogen activator
UK	urokinase
VCAM-1	vascular cell adhesion molecule-1

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Introduction

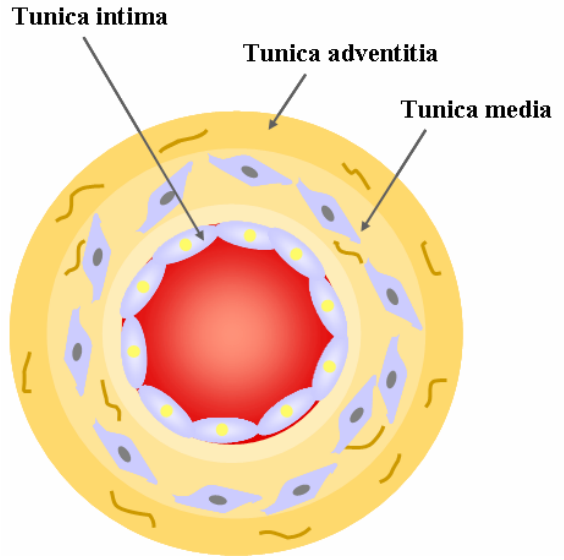
Cardiovascular disease (CVD) is a leading cause of morbidity and mortality in the world. Major risk factors associated with CVD have been identified, thus guiding researchers in creating a revolution in preventive medicine. This introduction serves to provide readers with a general overview of this disease, while this thesis work hopes to contribute towards the understanding of the protective effects to CVD.

Overview of atherosclerosis

Structure and function of blood vessels

All blood vessels, other than the capillaries, share a common basic three-layered structure. Namely, the intima, the media and the adventitia (Michiels 2003). The tunica intima is the innermost layer of blood vessels which consists of a thin lining of endothelial cells (ECs) overlying a thin layer of connective tissues. The tunica media is the middle layer of blood vessel walls, and comprises mainly of thin smooth muscle cells (SMCs) and elastic tissues which assist in the regulation of vessel volume and the blood pressure. The outermost layer, tunica adventitia, consists of connective tissues made up of fibroblasts and elastic fibers (Figure 1).

Figure 1



The intimal layer, of a healthy endothelium, is the main regulator of vascular homeostasis (Hirsch and Folsom 2004). The endothelium serves as an both anticoagulant and antithrombotic. ECs secrete a variety of molecules crucial for the regulation of blood coagulation, platelet functions and thrombosis. The major antiplatelet agents secreted by ECs include prostacyclin (PGI2) and nitric oxide (NO) (Lindemann, Kramer et al. 2007). Protein C/protein S pathway and thrombomodulin are also important for anticoagulation (Wakefield, Myers et al. 2008). The endothelium also participates in fibrinolysis by releasing tissue-type plasminogen activator (t-PA) and urokinase (UK) (Tesfamariam and DeFelice 2007).

ECs are exposed to three types of mechanical forces: pressure from hydrostatic forces of blood within the vessel, circumferential stress resulting from vasomotion of the vessel, and shear stress due to blood flow within the vessel.

Shear stress appears to play an important role in atherogenesis (Warkentin, Aird et al. 2003; Hurairah and Ferro 2004) because it stimulates the release of vasoactive substances and also because it changes gene expression, cell metabolism and cell morphology.

Atherosclerosis

Atherosclerosis — a progressive disease characterized by the deposition of intracellular and extracellular lipids, and the accumulation of fibrous elements in the arteries. It is the main cause of CVD.

Atherosclerosis begins early in fetal life (Wicker, Guelde et al. 1982) and progresses asymptomatically through adulthood (Chang, Binder et al. 2004), and later, it is clinically expressed as ischemic coronary syndromes, stroke, and peripheral artery disease.

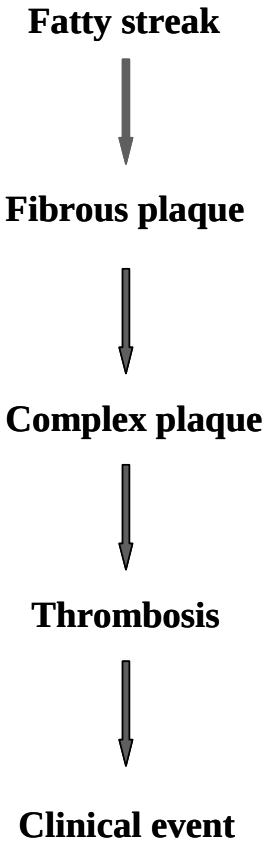
The progression of atherosclerosis include: fatty streak formation, fibrous plaque, and development into a complicated lesion.

Formation of fatty streaks involves the accumulation of lipid-laden cells beneath the endothelium. Most of these cells in a fatty streak are macrophages, together with some T cells and a small number of intima SMCs surrounded by deposits of lipid. The presence of fatty streaks causes little or no obstruction and expresses no clinical symptoms. They are present in the aorta of virtually every child. It may progress into an atheroma or eventually disappear.

The fibrous plaque is formed if the progresses unabated. The artery wall is thickened with compensation by gradual dilation (remodeling). Accumulation of more mononuclear cells, T cells, lipid-loaded foam cells, migration and proliferation of SMCs, and formation of fibrous tissue lead to further enlargement and restructuring of the lesion. The lesion comprises of a fibrous cap, together with collagen, elastic fibers and proteoglycans, overlying a core of lipid and nectotic tissues.

In a complicated lesion, the artery can no longer compensate by dilation and the growth of lesion extends inward, causing the narrowing of the vessel's lumen and the subsequent expression of clinical symptoms such as angina. An atherosclerotic plaque can become unstable and rupture, leading thrombus formation that occlusion of the vessel lumen. Ultimately, this may result in myocardial infarction (MI) and cerebral infarction (Figure 2).

Figure 2



Plaque rupture is largely induced by three major factors: a large central core of extracellular lipids and necrotic debris, a thin overlying fibrous cap (Fu, Davies et al. 1998) and a pronounced infiltrate of inflammatory cells (Faria-Neto, Chyu et al. 2006). Macrophages and T cells are the predominant cell types at the immediate site of rupture or erosion. The thinning of the fibrous cap and subsequent rupturing of the plaque leads to the release of inflammatory cytokines, digestive matrix metalloproteinases (MMPs), tissue factor (TF) and plasminogen activator inhibitor type-1 (PAI-1) mainly by tissue-invading macrophages and T cells (Plenz and Robenek 1998; Dzau, Braun-Dullaeus et al. 2002; Maron, Sukhova et al. 2002; Shah, Chyu et al. 2005)

Plaque rupture and endothelial erosion are two major events that (Corti and Badimon 2002) allow the blood to make contact with the thrombogenic material in the lipid core or the subendothelial region of the intima (Libby 2001), henceforth initiating thrombosis and the coagulation cascade.

Sites predisposed to lesion formation include branch points of arteries, where there are decreased shear stress and increased turbulence (Thornton, Vetvicka et al. 1994). At these sites, specific molecules are expressed on the endothelium. These molecules are responsible for the adherence, migration, and accumulation of monocytes and T cells. Such adhesion molecules act as receptors for glycoconjugates and integrins present on monocytes and T cells.

The thickness of the common carotid intima-media (IMT) as measured by ultrasound represents a marker for structural atherosclerosis (Juonala, Viikari et al. 2004).

Increased IMT is correlated with cardiovascular risk factors (Michiels 2003), severity of coronary atherosclerosis (Aikawa and Libby 2004) and is predictive of cardiovascular events in population groups.

The pathogenesis of atherosclerosis

Three main classical theories explaining the initiation of atherosclerosis have been put forward. Namely, the 'Response to injury' theory, 'Altered lipoprotein' theory and 'Clonal proliferation of smooth muscle cells' theory (Gordon, George et al. 2001).

In the 'Response to injury' theory, atherosclerosis was believed to be trigged by a 'injury' which refers to a loss of ECs from the vessel lining (Thornton, Vetvicka et al. 1994). Later studies, however, showed that the endothelial monolayer overlying the earliest lesions was actually intact (Fu, Davies et al. 1998).

Increased oxidative stress is considered to be a major factor involved in the pathogenesis of endothelial dysfunction, the initiation and progression of atherosclerosis and its adverse events (Yokoyama 2003)

Reactive oxygen specials (ROS) promotes cellular damage by reacting with NO, reducing vascular NO bioavailability, and exerting harmful effects by directly injuring of ECs and SMCs. Oxidation of LDL seems to play a central role in the atherogenic process.

Endothelial cell activation (ECA) is the earliest pathological signal of atherosclerosis. Upon activation, there is a loss of vascular integrity, increase expression of molecules associated with inflammatory responses (Tesfamariam and DeFelice 2007) such as adhesion molecules and chemotactic factors. These molecules are responsible for the capturing of monocytes and T cells and assistant in their migration into the arterial wall. For example, monocyte colony-stimulating factor (M-CSF) facilitates the phenotypic transformation of monocytes into macrophages while growth factors stimulates and induces the proliferation of SMCs. The endothelium then changes phenotype from antithrombotic to prothrombotic. This plays a critical role in the initiation of atherosclerotic lesions as well as the progression of vulnerable plaques (Hurairah and Ferro 2004). When the plaque ruptures eventually, local thrombosis occurs. With local obstruction, the thrombus can result in local ischemia (as in MI) or thromboembolism (as in stroke).

The candidates that initiate ECA include mechanical injury, oxidized low density lipoprotein (OxLDL), immunological injury, homocystinemia, hypercholesterolemia, chemical injury, hyperdislipidemia, hormone dysfunction, hypertension, smoking, diabetes and genetic factors (Steinberg and Witztum 2002).

Atherosclerotic development in germ-free apoE KO mice is not significantly different from that in apoE KO mice raised with ambient levels of microbial challenge suggests that infectious agents are not necessary for atherosclerotic development (Wright, Burton et al. 2000). However, they may play a role in accelerating disease progression.

Inflammation in atherosclerosis

Decades ago, the relationship between lipids and atherosclerosis dominated general thinking. However, hypercholesterolemia is crucial only in approximately 50 percent of patients with CVD. Despite using drugs to lower plasma cholesterol concentration (Shepherd, Cobbe et al. 1995), CVD continues to be the principal cause of death in the western world and with increasing prevalence in developing countries. Hence, other factors need to be taken into consideration.

Now atherosclerosis is generally viewed as a chronic inflammatory disease of the vascular wall (Li, Binder et al. 2004), that results from hyperlipidemia and a complex interplay of environmental, metabolic, and genetic risk factors. In addition, immune mechanisms can also modulate its progression.

Inflammation is involved in all aspects of atherogenesis (Maron, Sukhova et al. 2002; Libby 2008; Packard and Libby 2008). From the initial ECA to foam cell formation to the development of an atherosclerotic lesion, to the rupture of the "vulnerable" fibrous cap, all these eventually results in acute coronary syndrome and potentially death.

An inflammatory lesion is the site of interactions among ECs, monocytes and T Cells. Blood leukocytes adhere poorly to a normal endothelium. However, when the endothelial monolayer becomes inflamed, it expresses adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) that bind cognate ligands on leukocytes. Selectins such as P-selectin and E-selectin mediate leukocyte rolling and interaction with the inflamed luminal endothelium. Chemokines such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) recruit leukocytes into the arterial wall while integrins mediate firmer attachment.

Proinflammatory cytokines expressed within the atheroma provide a chemotactic stimulus to the adherent leukocytes, directing their migration into the intima. M-CSF can then induce monocytes entering the plaque to differentiate into macrophages and augment the expression of macrophage scavenger receptors (SRs) leading to the enhanced uptake

of modified lipoprotein particles and formation of lipid-laden macrophages. M-CSF and other mediators produced in plaques can promote the replication of macrophages within the intima as well (Thornton, Vetvicka et al. 1994). Activated macrophages in the intima can then release TF, MMPs, inflammatory cytokines, ROS, and other inflammatory molecules.

T cells, predominantly CD4+ T cells infiltrate into the intima (Hansson 1999). CD4+ T cells recognize antigens, such as OxLDL and heat-shock protein 60 (HSP-60), presented to them as processed fragments bound on major-histocompatibility- complex (MHC) class II molecules. This induces the expression of T helper 1 (Th1) cytokines such as interferonγ (INFγ), tumor necrosis factor (TNF) and interleukin-1 (IL-1) which causes the activation of macrophages and vascular cells, leading to inflammation. In apoE –knock out(apoE KO) mice lacking INFγ or its receptor, the development of atherosclerosis is inhibited (Whitman, Ravisankar et al. 2002). The cross reaction of different cytokines induces the production of substantial amounts of interleukin-6 (IL-6). IL-6, in turn, stimulates the production of acute-phase reactants, including C-reactive protein (CRP), serum amyloid A and fibrinogen (Torzewski, Klouche et al. 1998). These independent predictors of inflammation were thought to be early signs of atherosclerosis. Hence, the activation of a limited number of immune cells can initiate a potent inflammatory cascade, both in the forming lesion and systemically.

T helper 2 (Th2) cytokines produced by CD4+T cells can promote antiatherosclerotic immune reactions. However, they may also contribute to the formation of aneurysms by inducing elastolytic enzymes (Shimizu, Shichiri et al. 2004). Regulatory T cells modulate the process by secreting antiinflammatory cytokines, such as interleukin-10 (IL-10) (Nicoletti, Paulsson et al. 2000) and transforming growth factor β (TGF β) (Hessler, Robertson et al. 1979).

T lymphocytes join macrophages in the intima during lesion evolution. These leukocytes, as well as resident vascular wall cells, secrete cytokines and growth factors that promote the migration and proliferation of SMCs. Medial SMCs express specialized enzymes that can degrade the elastin and collagen in response to inflammatory stimulation. This degradation of the arterial extracellular matrix permits the penetration of SMCs through the elastic laminae and collagenous matrix of the growing plaque. Ultimately,

inflammatory mediators can inhibit collagen synthesis and evoke the expression of collagenases by foam cells within the intimal lesion. These alterations in extracellular matrix metabolism cause the thinning of fibrous cap, rendering it weak and susceptible to rupture.

Cross-talk between T cells and macrophages heightens the expression of the potent procoagulant TF (Thornton, Vetvicka et al. 1994). The main cell types involved in atherosclerosis, such as ECs (Bavendiek, Libby et al. 2002), macrophages (Brown, Schumacher et al. 2000), SMCs (Mach, Schonbeck et al. 1998), and platelets, express the proinflammatory cytokine CD40 (Mach, Schonbeck et al. 1998). T cells express CD40 ligand (CD40L) and CD40 ligation triggers the expression of adhesion molecules and secretion of numerous cytokines such as INFγ and MMPs. These cytokines are involved in extracellular matrix degradation (Mach, Schonbeck et al. 1998; Brown, Schumacher et al. 2000). Importantly, CD40 ligation also has a prothrombotic effect through the expression of TF which initiates the coagulation cascade when exposed to factor VII. Thus, when the plaque ruptures, TF can trigger a coagulation signaling pathway leading to thrombus formation that causes acute complications of atherosclerosis

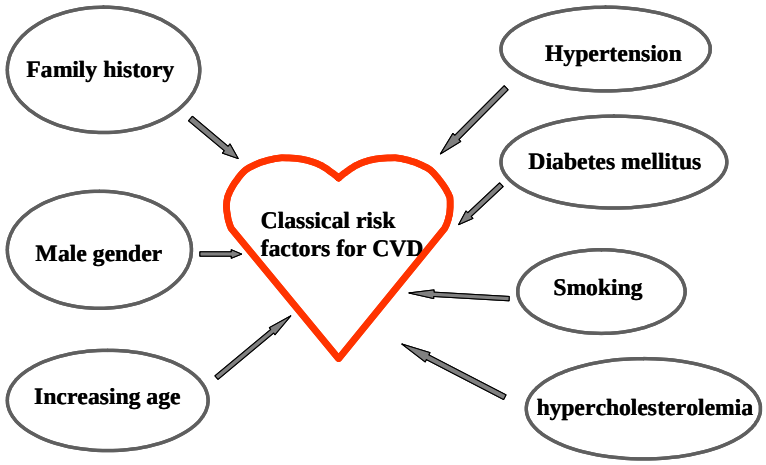
Initially the participation of leukocytes may be beneficial, but if the injurious agents are not removed by the inflammatory response, the response changes from being protective to injurious. This can then cause tissue damage and result in clinical events.

Risk factors for atherosclerosis

Risk factors investigation provides an opportunity to understand CVD and derive strategies for prediction, prevention, and treatment.

In 1940s, the town of Framingham was selected by U.S for researchers to find out which biological and environmental factors are associated with the development of CVD. 5,209 healthy residents between 30 and 60 years of age, both men and women, were enrolled as the first cohort of participants. This study is one of the most important epidemiological studies to investigate the major risk factors for CVD. Hypercholesterolemia, smoking, hypertension, family history, diabetes mellitus, male gender and old age were thus determined as classical risk factors for CVD (Figure 3).

Figure 3



People with a family history of CVD and with increasing age are more susceptible to CVD; man and menopausal women are also more prone to CVD as estrogen plays an important role in protection. In addition, anti-phosphorylcholine (anti-PC) antibody also contributes to protection against CVD, and will be discussed later. Angiotensin II (AII), together with its vasoconstricting properties, can instigate intimal inflammation. For example, AII elicits the production of ROS, from arterial ECs and SMCs. AII can also increase the expression of proinflammatory cytokines by arterial SMCs, such as IL–6,

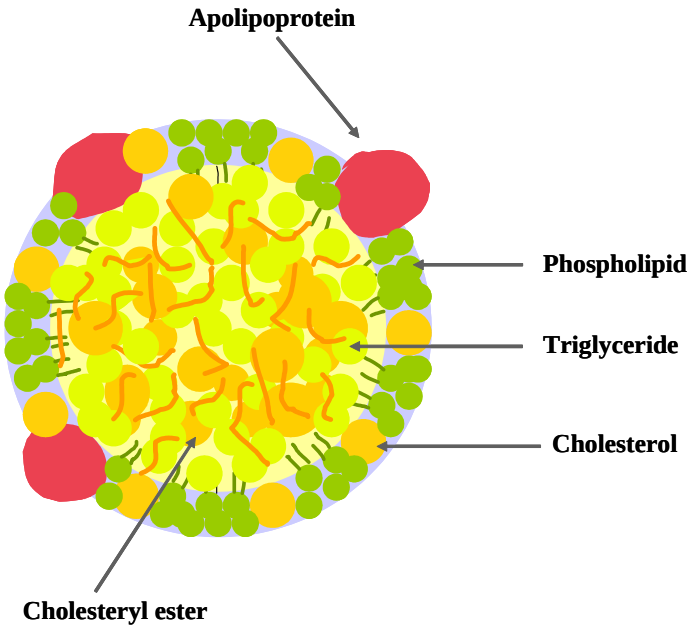
MCP-1 and o VCAM-1 expression on ECs (Maron, Sukhova et al. 2002). Hyperglycemia associated with diabetes by forming advance glycation end products (AGE) modify low density lipoprotein (LDL) can augment the production of proinflammatory cytokines by vascular ECs (Rudijanto 2007). Smoking is known to produce of oxygen-derived free radicals (Celermajer and Ng 2008) resulting in decreasa NO availability, oxidation of LDL and increase in insulin resistance (Barnoya and Glantz 2005; Heiss, Amabile et al. 2008).

Hypercholesterolemia, characterized by high cholesterol levels, in particular, high LDL-cholesterol level is a sufficient predictor for atherosclerosis (Steinberg and Witztum 2002).

However, the established classical risk factors could not fully explain the prevalence for CVD (Virani, Polsani et al. 2008). Thus, immune and inflammatory factors have been investigated and emerged as novel risk factors for CVD. Details are discussed below.

LDL

Figure 4



The general structure of LDL: An outer layer of phospholipids, polyunsaturated fatty acids of phospholipids act as a major oxidative target (Stremler, Stafforini et al. 1989; Chang, Binder et al. 2004; Xu, Yang et al. 2006), free cholesterol (FC), and apolipoproteins, with a core of cholesteryl ester (CE) with long-chain fatty acids and triglycerides (TG).

In a normal fasting man, the plasma concentration of LDL is 300-400 mg/100ml.

LDL is the major lipid carrier in plasma and 60-70% of total plasma cholesterol is carried in LDL. LDL consists of CE (29% of total mass), phospholipids (28%), protein (21%), FC (11%), and TG (9%) (Itabe 1998).

While LDL has an essential physiological role as a vehicle for the delivery of cholesterol to peripheral tissues, increased LDL cholesterol levels are associated with increased risk of CVD. LDL is taken up by cells via LDL receptors that recognize apolipoprotein B (apo B). The circulating level of LDL is determined mainly by its rate of uptake through the hepatic LDL receptor pathway.

LDL is a major cause of injury to the endothelium and underlying SMCs. When LDL is trapped in an artery they can undergo oxidation, transforming into OxLDL.

OxLDL

Oxidative modification of LDL is a quantitatively important event in the pathogenesis of the atherosclerotic lesion (Steinberg 1989; Witztum 1994).

Oxidation of LDL was the first observation of Brown and Goldstein in which incubation of native LDL with macrophages did not lead to foam cell formation unless the LDL was modified by chemical acetylation (Goldstein, Ho et al. 1979). Subsequently, studies by Henriksen and colleagues (Henriksen, Mahoney et al. 1981) demonstrated that LDL incubated with ECs had enhanced uptake by way of the acetyl LDL receptor of macrophages. Studies by Steinbrecher and coworkers (Steinbrecher, Parthasarathy et al. 1984) demonstrated that the LDL modification induced by ECs was the initiation of oxidation. Similar studies were performed by Chait and colleagues with respect to SMCs (Heinecke, Rosen et al. 1984). Chisolm and colleagues (Hessler, Robertson et al. 1979) demonstrated that OxLDL were cytotoxic to the ECs.

During oxidation of LDL, modification of the lipid moieties is initiated at sn-2 polyunsaturated fatty acid residues to generate hydroperoxides (Steinbrecher,

Parthasarathy et al. 1984; Smiley, Stremler et al. 1991), which are the primary products of lipid peroxidation reaction, and a variety of secondary products are considered as reactive molecular species, such as malondialdehyde (MDA) and the residual core-aldehydes of the OxPLs that contain the PC-headgroup, such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero- 3-phosphorylcholine (POVPC) that can modify other lipids and apolipoprotein B (apoB) (Steinbrecher 1987; Karten, Boechzelt et al. 1998). ApoB undergoes degradation, cleavage of its amide linkages, as well as derivatization of its lysine and other residues (Fong, Parthasarathy et al. 1987; Palinski, Rosenfeld et al. 1989), while short-chain aldehydes also form stable adducts with amino acid residues (Slatter, Paul et al. 1999). Both FC and EC are susceptible to oxidation, generating corresponding free and esterified oxysterols, respectively (Brown, Dean et al. 1996).

Levels of OxLDL are reported as the oxidized phospholipid content per particle of apoB (OxPL:apo B ratio) (Horkko, Bird et al. 1999). The OxPLs:apoB ratio remained an independent predictor of coronary artery disease.

After oxidation, OxLDL contains a variety of oxidation-specific neoepitopes on both lipid and protein moieties (Hörkkö, Binder et al. 2000). These epitopes are recognized by both adaptive T cell–dependent (TD) and innate T cell–independent type 2 (TI-2) immune responses. T cells recognize lipid antigens as complexes formed with antigen-presenting molecules, which belong to the CD1 family (De Libero and Mori 2006).

LDL modifications result in the formation of denser particles (Henriksen, Mahoney et al. 1981) with increased electrophoretic mobility (Steinbrecher 1987) , which are cytotoxic (Cathcart, McNally et al. 1989) and chemotactic for monocytes (Quinn, Parthasarathy et al. 1987) and inhibit macrophage motility (Quinn, Parthasarathy et al. 1985).

There are heterogeneous modifications of LDL. In vitro studies demonstrated that LDL could be oxidized under various conditions, such as metal ions medication (Heinecke, Rosen et al. 1984), culture cells (Babior 2000; Kobayashi, Matsuura et al. 2001), glycation (Berliner and Heinecke 1996; Rudijanto 2007), enzymatic degradation (Rankin, Parthasarathy et al. 1991; Cyrus, Pratico et al. 2001), aggregation (Tabas 1999)), all components of LDL undergo oxidation (Matsuura, Kobayashi et al. 2006).

There are supportive lines of evidence for oxidation of LDL in vivo. First, immunohistochemistry using antibodies against oxidized LDL (anti-OxLDL) recognize

materials in arterial lesions but not in normal artery of LDL receptor-deficient rabbits and 10% of CD4+ T cells cloned from human atherosclerotic plaques proliferate in response to OxLDL (Stemme, Faber et al. 1995). Second, LDL gently extracted from lesions of these rabbits is recognized by an autoantibody against malondialdehyde-LDL (anti-MDA-LDL). Third, the lipoprotein fraction gently extracted from atherosclerotic lesions (both rabbit and human) contains OxLDL, identified both by its physical properties and by its recognition by SRs (Frostegard, Svenungsson et al. 2005). Forth, anti-OxLDL can be demonstrated in rabbit and human sera. Firth, administration of antioxidants that can prevent oxidative modification of LDL slows the progression of atherosclerosis in several experimental animal models.

There is broad evidence which demonstrates that oxidation of LDL is involved in atherogenesis (Steinberg and Witztum 2002). First, OxLDL can be internalized by macrophages by SRs, resulting in the loading of macrophages with cholesterol and stimulation of foam cells formation (Matsuura, Kobayashi et al. 2006), which is the first step in atherosclerosis. Second, OxLDL stimulates ECs to express adhesion molecule, MCP-1 and M-CSF to recruit monocytes and T cells and transform monocytes to macrophages (Napoli, D'Armiento et al. 1997). Third, OxLDL can stimulate SMCs proliferation. Forth, OxLDL is cytotoxic for ECs (Hessler, Morel et al. 1983; Eva Hurt-Camejo, ncaron et al. 2001). Fifth, OxLDL may also regulate macrophage expression of pro-inflammatory genes potentially affecting plaque growth and/or stability (SRs, MMPs, and IL-6) by activating the ligand-dependent nuclear transcription factor peroxisome proliferator-activated receptor γ (PPARγ). Sixth, OxLDL inhibits vasodilation induced by NO (Palinski, Horkko et al. 1996). Seventh, OxLDL stimulates macrophages to express TF which promotes the pro-coagulant properties of vascular cells. Taking together, OxLDL is an independent risk factor for CVD.

Endothelium intima proteoglycans are thought to play a major role in the retention of OxLDL. The interaction between OxLDL and proteoglycans involves an ionic interaction between apoB and negatively charged sulphate groups on the proteoglycans (Skalen, Gustafsson et al. 2002).

Oxidation of the lipid-protein matrix of a plasma membrane is somewhat analogous to the oxidation of an LDL particle. Just as OxLDL can exert a number of effects, oxidation of

the plasma membrane of a cell may give rise to similar biological effects that could be relevant during apoptosis.

It is generally believed that LDL oxidation take place in the subendothelial space but not in circulation because of the presence of high concentrations of antioxidants and its rapid removal by the reticuloendothelial (RE) system, a process accelerated by the binding of autoantibodies (Frei, Stocker et al. 1988; Frostegard, Svenungsson et al. 2005).

OxPLs

Phospholipids make up the shell of LDL and are strctural phospholipids in the membranes of the cells. Nearly all of this phospholipid is phosphatidylcholine (PtC). Oxidation of phospholipids results in chain-shortened fragments and oxygenated derivatives of polyunsaturated *sn*-2 fatty acyl residues, which is often arachidonic acid (Boullier, Gillotte et al. 2000). A myriad of phospholipid products (platelet-activation factor (PAF) and PAF-like lipids) bind to and activate the human PAF receptor (Smiley, Stremler et al. 1991), and these PAF-like lipids are potent, selective inflammatory mediators (Stremler, Stafforini et al. 1989).

When LDL penetrate the ECs and undergo oxidation, a broad array of OxPLs are generated. Their accumulation in the vessel wall contributes significantly to the pathological consequences of developing atherosclerotic lesions (Stremler, Stafforini et al. 1989; Chang, Binder et al. 2004). They are toxic, pro-inflammatory and ultimately proatherogenic (Benveniste, Henson et al. 1972).

For example, oxidized cholesterol moieties can promote apoptosis and cell death. OxPLs can induce artery wall cells to secrete chemotactic molecules, such as MCP-1; activate ECs to express adhesion molecules for the attachment of monocytes and T cells; induce expression of growth factors, such as M-CSF, that facilitate the phenotypic transformation of monocytes into macrophages; and stimulate the proliferation of SMCs (Esterbauer and Ramos 1996). Synthesis of some of these oxidation products shows that they are able to activate cells expressing the PAF receptors (Tanaka, Iimori et al. 1994; Kern, Volk et al. 1998).

OxPLs are generated not only during atherogenesis but also in inflammation and apoptosis (Shaw, Horkko et al. 2001). The formation of PLs is a result of inflammation-induced oxidative stress. Inflammation is accompanied by increased oxidative stress.

Particularly during antibacterial defense, ROS generated by activated leukocytes not only kill the bacteria but also modify host molecules (Stremler, Stafforini et al. 1989; Horkko, Bird et al. 1999).

PAF can be inactivated by PAF-acetylhydrolase (PAF-AH). The role of PAF-AH in atherogenesis is currently under debate because it apparently has a dual function. Since much of the pro-atherogenic activity of OxLDL has been attributed to its increased lysophosphatidylcholine (LPC) content, a key proinflammatory mediators (Su, Hua et al. 2008; Wilensky, Shi et al. 2008), paradoxically, the ability of PAF-AH to hydrolyse PAF and related phospholipids also suggests an anti-inflammatory role (Tjoelker, Wilder et al. 1995)

In addition, OxPLs can inhibit the lipopolysaccharide (LPS) - induced upregulation of cell adhesion molecules in ECs (Sneddon, McLeod et al. 2006).

Antibodies against OxLDL (anti-OxLDL)

IgM and IgG anti-OxLDL antibodies developed in humans and in animal models of atherosclerosis (Fang, Kinlay et al. 2002), were reported to have different correlation (positive or negative) with the extent of atherosclerosis (Karvonen, Paivansalo et al. 2003; Licht, Dieker et al. 2004; Tsimikas, Brilakis et al. 2007).

Anti-OxLDL antibodies are present in part as immune complexes with OxLDL (Palinski, Ord et al. 1994). The titers of anti-OxLDL are well correlated with the content of OxLDL (Tsimikas, Palinski et al. 2001). The decrease in antibody titers is at least partially due to the result of the reduced presence of OxLDL in the aorta.

The titer of anti-OxLDL antibodies may be influenced not only by the amount of antigen, its immunogenic properties, and the tissue sites where it occurs, but also by complex regulatory mechanisms of the cellular and humoral immune system.

Anti-OxLDL antibodies as a risk marker are better than plasma LDL or OxLDL levels. OxLDL is cleared relatively rapidly from the plasma by the RE system (Joseph L. Witztum 1997) whereas plasma antibodies are expected to have a longer lifespan and to represent an integrated measure of the formation of OxLDL in the arterial wall.

Anti-OxLDL titers are an indicator of the rate of progression or severity of atherosclerosis (Palinski, Horkko et al. 1996). Elevated titers of antibodies to OxLDL directly correlate to atherosclerotic lesion progression.

Lipoprotein-containing immune complexes, by enhancing the release of proinflammatory mediators, can also play a pathogenic role in the development of atherosclerosis (Bidani, Roberts et al. 1980; Virella, Atchley et al. 2002).

The protective effect of anti-OxLDL against atherosclerosis has been demonstrated in animal studies. Several groups have investigated the effect of immunization with various forms of LDL in a variety of animal models of atherosclerosis. Immunization of hypercholesterolemic rabbits with homologous LDL and OxLDL significantly reduced the extent of atherosclerosis (Palinski, Tangirala et al. 1995; Nilsson, Calara et al. 1997; Freigang, Horkko et al. 1998; Nicoletti, Paulsson et al. 2000) Such a protection is associated with elevation of TD IgG anti-OxLDL.

Interestingly, immunization of LDL receptor knock out (LDLR KO) mice with MDA-LDL induced a robust IgG1 predominant anti-MDA-LDL response and concurrent secretion of large amounts of IL-5, which is consistent with the strong Th2-biased TD responses specific for MDA-LDL. In contrast, non-immunized cholesterol-fed mice generated autoantibodies to MDA-LDL predominantly of the Th1-dependent IgG2a isotype (Fossati-Jimack, Ioan-Facsinay et al. 2000; Binder, Hartvigsen et al. 2004). These findings suggest that the specific isotype of IgG responses (and/or associated cytokine responses) correlates with different, even opposing effects,

Anti-OxLDL antibodies may reduce lesion development by removing lipoproteins through Fc receptors (FcRs) by blocking their uptake that promotes lesion foam cells formation. These receptors are found on macrophages and other cells in the spleen and other lymphoid organs.

Anti-phospholipids antibodies

The anti-phospholipids (aPL) antibodies are most implicated in patients with antiphospholipid syndrome (APS) or systemic lupus erythematosus (SLE). Anti-PL usually of IgG class have thrombotic features. Several different mechanisms for the involvement of anti-PL in thrombogenesis have been proposed (Del Papa, Guidali et al. 1997).

For example, aPL antibodies may bind to the membranes of activated thrombocytes or activated vascular ECs and alter their function (Horkko, Miller et al. 1996). Patients with significant levels of anti-PL are prone to fetal loss (Bersot, Haffner et al. 2006), autoimmune thrombocytopenia (Rapaport and Le 1995), and thrombotic events (Rapaport and Le 1995; Bersot, Haffner et al. 2006)

The presence of aPL antibodies has been found to be a risk factor for various manifestations of coronary artery disease, such as angina and MI; as well as being associated with subclinical atherosclerosis (Vaarala, Manttari et al. 1995; Wu, Shoenfeld et al. 2003).

A protective role of these antibodies was found in one recent study (Roman, Shanker et al. 2003). In this case-control study, anticardiolipin (aCL) antibodies were found to be present less frequently in SLE patients with carotid plaque than in those without plaque. Furthermore, a monoclonal aCL antibody derived from a spontaneous murine APS/SLE model was reported to be able to protect from atherosclerosis when passively infused in atherosclerosis-prone mice (Nicolo, Goldman et al. 2003; Nicolo and Monestier 2004), confirming the lack of association between aCL antibody and carotid plaque or CVD as reported by other authors.

Other novel risk factors

Increased plasma concentrations of inflammatory markers, namely, CRP (Pasceri, Willerson et al. 2000), homocysteine (Rodgers and Kane 1986; Tsai, Perrella et al. 1994), IFNγ (Maron, Sukhova et al. 2002), CD40-CD40L (Mach, Schonbeck et al. 1998), PAF-AH (Carlquist, Muhlestein et al. 2007), HSP (Wicker, Guelde et al. 1982) and complement activation products (Tedesco, Fischetti et al. 1999), is a hallmark for CVD.

High density lipoprotein (HDL)

HDL particles may owe their protective role against atherosclerosis not only to reverse cholesterol transport, but also to provision of antioxidant enzymes, such as paraoxonase (PON) and PAF-AH (Frostegard 2005), and anti-inflammatory agents (Mackness, Arrol et al. 1991; Mackness, Mackness et al. 1996). This decreases endothelial adhesivity, and HDL-associated apolipoprotein A-1 (apoA1) has been shown to decrease TNF-α production through inhibiting contact-mediated activation of monocytes by binding to stimulated T cells (Hyka, Dayer et al. 2001).

Immune response in atherosclerosis

Atherosclerotic plaques contain significant amounts of T cells, many of which are in an activated state. Macrophages (Emeson and Robertson 1988), dendritic cells (DCs), natural killer T-cells, mast cells, few B cells (in the immediate adventitia surrounding lesions and in draining lymph nodes) (Moos, John et al. 2005; Galkina, Kadl et al. 2006), MHC class II molecules expressed on ECs and SMCs in the vicinity of activated T cells, immunoglobulins, C5b-9 complement complexes and CRP are found in atherosclerotic lesions. All these findings indicate local immunologic activation in the atherosclerotic plaque.

Adaptive immunity in atherosclerosis

Adaptive immunity results from somatic mutations in T and B cells, leading to the generation of an estimated 10^{18} T-cell and 10^{14} B-cell receptors, although its onset is delayed, they represent highly specific, high-affinity responses to newly recognized pathogens.

Adaptive immunity is an important modulator of disease development (Hansson 1999). T and B cells are not essential for lesion development but significant decreases in the extent of atherosclerosis are found in lymphocyte-deficient mice compared to immune competent mice (Dansky, Charlton et al. 1997; Reardon, Blachowicz et al. 2001), although this deficiency effect is site dependent (Reardon, Blachowicz et al. 2001).

T cells and atherosclerosis

T cells, representing the adaptive arm of the immune response, also play a critical role in atherogenesis.

Initial T-cell activation in response to atherosclerotic antigens such as OxLDL might occur in the regional lymph nodes (Robertson and Hansson 2006), possibly after antigen presentation by DCs trafficking from the plaque to the lymph node. After entering the blood, previously activated memory T cells bind cell-surface adhesion molecules that are expressed by ECs at the plaque surface, and then enter the plaque (Robertson and Hansson 2006). Macrophages in the plaque expressing MHC class II molecules might then present antigens to these T cells, leading to further activation. OxLDL uptake and

intracellular degradation by macrophages generate the T cell epitopes (Stemme, Faber et al. 1995).

10% of the clones derived from plaque T cells responded to oxidized LDL in an MHC class II-dependent manner (Robertson and Hansson 2006).

The specific cytokines expressed by lymphocytes can exert either pro-atherogenic or anti-atherogenic effects (Zhou, Caligiuri et al. 2001). Th1 cytokines dominate in mouse models of atherosclerosis and in human plaques. Th1 cytokines are believed to mediate proatherogenic responses. For example, IFNγ in particular is considered proatherogenic, via the activation of macrophages to augment many inflammatory mediators that promote lesion formation (Nicoletti, Caligiuri et al. 1999); downregulation of expression of ATP-binding cassette transporter A1 (ABCA1), which inhibit cholesterol efflux (Panousis and Zuckerman 2000); inhibition of production of proteoglycans by SMCs, which is predicted to weaken the fibrous cap; and lastly, inhibition of the production of the Th2 cytokine IL-10 (Nicoletti, Paulsson et al. 2000).

Th2 cytokines such as IL-10 and TGFβ can promote antiatherosclerotic immune reactions (Binder, Hartvigsen et al. 2004). However, they may also contribute to the formation of aneurysms by inducing elastolytic enzymes (Shimizu, Shichiri et al. 2004). Therefore, switching the immune response of atherosclerosis from Th1 to Th2 in late stage of atherosclerosis may not necessarily lead to reduced vascular disease.

Regulatory T cells modulate the atherosclerotic process by secreting antiinflammatory cytokines such as IL-10 and TGFβ (Chang, Binder et al. 2004).

B cells and atherosclerosis

B cells are typically characterized by their ability to produce antibodies. However, B cells possess additional immune functions, including the production of cytokines and the ability to function as antigen presenting cells (APC).

Several lines of evidence support the hypothesis that humoral immunity protects against atherosclerosis. First, B-cell-deficient LDLR KO mice developed increased atherosclerosis compared to wild type LDLR KO mice (Major, Fazio et al. 2002). Second, removal of the B cell–rich spleen enhances disease with decreased IgM anti-OxLDL antibodies, an effect that could be rescued by infusion of B cells from aged apoE

KO mice (Nicoletti, Paulsson et al. 2000). Third, injection of immunoglobulin preparations inhibits atherosclerosis (Nicoletti, Paulsson et al. 2000).

Few B cells are present in atherosclerotic lesions, but the antibodies produced by B cells largely localize in the lesions.

The B cell population contains functionally distinct subsets, and it is not known which population of B cells conferred this protection and whether this was due to humoral immunity or conceivably a newly emerging regulatory role of B cells as well (Mizoguchi and Bhan 2006). However, as the production of antibodies is the most important function of B cells, it is highly likely that antibodies play a major role in mediating this atheroprotection. This observed atheroprotective effects of B cells may in part be mediated by natural antibodies (NAbs).

Innate immunity in atherosclerosis

Innate immunity provides the first line of defense against highly conserved motifs in pathogens. It constitutes a rapid and blunt defense that can be mobilize in minutes to hours.

Innate immunity is characterized by a natural selection of germline-encoded receptors, It involves several cell types, most importantly macrophages, DCs and natural killer cells, which express a limited repertoire (about 100) (Miller, Chang et al. 2003) of highly conserved pattern recognition receptors (PRRs), such as SRs and TLRs. These receptors are preformed and present at birth and/or matured via positive selection during the neonatal period or shortly thereafter. They are available for almost immediate defense against a perceived pathogen. Such PRRs typically recognize a restricted pattern of ligands, called pathogen-associated molecular patterns (PAMPs), such as LPS, apoptotic surface phosphatidylserine (PS), CRP and oxidation-specific epitopes (Binder, Horkko et al. 2003).

By binding, engulfing, secreting cytokines and growth factors, innate immunity provide both targeted and generalized responses that help orchestrate a coordinated defense against “pathogens” (Gough and Gordon 2000).

In addition, NAbs and complement system are also integral mediators of innate immunity.

After endocytosis, macrophages and the other APC present epitopes to T cells, and thus serve as a vital link between innate and adaptive immunity.

Oxidation-specific epitopes could be bound by SRs, TLRs, CRP and NAbs (Miller, Chang et al. 2003; Miller, Viriyakosol et al. 2005). Hence these epitopes are an important PAMP recognized by innate immunity (Miller, Chang et al. 2003).

Atherogenesis involves cross talk between pathways involved in adaptive and innate immunity (Nicoletti, Caligiuri et al. 1999).

NAbs and atherosclerosis

Most of the antibodies produced by B-2 cells are encoded by genes that have undergone somatic rearrangement (Bendelac, Bonneville et al. 2001; Tuominen, Miller et al. 2006).

B-1 cells differ from B-2 cells in many ways, such as surface phenotype, anatomical location, restricted use of V_H genes that are minimally mutated and reflective of germ line usage, and importantly, their capacity for self renewal (Hardy, Carmack et al. 1994).

Burnet in 1959 proposed the clonal selection theory, which postulates that autoreactive clones are deleted during ontogeny. However, recent studies have demonstrated that NAbs are present in measurable quantities in the sera of normal individuals of different species (Karsenti, Guilbert et al. 1977; Lutz and Wipf 1982).

These NAbs are reactive towards highly conserved molecules, such as serum proteins, cell surface structures and intracellular structures. Thus, it appears that the presence of autoantibodies is not restricted to a pathological state, but also paticipates in normal physiology.

NAbs are an essential layer of innate immunity (Baumgarth, Tung et al. 2005). They are germ line encoded antibodies secreted by B-1 cells (Kearney 2000), mainly IgM, forming a crucial first line of defense against infectious pathogens. Also they play key housekeeping roles against endogenous pathogens (Holmberg and Goutinho 1985; Avrameas 1991).

B-1 cells are positively selected in fetal life, or shortly thereafter. Since NAbs are present even in germfree mice and in human cord blood (Baumgarth, Tung et al. 2005), this strongly implies that endogenous antigens must be the primary selecting factor (Harkewicz, Hartvigsen et al. 2008). Dying cells may be the initial stimulus for the production of NAbs (Petri, Roubenoff et al. 1996). B-1 cell clones can be expanded later

by antigen exposure leading to increased IgM levels in plasma (Hörkkö, Binder et al. 2000; Kearney 2000; Kenny, Derby et al. 2000). It is now understood that antigen selection during fetal and neonatal period leads to positive selection (Kenny, Derby et al. 2000).

Because NAbs have been conserved by natural selection, they must possess advantageous properties in maintaining homeostasis such as their crucial role in immediate host defences against pathogens in a general context (Binder, Chou et al. 2008).

NAbs mainly belong to the IgM class, IgG and IgA natural antibodies have also been described (Adib, Ragimbeau et al. 1990; Avrameas 1991).

T-15 (Briles, Nahm et al. 1981), an immunoglobulin A myeloma protein, has specificity to PC covalently linked to cell-wall polysaccharide (C-PS) of pathogens and provide optimal protection to mice from lethal infection with *Streptococcus pneumoniae* (Briles, Forman et al. 1982; Binder, Hartvigsen et al. 2004).

Cholesterol-fed apoE KO mice have very high autoantibody titers to OxLDL, in particular IgM. EO6 was cloned from the spleens of these mice with specificity for Cu-OxLDL (Palinski, Horkko et al. 1996). The structure and specificity of EO6 are identical to T15.

T15/EO6 antibodies, secreted by B-1 cells, arise spontaneously in the perinatal period. They are found at low titers in normal mice but expanded during atherogenesis, presumably as a result of the extensive burden of oxidation- specific antigens that occur with the development of atherosclerosis (Palinski, Horkko et al. 1996; Hörkkö, Binder et al. 2000; Kearney 2000).

IL-5 links adaptive and natural immunity specific to epitopes of OxLDL. IL-5 also protects from atherosclerosis (Binder, Hartvigsen et al. 2004), in part by stimulating the expansion of atheroprotective natural IgM anti OxLDL antibodies.

NAbs are low affinity polyreactive molecules that react with both self and non-self antigens. Broad specificity and low-affinity binding of these NAbs to self antigens helps to prevent autoreactive clones from reacting vigorously with self antigens (Notkins 2004; Shoenfeld, Wu et al. 2004), therefore preventing the development of antoimmune diseases.

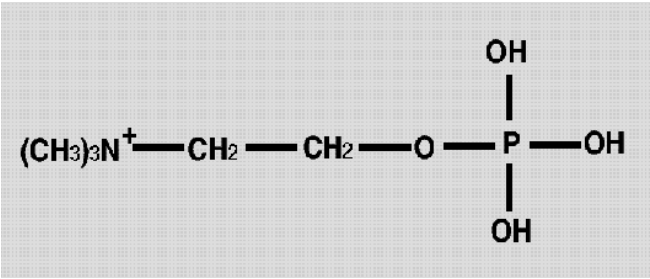
There is a difference between physiological natural autoimmunity and disease-associated processes. It has been established, mostly through the work of Weigert and colleagues (Shan, Shlomchik et al. 1994), that typical disease-associated autoantibodies undergo affinity maturation through antigen selection, followed by extensive somatic mutation. This allows the demarcation of physiological and pathological autoimmunity.

There are possible effector mechanisms of NAbs in atherosclerosis, such as, First, NAbs would bind to OxPLs present in OxLDL or apoptotic cells and neutralize most of their proinflammatory properties. These properties could promote atherogenesis through the activation of ECs (Binder, Hartvigsen et al. 2004; Harkewicz, Hartvigsen et al. 2008). Second, NAbs could block the uptake of OxLDL by macrophages and thus prevent foam cell formation in vivo (Frostegard, Svenungsson et al. 2005). Third, the formation of circulating immune complexes may be atheroprotective by preventing LDL from entering vulnerable sites of the artery wall. Forth, NAbs could recognize and remove self-antigens such as senescent cells, cell debris, and apoptotic cells (Huber, Vales et al. 2002; Binder, Hartvigsen et al. 2004).

The spleen is central to the generation and maintenance of the B-1 cell pool (Wardemann, Boehm et al. 2002). It is also a major source of NAbs products. Splenectomy of ApoE KO mice, which has lower anti OxLDL antibody titers, was shown to result in increased atherosclerosis,

Phosphorylcholine (PC)

Figure 5



PC was first detected in 1967 in the Gram-positive bacterium *Streptococcus pneumoniae,* where it was found to be associated with a polysaccharide component of the cell wall

(Tomasz 1967), subsequently shown to be teichoic acid (Brundish and Baddiley 1968). In addition, it was later found in cell membrane lipoteichoic acid (Briles and Tomasz 1973).

PC is also a component of a variety of other Gram-positive organisms, including *Clostridium* spp. (Sanchez-Beato, Ronda et al. 1995), *Lactococcus* spp. (Gillespie 1996), and *Bacillus* spp. (Gillespie 1996).

PC appears to play a crucial role in the maintenance of normal cell shape, size and physiology (Horne and Tomasz 1993). It is also necessary for normal growth and cell division.

PC is an antigenic component on the cell surface of many commensal and pathogenic bacteria that reside in the upper airway (Goldenberg Hb Fau - McCool, McCool Tl Fau - Weiser et al. 2004).

Oxidative modification of PtC in the membranes of LDL induce conformational changes to reveal PC epitopes (Boullier, Gillotte et al. 2000). (Figure 5)

Most biological cell membranes appear to have a different phospholipid composition in their inner and outer leaflets. At least in plasma membranes, transverse lipid segregation is firmly established. In erythrocytes, the best documented system, PS, phosphatidylethanolamine (PE), and probably phosphatidylinositol (PI) are located mainly in the inner monolayer while PtC and sphingomyelin (SM) are essentially in the outer monolayer (Gleason, Pierce et al. 1981). So when cells are apoptotic PC epitopes are easily exposed.

PC epitope becomes accessible for binding by SRs after oxidation-induced conformational changes occur in the PtC molecule in OxLDL or apoptotic cells. PC mediates the uptake of OxLDL by macrophages thus inducing foam cells formation, which is the hallmark of the early fatty streak.

Molecular mimicry in vivo between the PC moiety of the cell wall polysaccharide of bacterial pathogens and the PC moiety of OxPLs, such as POVPC, present on OxLDL and on apoptotic cells (Chang, Binder et al. 2004). So PC epitopes shared by oxidation, apoptosis and infection act as a PAMP recognized by PRRs.

PC provides an attractive model system for studying mechanisms of mimicry of bacterial antigenic epitopes and the quality of an immune response against bacterial infection and oxidative effects.

Anti-phosphorylcholine antibodies

Anti-PC antibodies were first found to render protection against *Streptococcus pneumoniae* infection in mice.

Mice that are experimentally or genetically unable to produce anti-PC antibodies are highly susceptible to bacterium infection (Briles, Nahm et al. 1981; Yother, Forman et al. 1982). Immune responses to PC regardless of immunoglobulin isotype, IgM , IgG3 and some IgG1 (Claflin, Lieberman et al. 1974) as well as PC binding CRP (Mold, Nakayama et al. 1981) can protect mice from fatal infection by the virulent *Streptococcus pneumoniae.*

In BALB/c mice, anti-PC antibodies are associated with different idiotypes, such as M167, MOPC 603, 511. and T15 (Claflin and Davie 1974; Lieberman, Potter et al. 1974). All the idiotypes render protection against bacterial infection with T15 having the strongest effect. The T 15+ anti-PC antibodies are dominant in normal BALB/c sera and in the antibodies in mice immunized with C-PS or PC conjugated - keyhole limpet hemocyanin (PC-KLH) (Mond, Vos et al. 1995).

The response to PC is restricted to the T15 idiotype regardless of the carrier used to induce the antibody response. This finding suggested that the binding site pocket for PC is essentially preserved, whereas that for the carrier is variable (Andres, Maddalena et al. 1981).

IgM anti-PC antibodies are atheroprotective in mice. Active immunization with *Streptococcus pneumoniae*, which bears PC on its cell wall, reduced atherosclerosis in hypercholesterolemic mice and this effect was attributed to an immune response towards PC (Miller, Chang et al. 2003). Passive infusion of IgM anti-PC into apoE KO mice inhibited the accelerated vein graft atherosclerosis, although this failed to inhibit progression of lesions in native atherosclerosis (Faria-Neto, Chyu et al. 2006).

Anti-PC antibodies are divided in two groups according to animal studies by the antibodies response towards PC-KLH. Group I antibodies are mainly IgM. They bind strongly to OxLDL, PC analogs such as p-nitrophenyl PC (NPPC) or aminophenyl PC, free PC, early stages of apoptotic cells and *s.pneumococcal* (Chang, Brown et al. 1982; Chang, Brown et al. 1982). Group II antibodies are mainly IgG. They bind to OxLDL, PC analogs (Andres, Maddalena et al. 1981; Bruderer, Stenzel-Poore et al. 1989) and later

stages of apoptotic cells and bind weakly to PC and *s.pneumococcal* (Kenny, Guelde et al. 1994; Fischer, Longo et al. 1995; Guo, Burger et al. 1997). Neither of these two groups binds to native LDL.

Anti-PC antibodies are found in normal serum (Harnett and Harnett 1999) and they represent a significant component of natural immunity (Riesen, Braun et al. 1976). In contrast to murine anti-PC response, human anti-PC is overwhelmingly IgG, with more than 80% of all immunoglobulin being IgG2. Nevertheless, a large proportion of this human IgG2 does bear the T15 idiotype (Brown, Schiffman et al. 1984). IgG2 has been shown to include majority of the IgG antibodies against carbohydrate antigens.

Anti-PC antibodies are detectable in the serum of normal individuals at mean concentrations of 320μg/ml for the IgG class and 110μg/ml for the IgM class (Nishinarita, Sawada et al. 1990).

Anti-PC antibodies are present in most adults under the age of 60 years. However, they are absent in very young children and are found at low levels in the elderly and IgG2-deficient adults, which are known to respond poorly to carbohydrate antigens.

Human anti-PC antibodies share idiotypes and are self-binding (Halpern, Kaveri et al. 1991). The self-binding site involves structures of the hypervariable region of the heavy chain. Human anti-PC self-binding antibodies express the murine T15 idiotype, indicating mouse-man idiotypic cross-reactivity.

The fluctuation of anti-PC antibodies levels in healthy individuals over time is small (Diaz Padilla, Ciurana et al. 2004). There were no significant differences in mean concentration of IgM anti-PC after repeated freeze–thaw cycles.

Some anti-DNA antibodies may cross react with PC (Sharma, Isenberg et al. 2001). Although it has been suggested that anti-PC antibodies generated in response to pneumococcal infections may mutate to anti-DNA antibodies thus leading to disease (Ray, Putterman et al. 1996), vaccination with pneumococcal polysaccharide failed to induce anti-DNA antibodies in humans (Sharma, Isenberg et al. 2001).

PC-bearing dental plaque microorganisms may induce an antibody response to PC that could influence the inflammatory response associated with atherosclerosis (Schenkein, Berry et al. 2001).

Sera of patients, containing high titers of IgM anti-PC also bound to apoptotic cells (Padilla, Ciurana et al. 2004). These data suggest that humans also have PC-specific IgM antibodies with microbial/OxLDL/apoptotic cell cross-reactivity.

The maintenance of T15 antibodies as the dominant anti-PC idiotype is under strict regulation. Effector mechanisms involves helper T cells (Bottomly and Mosier 1979), suppressor T cells (Rowley, Kohler et al. 1976), and auto-anti-idiotype (Cerny, Caulfield et al. 1980).

Generally, whether the antibody response is protective or not is dependent on the idiotypes (Briles, Forman et al. 1982; Casadevall, DeShaw et al. 1994) and the isotypes (Yuan, Casadevall et al. 1995; Yuan, Spira et al. 1998). In particular, isotype changing can dramatically affect the outcome of the response, shifting it from protection to disease enhancement.

But, mice immunized with PC-KLH had an increase in both IgG and IgM titers which helps to protect mice against atherosclerosis. Theoretically, the presence of oxLDL/IgG immune complexes could lead to the cross-linking of macrophage FcγRs and activation of proinflammatory responses. In addition, passively administered IgM, IgG2a and IgG3 of anti-PC antibodies show varying levels of protection against lethal infection in xid mice (Briles, Claflin et al. 1981). The presumption is that different IgG isotypes bind to different FcγRs with different affinities and thus have different functional activities. Hence, the IgG isotypes may also have atheroprotective effect.

C-PS was injected in rabbits and mice, but only the mouse antibodies were opsonophagocytic (Szu, Schneerson et al. 1986). This result suggests that the induction of anti-PC antibodies might be protective, but in a species-dependent manner. This result is also supported by Pneumococcal vaccination which does not increase circulating levels of IgM antibodies to OxLDL in humans (Damoiseaux, Rijkers et al. 2007).

The protective effects of anti-PC antibody are likely to be due to their abilities to enhance the phagocytosis of pneumococci, which are subsequently killed by the ingesting phagocytes (McDaniel Ls Fau - Benjamin, Benjamin Wh Jr Fau - Forman et al.). In addition, the complement system is required to provide in vivo protective effects.

In our group's investigation, we found that IgM anti-PC antibodies are protective factors for atherosclerosis in patients with hypertension (Matsuura, Kobayashi et al. 2006). IgG

anti-PC antibodies are atheroprotective in SLE (Su, Hua et al. 2008). IgM anti-PC could mask the PC epitopes on OxLDL that are recognized by macrophage SRs; IgG anti-PC antibodies have an anti-inflammatory effect to ECs activated by OxPLs. Diet has also been shown to have an effect on IgM anti-PC levels (Frostegard, Tao et al. 2007).

Natural or induced antibodies reacting with PC might dynamically orchestrate protective host responses in infection, autoimmunity, and atherosclerosis (Binder and Silverman 2005).

Macrophages and foam cells formation in atherosclerosis

Macrophages in atherosclerosis

In a plaque, 40% of the cells are macrophages (Nicoletti, Caligiuri et al. 1999) while 10% are CD3+ T cells.

Macrophage, a central mediator of cellular innate immunity, by means of their scavenging functions protect the body from infection (Nicoletti, Paulsson et al. 2000). But the same mechanism also involve macrophages to initiate the oxidation of LDL and rapidly take up OxLDL through specific SRs, leading to foam-cell formation. This is a key event in atherosclerosis progression.

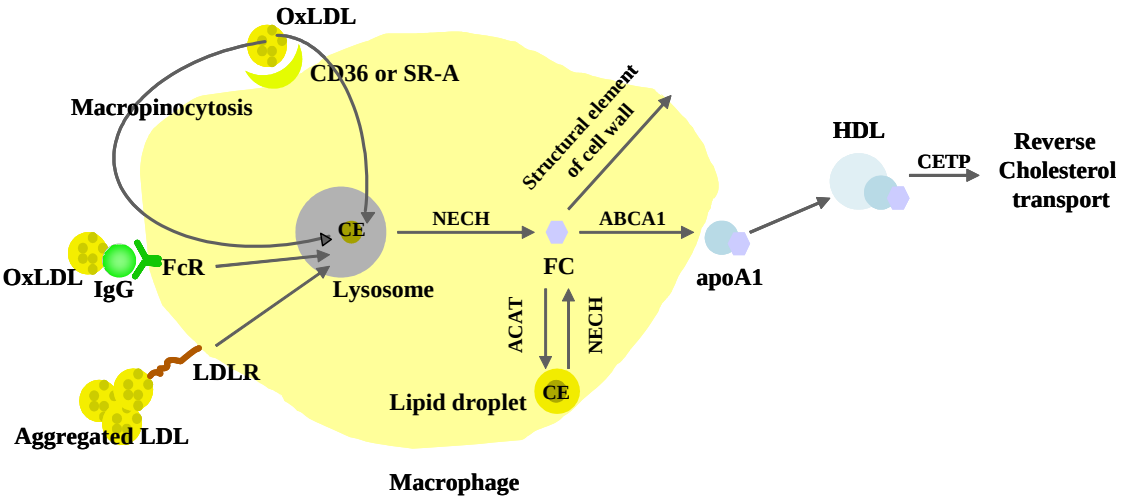
Macrophages also collaborate extensively with T-cells through cell–cell and cytokine-mediated interactions to coordinate the evolution of inflammatory responses and establish acquired immunity (Gordon, Clarke et al. 1995).

Hypercholesterolemic mice become extremely resistant to atherosclerosis when they are bred to macrophage-deficient mice (Scott, Briles et al. 1987).

Concerning atherosclerosis, macrophages have bifacial functions. On one hand, they participate in the clearance of OxLDL and the efflux of lipoprotein-derived cholesterol to apoA1 for reverse cholesterol transport, on the other hand, they contribute to foam cells formation.

OxLDL metabolism and foam cells formation

Figure 6



As shown in Figure 6, OxLDL taken up via SRs is delivered to lysosomes, where CE are hydrolyzed to FC and fatty acids by cholesterol esterases such as neutral cholesteryl ester hydrolase (NCEH) (Goldstein, Ho et al. 1979; Brown and Goldstein 1983).

Some of the FC are transported from the lysosome to cell membranes (Mathur, Albright et al. 1988; Slatter, Paul et al. 1999), serving as structural elements. The remaining FC are transported by ABCA1 (goldstein 1986) from cells to HDL acceptors , apoA1. Once the FC have been taken up by HDL, it is esterified to CE by lecithin-cholesterol acyltransferase (LCAT). HDL can subsequently exchange CE for TG carried by other lipoproteins via cholesterol ester transfer protein (CETP). Alternatively, HDL can selectively deliver CE to the liver for excretion by binding to the HDL receptor SR-B1 for the synthesis of bile acids in the liver, steroid hormones in ECs (e.g., adrenal gland, ovary, testes), and vitamin D in skin.

In the absence of an acceptor, excess FC inside the macrophages is converted to CE by acyl coenzyme A, such acyl coenzyme A:acylcholesterol transferase (ACAT) (Jerome,

Cash et al. 1998; Brown, Schumacher et al. 2000). CE storage in the lipid droplets gives the macrophages a foamy appearance (Torzewski, Klouche et al. 1998).

Acid hydrolases in lysosome are normally capable of complete and efficient degradation of lipoproteins to their constituent components (FC, fatty acids, glycerol, amino acids, etc.) In contrast to LDL, OxLDL is much less rapidly degraded. Oxidation of LDL clearly increases LDL uptake (Steinberg 1989) and appears to decrease lysosomal protein and CE hydrolysis. It has been recognized that apoB of OxLDL is more resistant to proteolysis by lysosomal enzymes (cathepsins) than native apoB (Steinbrecher and Lougheed 1992; Jessup and Kritharides 2000). Also, together with a large pool of CE which are resistant to esterases remain trapped in the lysosomes (Mander, Dean et al. 1994) in the form of particulate material such as ceroid. FC in the lysosomes also encounter a defect of in intracellular traffic mechanisms, specifically Golgi and trans-Golgi network (Jerome, Cash et al. 1998) hence resulting in FC accumulation within lysosomes (Aviram 1993). In addition, OxLDL upregulates ACAT-mediated reesterification (Roma, Catapano et al. 1990). All these may influence the physiology of macrophages and their subsequent roles in forming foam cells and the development of fatty streaks in early atherosclerosis.

Scavenger receptors and OxLDL uptake mediation

SRs were discovered in 1979 (Goldstein, Ho et al. 1979), and were originally named by Brown and Goldstein laboratory because they bound and internalized modified LDL, but not native LDL, contributing to foam-cell formation. The first such receptor identified is the “acetyl LDL receptor”, now known as SR-A.

So far, six different classes of such receptors have been identified (Krieger 1992; Pluddemann, Neyen et al. 2007). SR-A(SR-AI, SR-AII, MARCO), SR-B (CD36, SR-BI), SR-C (DSR-CI), SR-D(CD68), SR-E (LOX-1), SR-F (SREC).

In addition to macrophages, other cells such as ECs and SMCs exhibit SRs activity (Briles, Nahm et al. 1981; Pitas 1990).

Normal functions of SRs are the recognition and internalization of pathogens and apoptotic cells (Moore and Freeman 2006), crucial mechanisms in host defence and the phagocytosis of apoptotic cells which results in immunosuppressive and anti-inflammatory responses (Savill, Dransfield et al. 2002). However, the uptake of bacteria and bacterial components via SRs causes mostly pro-inflammatory responses.

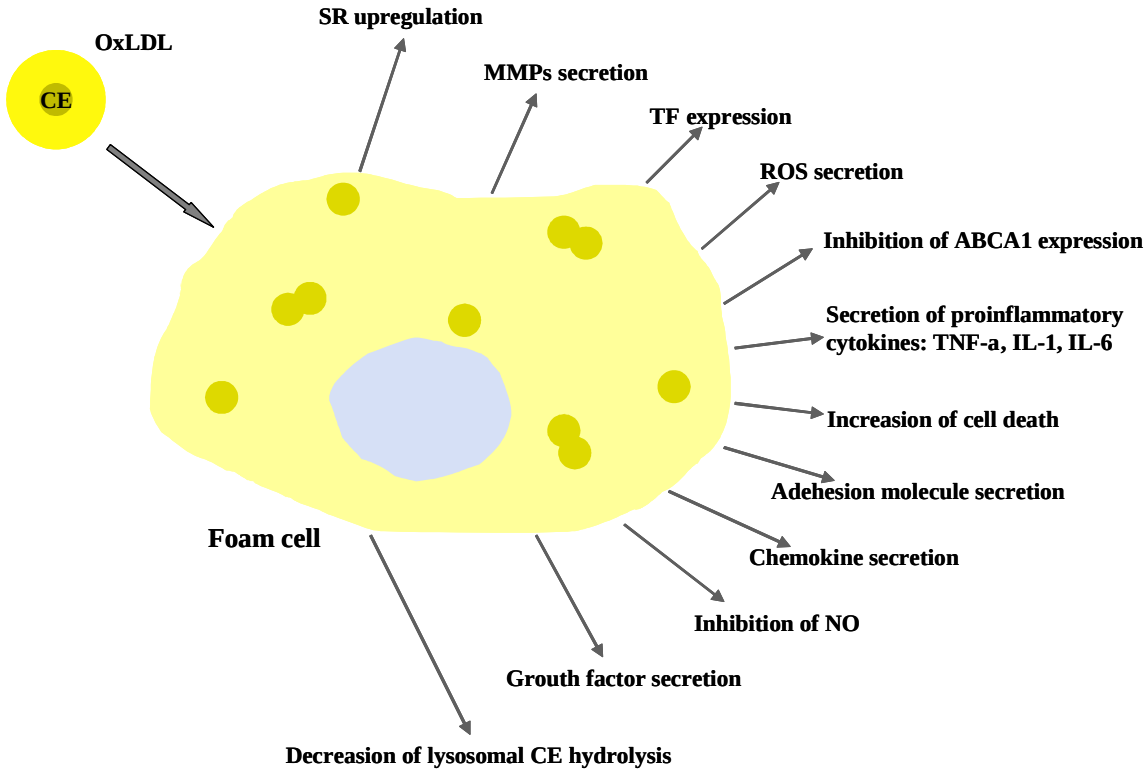
Unlike low density lipoprotein receptors (LDLRs), SRs are upregulated by OxLDL internalization (goldstein 1986; Steinberg 1989). Although the clearance of highly cytotoxic OxLDL may prevent the endothelium from exhibiting adhesion, the unregulated uptake of OxLDL by macrophages within the arterial wall leads to massive accumulation of CE which is characteristic of macrophage foam cells (de Villiers and Smart 1999; Asanuma, Oeser et al. 2003). Foam cells formation is most prominent and a pathologically important response in atherosclerosis.

Aggregated LDL,enzymatically modified LDL, immune complexes of LDL could all enter the macrophage by means of the SRs or FcR involve in foam cells formation.

Foam cells are capable of oxidizing LDL even when maximally loaded with cholesterol (Rosenfeld, Khoo et al. 1991).

Macrophage-derived foam cells have proinflammatory effects, such as expression of inflammatory cytokines (Huang, Jaffa et al. 1999; Huang, Schafer-Elinder et al. 1999; Petit, Lesnik et al. 1999; Hamilton, Byrne et al. 2001); toxicity (Li, Yuan et al. 1998); increased expression of MMPs (Huang, Mironova et al. 1999); overexpression of procoagulant TF (Lamb, Avades et al. 2001); inhibition of inducible NO synthase expression (Huang, Li et al. 1999); and influence on macrophage lipid metabolism and accumulation (Stengel, Antonucci et al. 1998). (Figure 7)

Figure 7



In addition, the death of lipid-laden foam cells leads to the formation of a necrotic, cholesterol-rich core covered by extracellular matrix proteins secreted by SMCs. This builds up a fibrous cap and leads to the development of fatty streak (Toshima, Hasegawa et al. 2000).

The preincubation of macrophages with minimally modified LDL (mmLDL) leads to an increased uptake of OxLDL by receptors such as CD36. Two OxPL components, i.e. 9- or 13-hydroxyoctadecadienoic acid (9-HODE and 13-HODE) activate PPARγ and result in the up-regulation of the SRs (Wu, Shoenfeld et al. 2003), thereby promoting foam cell formation.

SR-A and CD36 account for up to 75% - 90% of the uptake of OxLDL by macrophages in vitro and thus probably play a key role in foam-cell formation in vivo (Kunjathoor, Febbraio et al. 2002; Rahaman, Lennon et al. 2006).

CD36 recognizes a broad variety of ligands including OxLDL (Greenberg, Sun et al. 2006), anionic phospholipids (Ryeom, Silverstein et al. 1996), apoptotic cells (Manson, Mauri et al. 2005), long-chain fatty acids (Abumrad, el-Maghrabi et al. 1993), ß-amyloid (Moore, El Khoury et al. 2002) and AGE products (Ohgami, Nagai et al. 2001). It also functions in the recognition and removal of *Plasmodium falciparum*-infected erythrocytes (Oquendo, Hundt et al. 1989).

SRs may be beneficial during the initial stages of atherogenesis given their ability to clear potentially deleterious modified lipoproteins that accumulate in the artery wall. But unregulated SRs promote disease-causing foam cells formation and chronic inflammation. In some conditions, it could be protective if the concentration of OxLDL generated is minimized.

PC play a major role in the binding of OxLDL to CD36 present either in the lipid phase or covalently attached to the apoprotein (Bird, Gillotte et al. 1999; Gillotte, Horkko et al. 2000; Podrez, Poliakov et al. 2002; Boullier, Friedman et al. 2005). PC act as a pattern recognition ligand for SRs. Therefor PC is a perfect therapeutic target for inhibiting foam cells formation.

It was approved by the studies. Such as the human derived anti-OxLDL antibodies blocks uptake of OxDL by macrophages (Binder, Horkko et al. 2003); EO6, specific for PC or PC-protein adducts inhibit macrophages uptake of OxLDL (Frostegard, Svenungsson et al. 2005).

On the whole, macrophage SRs, such as CD36 and SR-A play a central role in foam cell formation and atherogenesis by mediating the uptake of OxLDL. SRs mediate internalization, degradation and antigen presentation of ligands (Nicoletti, Caligiuri et al. 1999) and they connect the innate and adaptive immunity.

FcγRs, another class of receptors that contribute to foam cells formation, act as trigger molecules for the production of cytokines (Virella, Atchley et al. 2002), proteolytic enzymes (Huang, Fleming et al. 2000) and reactive oxygen intermediates (Kiener, Rankin et al. 1995). In addition to the direct clearance of LDL immune complexes (Morganelli, Rogers et al. 1995), FcγRs are involved in the upregulation of LDL and SRs (Kiener, Rankin et al. 1995), affecting the uptake of modified LDL through non– FcγRs -mediated

pathway. FcγRs deficiency confers protection against atherosclerosis in apoE KO Mice (Hernandez-Vargas, Ortiz-Munoz et al. 2006).

However, the mechanism behind the SRs inhibiting and decreasing clearance of modified lipoprotein from the intima remains complicated, since the fate of these proinflammatory lipoproteins in the artery wall remains unknown.

On the other hand, there are conflicting results on the impact of SRs in mouse models of atherosclerosis (Babaev, Gleaves et al. 2000; Febbraio, Podrez et al. 2000; Moore, Kunjathoor et al. 2005). The following assumptions have been raised.

First, there are alternative ways to produce foam cells. For example, aggregated LDL could enhance uptake via LDLRs (Khoo, Miller et al. 1992); LDL immune complexes could increase uptake both by LDLRs and FcγRs (Griffith, Virella et al. 1988); mmLDL, itself is not a ligand for SRs, but it could stimulate SRs expression and prime macrophages for foam cell generation. In addition, macropinocytosis of OxLDL taken up in the fluid phase without receptor-mediated binding is a novel endocytic pathway that generates macrophage foam cells (Kruth, Jones et al. 2005).

Second, in the presence of mild hypercholesterolemia, only a few receptors are operative and sufficiently contributory to foam cells formation. Nevertheless, when plasma cholesterol levels are exceedingly high, all of these receptors are operative and individually sufficient to drive foam cell formation. In this latter scenario, deletion of any one may not influence lesion formation. Alternative pathway could conceivably mediate sufficient uptake of modified LDL, leading to foam cell formation.

Third, there are site dependent lipid accumulation in macrophages of the aorta and peritoneum. Peritoneal macrophages are sensitive for SR deficiency.

Forth, SRs are also involved in anti-atherogenic function, such as SR-BI, which mediate reverse cholesterol transport by HDL (Riesen, Braun et al. 1976; Acton, Rigotti et al. 1996).

Hence, the role of SRs in atherogenesis still needs to be clarified.

Treatment strategy of atherosclerosis

Inflammation as a therapeutic target in atherosclerosis

Powerful antiinflammatory agents could represent attractive treatments for CVD.

The treatment of hypercholesterolemia and hypertension was expected to eliminate CVD by the end of the 20th century.

Statins, aspirin, β-adrenergic blocking agents, angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers were used to ameliorate CVD.

Inflammation as a therapeutic target emerged, such as, Lipid-lowering statins (Bersot, Haffner et al. 2006), HDL-elevating drugs (Pal and Pillarisetti 2007), CETP inhibitors (Faria-Neto, Chyu et al. 2006), ACAT inhibitors (Meuwese, Franssen et al. 2006), Lipoprotein-associated phospholipase (Lp-PLA2) inhibitor (Macphee, Benson et al. 2005) and PPARγ agonists (Li, Binder et al. 2004) are already in the market.

CVD is an inflammatory disease and redundant factors in inflammation could be involved. Thus inhibiting a narrow-spectrum of factors may not effectively modify the disease process, while broad blockade of these mediators will impair host defences.

Immune modulation as a treatment strategy for atherosclerosis

The immune response plays an important role in the pathogenesis of atherosclerosis. In this regard, immune-based therapies can mitigate the disease process.

The existence of antigens such OxLDL in the atheromatous plaque and its association with atherosclerosis progression, have provided us the opportunity to test a novel antigen-specific immunologic approach for treating the atherosclerotic process.

Immunization with OxLDL ameliorates atherosclerosis in several animal models (Palinski, Tangirala et al. 1995; Nicoletti, Paulsson et al. 2000). This effect is associated with the increased titer of IgG anti-OxLDL and TD B cell response, (Nicoletti, Paulsson et al. 2000) therefore, involving both T-cell as well as B cell activation.

Immunization with OxLDL also leads to a reduction of neointimal formation after balloon injury (Nilsson, Calara et al. 1997). Such protective effects might involve Fc-dependent removal of OxLDL from the circulation or neutralizing the effects of OxLDL systemically or locally.

Immunization reduces the progression of atherosclerosis rather than preventing the initiation of atherogenesis (Hörkkö, Binder et al. 2000).

Application of anti-PC immunization strategy in humans

To develop a generalized and safe vaccine, identification of specific immunogenic oxidation-specific epitopes is required to provide the atheroprotective immunity. Fortunately, PC is an ideal epitope that could be widely administered.

PC is antigenic, and PC targeting immunization reduced atherosclerosis in apoE KO mice (Nicoletti, Paulsson et al. 2000). They do so by increasing in the number of splenic mature B cells and homing to plaques; reduction of expression of MHC class II antigens and raising both anti-PC and anti-OxLDL antibody titers. PC-immune serum was able to reduce macrophage-derived foam cell formation in the presence of OxLDL in vitro.

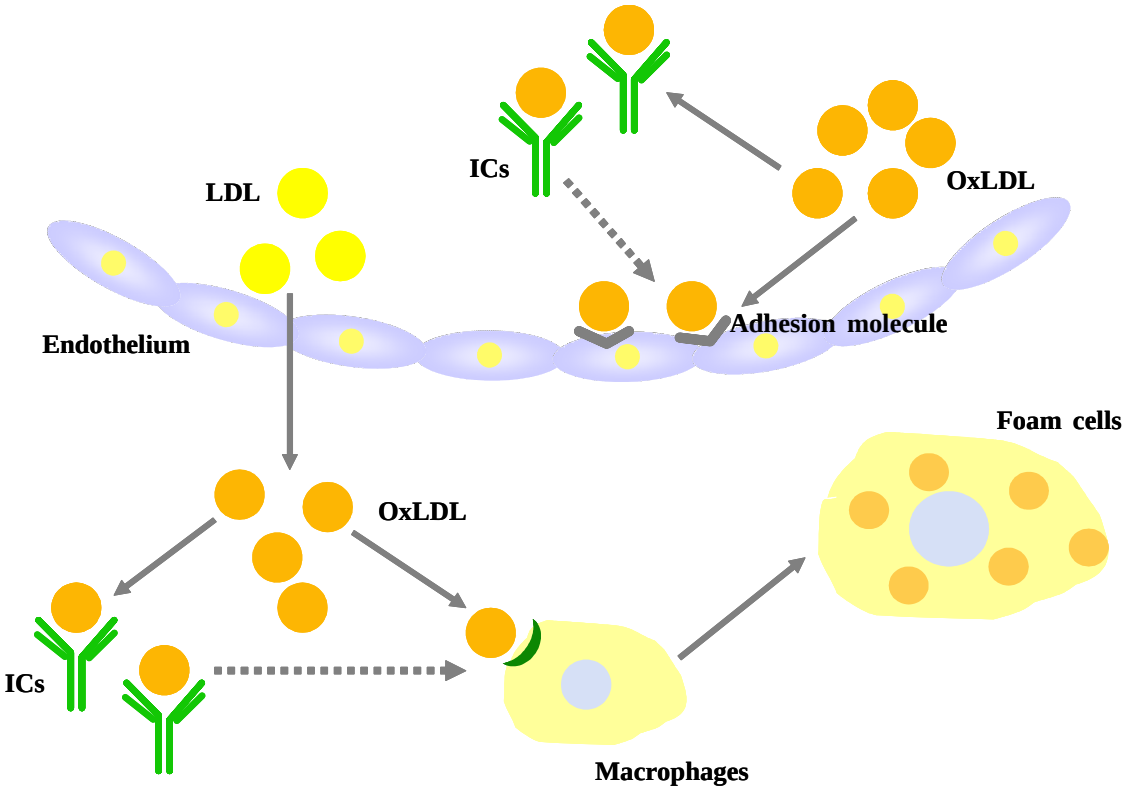
However, this positive immunization has its disadvantages. The immune response to PC in human is different. Unlike in mice, IgG antibodies—in particular, IgG2—dominate the anti-PC response in humans (Brown, Schiffman et al. 1984). The children, elder and IgG2 deficient patients have low response to PC. Even when an anti-PC response was induced, it may not have raised the levels of anti-PC antibody sufficiently higher than the naturally occurring levels to cause a marked difference in protection (Briles, Nahm et al. 1981).

An immunization strategy that enhances anti-PC antibodies in mice is atheroprotective. Given that passive immunization with monoclonal IgM anti-PC reduces accelerated vein graft atherosclerosis in apoE KO mice (Faria-Neto, Chyu et al. 2006). In the same way, Pneumococcal vaccination also decreases atherosclerotic lesion formation (Miller, Chang et al. 2003).

In contrast, Pneumococcal vaccination does not increase circulating levels of IgM anti-OxLDL in humans (Damoiseaux, Rijkers et al. 2007). A conceivable reason is that current pneumococcal vaccines are made from multiple serotypes (usually 17 or, more recently, 23), which are covalently coupled to a variety of immunogenic carrier proteins, including diphtheria toxoid tetanus toxoid, and meningococcal outer membrane complex. In turn, this leads to classic MHC-II responses to antigens other than PC.

So ideally, specific anti-PC immunization with anti-PC natural antibody might orchestrate protective host response in atherosclerosis (Binder and Silverman 2005) by anti-inflammation and inhibition of foam cells formation. (Figure 8)

Figure 8



Other forms of immunization concerning atheroprotection are: oral and nasal immunization with HSP65 (Maron, Sukhova et al. 2002); immunization of cholesterol-fed rabbits with CETP which attenuates disease progression (Rittershaus, Miller et al. 2000) accompanied by increased levels of HDL cholesterol; and immunization with human apoB100 peptides leading to a reduction in lesion size (Fredrikson, Soderberg et al. 2003).

Autoimmunity

Autoimmunity is a process whereby the immune system reacts against the body's own tissues. It occurs due to failure to distinguish between self and nonself by the immune system.

Most autoimmune responses represent a pathological aberration of the immune system with the presence of atuo-reactive T cells and autoantibodies.

Molecular mimicry might also be involved in autoantibody production (Jerome, Cash et al. 1998). For example, antibodies against double-strandedDNA(dsDNA).

Autoimmunity is demonstrated to be protective in some situations. Such as NAbs as I have discussed previously.

For therapeutic purposes, utilizing active immunization with relevant autoantigens to stimulate the appropriate immune response (e.g., active immunization with OxLDL to protect against atherosclerosis) or utilizing passive transfer of protective antibodies (e.g., IgM anti-dsDNA, anti-CL) can be useful treatments for patients with SLE and patients with atherosclerosis, respectively.

Systemic lupus erythematosus

SLE is often characterized as a prototypic autoimmune systemic disease whereby many different organ systems are affected. In particular, the kidney, neural system, cardiovascular system and hematologic system. Manifestations include nephritis, arthritis, pleuritis, pericarditis, and vasculitis.

From an immune point of view, SLE is a chronic inflammatory, autoimmune disease that produces autoantibodies of different specificities (Cederholm, Svenungsson et al. 2005). The presence of high titres of autoantibodies against nuclear components (antinuclear antibodies), elevated circulating immune complexes and complement consumption (Munoz, Gaipl et al. 2005) are some of the main characteristics of this disease.

The loss of immune tolerance, increased antigenic load, excessive T cell help and defective B cell suppression ultimately leads to B cell hyperactivity and the production of pathogenic autoantibodies (Mok and Lau 2003).

80% of SLE subjects had IgG anti-MDA-LDL antibody, while 40% of patients had elevated IgG anti-CL titers. Both antibodies had been found to cross react (Palinski, Horkko et al. 1996).

SLE is also a Th2 predominant female disease (Cervera, Khamashta et al. 1993). First onset of SLE before puberty and after menopause (Formiga, Moga et al. 1999) is uncommon.

Estrogen plays an important role in disease progression by prolonging the survival of autoimmune cells, Estrogens facilitate humoral responses, leading to increased B cell proliferation and antibody production (Kanda, Tsuchida et al. 1999). Lupus T cells are more sensitive to estrogens as shown by enhancement of CD40 ligand (CD40L) expression (Rider, Jones et al. 2001), increasing Th2 cytokine production, such as IL-10 to stimulate B-cell proliferation and differentiation (Mok and Lau 2003) and autoantibodies production by B cells.

An increased rate of formation, and/or a defective clearance of apoptotic cells (Martin Herrmann 1998; Licht, Dieker et al. 2004) has been postulated as a potential mechanism responsible for autoantibodies production (Mok and Lau 2003).

In normal healthy subjects, overproduction of antibodies is prevented by an idiotype network. This network is probably defective in patients with SLE, leading to dysregulation of autoantibody production (Wu, Shoenfeld et al. 2003).

SLE and CVD

SLE is associated with increased morbidity and mortality due to CVD. Up to 30% of deaths in SLE patients attributed to CVD (Aranow and Ginzler 2000).

In 1997, Manzi *et al*. (Manzi, Meilahn et al. 1997) compared women with SLE with age-matched controls in the Framingham cohort: 40 percent of patients with SLE had atherosclerotic plaques. Women with SLE had a relative risk of atherosclerosis of 5, as compared with the general population, and with those younger than 45 years, the risk was a staggering 50.

The prevalence of atherosclerosis significantly increased is not attributable to traditional risk factors for CVD (Hahn 1998), but associated with additional risk factors closely related to inflammation and autoimmunity. (Table 1)

Factors that increase risk in the general population also increase the risk among patients with SLE. However, SLE itself is an independent risk factor (Petri, Roubenoff et al. 1996). SLE itself may be atherogenic through chronic activation of immune system and the inflammatory process (Svenungsson, Jensen-Urstad et al. 2001).

Table 1

SLE-related risk factors to CVD
Dyslipidemia
lipid peroxidation
Autoantibodies (anti-CL, anti-OxLDL)
Circulating immune complexes
Dysfunction of complement system
Systemic inflammation (CRP, IL-6, CD40/CD40L, adhesion molecules)
Abnormal coagulation factors (fibrinogen, PAI-1, homocysteine)
Decreased binding of Annexin A5 to ECs

SLE-related factors are likely to be involved in all stages of atherogenesis from the formation of the atherosclerotic lesion to its rupture.

The "lupus pattern" of dyslipidemia was characterized by elevated levels of LDL and TG, and lower HDL levels.

Dyslipidemia could certainly contribute to enhanced OxLDL levels. Indeed, high levels of OxLDL were identified in this disease (Frostegard 2005). Furthermore, antibodies to OxLDL epitopes were described in SLE (Vaarala, Alfthan et al. 1993; Frostegard, Svenungsson et al. 2005).

Processes critical to the pathogenesis of SLE, such as immune complex formation and complement activation, are involved in endothelium injury and local inflammations. Upregulated CD40–CD40L interactions in SLE could potentially promote inflammatory processes while homocysteine is involved in injuring the endothelium thus contributing to local inflammation and plaque vulnerability. CRP activates the complement and not only increases the inflammatory response, but also enhances uptake of OxLDL into macrophages through FcγRs and stimulate macrophages to express TF leading to plaque formation and thrombosis.

Annexin A5 is an anticoagulant protein with its activity being Ca^{2+} dependent binding to ECs. This binding could be interfered by aPL. SLE related CVD patients had a decreased Annexin A5 binding to ECs (Su, Hua et al. 2008).

The treatments of SLE are related to CVD. Long-term treatment with corticosteroids was not associated with a significantly increased risk for CVD. Furthermore, Roman et al. found that patients without plaque had a significantly higher mean daily dose of prednisone and more frequent use of hydroxychloroquine and cyclophosphamide than did those with plaque. this suggests that more aggressive control of disease activity might help prevent atherosclerosis (Roman, Shanker et al. 2003).

The understanding of SLE-related risk factors for enhanced atherosclerosis could shed more light on disease mechanisms, leading to new therapeutic strategies for the treatment of CVD in SLE patients.

SLE is an excellent model to consider how autoimmunity and atherosclerosis may coexist and how autoimmunity may influence the atherosclerotic process (Frostegard, Svenungsson et al. 2005).

NAbs against OxLDL-epitopes like PC play a protective role in SLE and SLE related CVD, such as prevention of foam cells fomation and reduction of inflammatory effects stimulated by aPLs to ECs (Su, Hua et al. 2008).

When considering strategies for preventing premature CVD in SLE, modifying immune and inflammatory risk factors will likely become a major component of the program in addition to modifying the current traditional risk factors.

Aims

The following specific aims have been addressed:

- To determine the importance of anti-PC antibodies and anti-OxLDL antibodies in the development of atherosclerosis.
- To investigate anti-PC antibodies in SLE patients with and without CVD.
- To determine anti-PC antibodies in the prediction of CVD.
- To characterize the role of anti-PC on macrophage uptake of OxLDL.
- To determine the anti-inflammatory role of anti-PC antibody in CVD

Methodological considerations

Study groups

Hypertension cohort (paper I)

226 subjects with established hypertension (diastolic pressure >95 mmHg) prior to their entry into the Swedish component of the European Lacidipine Study on Atherosclerosis (ELSA). Samples were collected following a 4-week washout period with no medication to minimize the effects of treatment on the measured parameters. 115 subjects were subsequently assigned to treatment with beta-blocker atenolol, and 111 of the subjects were assigned to treatment with calcium antagonist lacidipine. The study was approved by the Ethics Committee of Karolinska Hospital and was conducted in accordance with the Helsinki Declaration. All subjects gave informed consent.

SLE cohort (paper II)

26 women with SLE who had survived one or more manifestations of CVD (myocardial infarction, angina pectoris, thromboembolic stroke or intermittent claudication), 26 age-matched women with SLE without clinical manifestations of CVD and 26 age-matched control women who were recruited randomly using the Swedish population registry. Details of the recruitment and clinical characteristics of the three groups have been reported (Svenungsson, Jensen-Urstad et al. 2001). All patients fulfilled the 1982 revised criteria of the American Rheumatism Association for classification of SLE. The study was approved by the local ethics committee of Karolinska Hospital. All subjects gave informed consent before entering the study.

60-years old cohort (paper III, IV)

Between July 1st 1997 and June 30th 1998, every third man and woman, reaching the age of 60 years and living in the County of Stockholm, were invited to participate in a health screening for cardiovascular diseases. The response rate was 78% and a total number of 4232 subjects (2039 men and 2193 women) participated in the investigation. The study base of 4232 subjects was matched with that of the national cause of death registry (fatal events until December 31, 2003) and the national in-hospital registry (non-fatal events until December 31, 2005). Through these matching procedures 211 incident cases of CVD were recorded. For each case three controls were randomly selected, matched for

gender and age (+/- 60 days). Thus, a nested case-control design (211 cases and 633 controls) was applied for the epidemiological and statistical analyses to assess relative risks for future events.

Carotid ultrasound (paper I, II, IV)

The right and left carotid arteries were examined with the duplex scanner. The IMT was determined in the far wall, as the distance between the leading edge of the lumen intima echo and the leading edge of the media-adventitia echo. The outcome measurement as a surrogate indicator for atherosclerosis was the change in mean maximum IMT of the four far walls in the distal common carotids and carotid bifurcations bilaterally. A plaque was defined as a local intima-media thickening, with a thickness greater than 1 mm.

Enzyme-Linked Immunosorbent Assay (ELISA)

Determination of antibodies against PC (paper I, II)

IgG and IgM antibodies to PC-BSA or PC-KLH were determined by ELISA. Pooled serum from 17 antiphospholipid syndrome patients was used as internal standard and tested on every plate. F96 microtiter polysorp plate was coated with PC-BSA or PC-KLH (10µg/ml) 50µl/well in PBS. Coated plates were incubated overnight at 4 ˚C. After five washings with PBS, the plates were blocked with 2% BSA-PBS for 2 h at room temperature and washed as described above. Serum samples were diluted (1:30) in 0.2% BSA-PBS and added at 50µl/well. Plates were incubated overnight at 4 ˚C and washed as described above. Alkaline phosphatase-conjugated goat anti-human IgG (diluted 1:9000 in the sample buffer) and alkaline phosphatase-conjugated goat anti-human IgM (diluted 1:7000 in the sample buffer) were added at 100µl/well and incubated at 4 ˚C overnight. After five washings, colour was developed by adding alkaline phosphatase substrate (PNPP) at 100µl/well and incubating the plates for 60 min at room temperature in the dark. The plates were read with ELISA Multiscan Plus spectrophotometer (Molecular Devices Emax, San Francisco) at 405 nm. All samples were measured in duplicates a single assay and the coefficient of variation was below 15%.

Determination of antibodies against OxLDL and MDA-LDL (paper I)

OxLDL or MDA-LDL was diluted to 2 μg/ml in coating buffer (carbonate–bicarbonate buffer 50mM pH 9.7), and 100 μl/well was used to coat ELISA plates (Costar 2581). The plates were kept at 4 °C overnight, washed four times with PBS and then blocked with 20% adult bovine serum in PBS (20% ABS-PBS) for 2 h in room temperature. They were then incubated with 100μl serum, diluted 1:30 in 20% ABS-PBS at 4 °C overnight. The procedure for detecting antibodies, substrate addition was the same as that for anti-PC detection.

Determination of antibodies against PS and BSA (paper II)

IgG and IgM antibodies to PS and BSA were determined by ELISA. F96 microtiter polysorp plates (Roskilde, Denmark) were coated with BSA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at 2μg/ml; while Immulon 1B plates (Thermo Labsystems, Franklin, MA, USA) were coated with PS (Sigma) at 160μg/ml, and incubated overnight at 4°C. After five washings with PBS, the plates were blocked with 2% PBS–BSA for 2 h at room temperature. Serum samples were diluted 1:30 in 0.2% BSA–PBS, added to the coated plates at 50μl/well and the plates were then washed as described earlier. Subsequently, the procedure for detecting antibodies, substrate addition was the same as that for anti-PC detection.

Determination of IgM anti-PC antibodies with ELISA kits (paperIII)

To quantify the IgM anti-PC levels of patients in the studied cohort, sera were tested with an indirect non-competitive enzyme immunoassay (CVDefine®, Athera Biotechnologies AB, Stockholm, Sweden) according to the manufacturer's instructions. First, IgM anti-PC of serum samples were allowed to bind to PC-coated wells of a microplate. Then, enzyme-labeled conjugate (horse-radish peroxidase-labeled anti-IgM) was allowed to bind to the antigen-antibody complex, forming an enzyme-containing conjugate-antibody-antigen complex. This complex converted the added substrate into a coloured solution which was quantified by absorbance measurements at 450 nm with a reference wavelength of 620 nm. The rate of colour formation from the chromogen is a function of the amount of conjugate complexed with the bound antibody and is thus proportional to the initial concentration of IgM anti-PC in the samples. The IgM anti-PC levels were

expressed as arbitrary units (U/ml) estimated from a six point calibrator curve containing IgM anti-PC levels ranging from 0 – 100 U/ml.

Detection of IgG anti-PC antibodies with ELISA kits (paper IV)

For detection of IgG anti-PC, a research prototype (Athera Biotechnology AB, Stockholm, Sweden) was used and serum samples were pre-diluted 1:100 before testing. Microtiter wells coated with PC-BSA were incubated for 30 minutes with 100 µl of diluted serum samples. After washing, the wells were incubated for 30 minutes with 100 µl of horse-radish peroxidase-labeled rabbit anti-human IgG conjugate at 1:3000 dilution (Dako Denmark A/S, Glostrup, Denmark). Thereafter the wells were washed and incubated in the dark with 100 µl substrate (TMB). Finally, after 10 minutes the color reaction was stopped by addition of 50 µl of 0.5 M H_2SO_4 and absorbance was measured at 450 nm with a reference wavelength of 620 nm. All incubations were carried out at room temperature. IgG anti-PC levels in serum were expressed as arbitrary units (U/ml) determined from a calibrator curve containing 100, 50, 25, 12.5, 6.25 and 0 U/ml of IgG anti-PC. The within and between variability of IgG anti-PC was 1.8% and 1.9% respectively.

Specificity of anti-PC antibodies (paper I)

In order to investigate the specificity of anti-PC antibodies, absorption assays were performed. At a dilution giving 50% of maximal binding to PC-BSA, sera were preincubated with different concentrations of PC-BSA and other antigens (competitors) as indicated. After vortexing, the tubes were incubated at 4^0C overnight and centrifuged at 13,000 rpm for 30 min (4^0C). The supernatants were tested for antibody binding to PC-BSA as described. The percentage of inhibition was calculated as follows:

percent inhibition = (OD without competitor − OD with competitor)× 100 /(OD without competitor)

Characterization of purified anti-PC antibodies (paper II, III)

The binding specificity of the purified IgG anti-PC or IgM anti-PC antibodies was measured by competition ELISA. The purified anti-PC was preincubated with indicated concentrations of competitors. The preincubated were added to microtiter plates (Nunc, Roskilde, Denmark) coated with PC-KLH (10 µg/ml) and the amount of bound antibody

was detected with alkaline phosphatase-conjugated goat anti-human IgG. The colour was developed by adding the alkaline phosphatase substrate. The percentage of inhibition was calculated with (OD without competitor – OD with competitor) x 100%/OD without competitor.

Cell culture

Pooled human umbilical vein endothelial cells (paper II)

Pooled human umbilical vein endothelial cells (HUVECs) at passage 2 were purchased from Cascade Biologics, Inc (Portland, OR). Cultures were maintained in EGMTM phenol red-free medium (Clonetics, San Diego, CA), containing 2% FBS and supplements, at 37°C under humidified 5% CO_2 conditions. All experiments were performed at passages 3 to 5. HUVECs were seeded at $6x10^4$ cells/2mL density on 6-well plates (NUNC Inc, Naperville, Ill). The endothelial cells were left overnight for attachment to occur before they were ready for stimulation.

Macrophages differentiated from THP-1 cells (paper III, IV)

Human monocyte-derived THP-1 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were cultured in RPMI-1640 medium, 10% FBS supplemented with 1mM sodium pyruvate, 0.05mM β-mercaptoethanol, penicillin (100U/ml), and streptomycin (100μg/ml) in a humidified cell incubator with 5% CO_2 at 37 °C. THP-1 monocytes were differentiated by incubation with 0.1μg/ml PMA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 1-5 days.

Extraction of anti-PC antibodies

Column coupling (paper II, III, IV)

PC-KLH (Biosearch Technologies Inc., CA, USA) was diluted in coupling buffer (0.2 M NaHCO3, 0.5 M NaCl, pH 8.3) to 1 mg/ml and then coupled to a HiTrap NHS column (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instruction. The coupled column was stored at 4°C.

Purification of IgG anti-PC antibodies (paper II, IV)

Human pooled immunoglobulin (Baxter Medical AB Torshamnsgatan 35 Stockholm, Sweden) was diluted in binding buffer (20 mM Na_2HPO_4) to 50 mg/ml and filtered through a 0.45 μm filter before passing through a pre-coupled PC-KLH sepharose gel column. Washing and further elution were performed according to the manufacturer's recommendation. Shortly, anti-PC IgG was eluted using 0.1 M glycine–HCl buffer, and the pH value was neutralized to 7.0 with 1 M Tris–HCL (pH 9.0). The purified fractions were desalted using PD-10 columns (Amersham Pharmacia Biotech AB, Warrenale, PA, USA). The antibody was concentrated by centrifugation with Centriprep Centrifugal Filter (Millipore, USA, Billerica, MA, USA), then stored at 4°C after being filtered through a 0.22 μm filter.

Purification of IgM anti-PC antibodies (paper III)

Purified human IgM (Sigma) was used to purify IgM anti-PC. The IgM was diluted in binding buffer (20mM Na_2HPO_4, 0.8M $(NH_4)_2SO_4$, pH7.5), and then filtered through a 0.45 μm filter. The purified IgM was applied on the PC-KLH Sepharose™ colum and subsequent washing and elution steps were performed according to the manufacturer's recommendation. The anti-PC IgM was eluted by 20mM Na_2HPO_4 pH7.5 buffer.

Labelling OxLDL with DiI (paper III, IV)

OxLDL (Industrylane Frederick) was incubated with DiI (Molecular Probes Engene, Oregon, USA) in lipoprotein-deficient serum (Sigma) at 37°C for 15 hours. Then OxLDL was dialyzed against saline-EDTA buffer for 6 hours.

Flow cytometry analysis

Inhibition of inflammation by anti-PC (paper II)

HUVEC cells were incubated with PAF (1μg/ml) and either of anti-PC IgG(10μg/ml) or commercially available human Ig Gammagard®S/D (Baxter, Inc.) (10μg/ml). After 24 h of incubation, detached floating cells were washed away and the remaining cells were harvested into BD Falcon tubes (Becton Dickinson, San Jose, CA, USA). After centrifuging at 410*g* for 5mins, cells were resuspended in 300 μl FACS buffer (1% FBS–

PBS) and stained with PE-conjugated anti-CD54 and FITC-conjugated anti-human CD106 for ICAM and VCAM analysis.

Uptake of DiI-labeled OxLDL with anti-PC antibodies (paper III, IV)

The macrophages were seeded in a 6-well cell culture plate at a density of 1 x 10^6 cells/well and cultured in DMEM containing 10% FBS at 37 °C, overnight. Cells were washed three times with serum free medium before incubation with DiI-OxLDL (5μg/ml) and anti-PC (5μg/ml) in DMEM with 10% lipoprotein-deficient serum at 37 °C for 6h. Thereafter, the cells were washed 4 times with 0.2% cold BSA/PBS, once with cold PBS and harvested in PBS containing 0.1% BSA and 0.01% NaN_3. For data acquisition and analysis, FACSCalibur (BD Biosciences, San Jose, CA, USA) with Cell Quest software was used. For each sample, a minimum of 10.000 cells were analyzed.

Uptake of DiI-OxLDL with IgG anti-PC and Fcγ receptor antibodies (paper IV)

To determine whether FcγR together with IgG anti-PC engagement was responsible for the uptake of OxLDL by macrophages, the macrophages were incubated with monoclonal anti-CD64 antibodies (R&D Sweden) at 50μg/ml or anti-CD32 antibodies (R&D Sweden) at 50μg/ml for 1h at 4^0C. At the end of this preincubation, DiI-OxLDL (5μg/ml) and IgG anti-PC (5μg/ml, 50μg/ml) were added for 5h at 37^0C. After incubation, the cells were washed twice with ice-cold 0.2% BSA-PBS, once with ice-cold PBS before harvested. The cellular fluorescence was monitored in FACSCalibur. For each sample, a minimum of 10.000 cells were analyzed.

The specificity of uptake of DiI- OxLDL (paper III)

The THP-1 cells were incubated with DiI-OxLDL (5μg/ml) in the presence of either a 8-fold excess of unlabeled OxLDL (40μg/ml) or unlabeled native LDL (40μg/ml) at 37 °C for 6h. After incubation, the cells were harvested and analyzed with the same method as described above.

Confocal microscopy analysis

Uptake of DiI-labeled OxLDL with anti-PC antibodies (paper III, IV)

1 x 10^6 macrophages were grown overnight on culture slides (Nunc, Naperville, New York) in DMEM (Invitrogen, USA) with 10% FBS. After three washes with DMEM

without FBS, the cells were incubated with Dil-OxLDL (5µg/ml in DMEM with 10% lipoprotein-deficient serum) at 37 °C for 6h. Thereafter, the cells were washed five times with 0.2% BSA/PBS and once with PBS. The cells were then fixed with 4% paraformaldehyde in PBS for 30 mins. After three washes with PBS, the mounting gel and cover slips were applied on the slides.

Statistical analysis (paper I, II, III, IV)

The statistics were computed using StatView software (SAS Institute AB, Göteborg, Sweden). Antibody levels were dichotomized at the 75th and 90th percentile or determined as continuous variables as indicated. The association between antibodies and the progression of atherosclerosis was determined either by estimating increases in IMT (yes or no) using logistic regression analysis or the calculation of odds ratios (ORs) and 95% confidence intervals (CI).

Correlation analysis was performed using simple regression for normally distributed variables, and Spearman's correlation analysis for non-normally distributed variables. Skewed continuous variables were logarithmically transformed to attain a normal distribution. Study groups were compared using ANOVA for continuous variables with Fischer's protected least significant difference test as post hoc test and chi-squared for categorical variables where comparisons between two groups were made using Fischer's exact test. The significance level was determined at $P < 0.05$.

Descriptive analyses of demographic data, biochemistry and anthropometry were performed for cases and controls, respectively with values expressed as medians (ranges) or proportions respectively. Statistical differences between cases and controls were assessed through non-parametric tests. Relative risks (RR) with 95% confidence intervals (CI) were calculated applying conditional logistic regressions. Analyses were run both crude and adjusted for traditional risk factors.

Results and discussion

The role of high levels of IgM to PC, OxLDL and MDA-LDL in the prediction of atherosclerosis (paper I)

The relationships between antibody levels of IgM anti-PC, IgM anti-OxLDL, IgM anti-MDA-LDL and changes in IMT were independent of age, smoking habits, treatment with atenolol or lacidipine and blood lipids. IgM anti-PC was negatively associated with changes in IMT at both 75th and 90th percentile, while IgM anti-OxLDL and IgM anti-MDA-LDL only showed significance at the 90th. IgG anti-PC was somewhat lower in subjects with increase in IMT but this difference did not reach statistical significance.

There were striking differences between men and women. IgM of anti-PC, anti-MDA-LDL and anti-OxLDL, were significantly higher in women than in men. In contrast, there were no differences between men and women in IgG levels of these autoantibodies.

To conclude, high levels of IgM of anti-PC, anti-OxLDL and anti-MDA-LDL predict a decreased rate of progression of carotid IMT in hypertensive patients.

The correlation between anti-OxLDL antibodies and atherosclerosis is heterogenous, Observation of the present study showing that IgM anti-OxLDL and IgM anti-MDA-LDL are inversely related to subsequent changes in carotid IMT support the idea that some anti-OxLDL antibodies may in fact confer atheroprotective properties. However, OxLDL is a very complicated compound and immune reactivity to it may be difficult to standardize. PC therefore appears to be a more promising target for diagnostic and therapeutic intervention. Furthermore, IgM anti-PC correlated with IMT-changes when analyzed as a continuous variable, which was not the case with IgM anti-OxLDL or IgM anti-MDA-LDL. This may indicate that IgM anti-PC has a more robust protective effect than the other antibodies tested.

The cause for the low antibody levels against these antigens in individuals with increased atherosclerosis could be consumptive into the atherosclerotic lesions, which are known to contain OxLDL epitopes, or immune complexes containing PC or OxLDL.

In this study, Men had significantly lower levels of IgM autoantibodies to these antigens as compared to women. This finding could provide a novel mechanism explaining why men have a higher risk of atherosclerosis and CVD than age-matched women.

IgM anti-PC could be novel markers of disease (indicating decreased risk at high levels), while OxLDL-related antibodies may be too complex and heterogeneous for this purpose. Whether passive or active immunization induces elevation of protective antibodies such as IgM anti-PC will be of value to humans.

Lower levels of IgG anti-PC in SLE and SLE-related CVD (paper II)

SLE cohort consisted of 26 women with SLE with a history of CVD (SLE cases), 26 age-matched women with SLE but without clinical manifestations of CVD (SLE controls) and 26 age-matched control women (controls). IgG anti-PC were decreased among SLE-cases and SLE-controls as compared to controls. IgM anti-PC values in the lowest 25th percentile and in the lowest 10th percentile were more common among SLE cases as compared with controls. Among SLE controls, IgG anti-PC-BSA were negatively associated with organ damage (SLICC) and disease activity (SLEDAI). Among SLE cases, IgG anti-PC-BSA and IgG anti-PC-KLH were negatively associated with SLICC and IgG anti-PC-BSA was negatively associated with SLEDAI.

IgG anti-PS and IgG anti-BSA were raised among SLE cases as compared with other groups ($P<0.05$). they did not cross-react with anti-PC.

SLE is a chronic inflammatory disease characterized by a perturbed immune response against self with production of high levels of an array of autoantibodies believed to contribute to the clinical symptoms in SLE. Surprisingly, antibodies against PC are low in the SLE patients in our study.

Apoptic function is generally believed to be disturbed in SLE, and lipid peroxidation could be one important factor not only in SLE-related CVD but also in SLE in general. Anti-PC recognizes apoptotic cells and also OxLDL. Decreased levels of anti-PC could thus predispose to defective clearance of obnoxious particles such as OxLDL. Anti-PC have been reported to be self-binding, to participate in immunological networks and to be able to present antigen to T cells. A perturbation of anti-PC could play a role in the immune dysregulation present in SLE.

Since anti-PC were also low among SLE controls, clearly other traditional and nontraditional factors also contribute to CVD in SLE.

Anti-PS were raised among SLE cases and differed significantly from SLE controls and controls. Anti-PS thus appears to be an important antibody in predisposition towards CVD in SLE, most likely through its pro-thrombotic effects.

Anti-BSA have been discussed as a possible marker and underlying aetiological agent in insulin-dependent diabetes mellitus. Anti-BSA can also reflect a defect in immunological tolerance and a predisposition to autoimmunity. We report here that IgG anti-BSA was significantly raised among SLE cases as compared with SLE controls, which were in their turn significantly higher than among controls. This finding is compatible with an aberrant immune reactivity against BSA in SLE.

Anti-inflammatory effect of IgG anti-PC (paper II)

IgG anti-PC were subtracted from the human Ig and the specificity of IgG anti-PC was confirmed by competition ELISA. PAF-induced expression of adhesion molecules by ECs could be inhibited by IgG anti-PC, suggesting an atheroprotective role of anti-PC.

SLE is characterized by vascular inflammation in many organs through the expression and up-regulation of adhesion molecules. ICAM-1 and VCAM-1 appear to be the predominant adhesion molecules at inflammatory sites. PAF is an important pro-inflammatory phospholipid, which shares many of its effects with OxLDL, having PC as a major epitope that binds to the PAF receptor. Inhibition of PAF by interfering with the PAF receptor leads to decreased atherosclerosis.

In the present study, IgG anti-PC can suppress PAF-induced up-regulation of these adhesion molecules, indicating that IgG anti- PC has anti-inflammatory effects by means of inhibiting adhesion molecule expression on ECs. This could be one mechanism explaining the anti-atherosclerotic effects of anti-PC. Low anti-PC levels could, in principle, predispose to vascular inflammation in SLE.

Prediction of an increased risk for CVD by low levels of IgM anti-PC among 60-year-old men (paper III)

There were strong association between the lowest quartile of IgM anti-PC value and the risk of CVD in men. Subjects within quartile 1 (with values below 29.7 U/l) had a significantly increased RR of 1.96 (CI 1.09 – 3.55) independent of traditional risk factors including hsCRP. No excess risks were noted among women.

Little is known about how levels of IgM anti-PC are established in humans. In this present study, we demonstrated that low anti-PC among 60-year-old men confer an increased risk for CVD independent of traditional factors. CRP are usually thought to be a risk marker for CVD, but CRP became a weaker marker after adjustments for traditional risk factors in this study. It is possible that low levels of IgM anti-PC could be a novel risk marker related to risk for CVD.

Data obtained indicate that low IgM anti-PC could be a risk marker in men only, but a larger series of incident cases of women are needed to affirm this finding. The data from the present cohort showed higher values in women as compared to men. It is thus possible that higher levels of these antibodies contribute to a lower burden of CVD seen in women as compared to men.

Inhibition of uptake of OxLDL in macrophages by IgM anti-PC (paper III)

The binding and degradation of Dil-OxLDL in macrophages differentiated from the THP-1 cell line were inhibited by purified IgM anti-PC for up to 76% as compared to the buffer control. Unpurified IgM had no effect.

OxLDL is taken up through specific SRs, which contribute to foam cells formation. Inhibition of the scavenger function is generally believed to be atheroprotective by preventing foam cells formation in the vascular wall, which is a key process in atherosclerosis.

Our finding accords with a recent report whereby murine natural IgM antibody to OxLDL that recognized PC had the capacity to inhibit macrophage uptake of OxLDL. This is achieved with antibodies binding to PC on OxLDL, which mediates the uptake by SRs such as CD36.

One mechanism by which low IgM anti-PC levels could predispose to CVD could be the decreased efficiency to inhibit uptake of OxLDL by macrophages, leading to increased foam cell formation and ensuing growth of atherosclerotic plaques.

The role of IgG anti-PC in prediction of CVD (paper IV)

IgG anti-PC in the lowest quartile was not significantly associated with a risk for CVD.

In the third paper we demonstrated that low levels of IgM anti-PC significantly and independent of other risk factors predict high risk of CVD, but the role of IgG anti-PC are

not known. We report here, that in contrast to IgM anti-PC, low levels of IgG anti-PC were not significantly related to increased risk of CVD since the association did not reach statistical significance.

This result is in line with the result of my first paper, whereby high levels of IgM anti-PC were protective factors, associated with less atherosclerosis development. For IgG anti-PC these associations did not reach statistical sigificance but trendwise pointed in the same direction.

Effect of uptake of OxLDL in macrophages by IgG anti-PC (paper IV)

IgG anti-PC, extracted from pooled immunoglobulins, only had a slight effect as compared to IgM anti-PC on the ptake of OxLDL in macrophages. However, pre-incubation with antibodies against FcRs enhanced uptake inhibition.

This difference could contribute to the lower capacity of IgG anti-PC as compared to IgM to predict development of atherosclerosis, since uptake of OxLDL in the artery wall is a major component of atherosclerotic plaques. Interaction with FcRs could be the cause of this lesser effect.

FcRs, taking up LDL-containing immune complexes, could play an important role in foam cells formation. Furthermore, FcRs expression are increased in patients with CVD. FcR deficient mice develop less atherosclerosis than controls.

IgG anti-PC, by forming immune complexes with OxLDL could be taken up through FcRs. Antibodies against FcγRI (but not FcγRII) promoted uptake inhibition by IgG anti-PC.

Conclusions

It is now widely accepted that inflammation and immune response are both involved in atherosclerosis. This concept started when autoantigens and immune cells, such as OxLDL and activated T cells and macrophages were detected in atherosclerotic lesions. Based on this concept, immunomodulation is appretiated to be a useful tool for the development of atherosclerosis.

PC is an antigenic epitope of OxLDL. There is molecular mimicry between OxLDL, *Streptococcus pneumoniae* and apoptotic cells. It is a specific target for scavenger cells to develop into foam cells which is a hallmarker of atherosclerosis.

Anti-PC antibodies are evolutionarily conserved to maintain homeostasis. For example, they have crucial role in immediate host defences against pathogens in a general context. Protective effects of anti-PC antibody were also due to their abilities to enhance the clearance of OxLDL and apoptotic cells.

The study of this thesis shows that anti-PC antibodies could be a novel marker for atherosclerosis. We have shown that high titers of IgM antibodies against PC together with IgM anti-OxLDL and IgM anti-MDA-LDL are associated with a favourable outcome in atherosclerosis measurements (carotid IMT) in individuals with hypertension. In addition, IgG anti-PC antibodies are low in autoimmune disease patients, such as SLE and SLE with CVD. This is possibly due to the defective clearance of apoptotic cells by low anti-PC antibody levels. Low levels of IgM anti-PC are associated with an increased risk of CVD in men in a population based cohort. Low levels of IgM anti-PC could thus be a novel risk marker among men. IgG anti-PC in contrast to IgM anti-PC is not significantly associated with the development of CVD.

One underlying mechanism of the atheroprotective effect of anti-PC antibodies could be that these antibodies inhibit the uptake of OxLDL in macrophages and thus decrease foam cell formation. The reason IgG anti-PC is much less effective in inhibiting uptake of OxLDL in macrophages could be due to its interaction with the FcRs. With the inhibition of FcRs, IgG anti-PC has an increased capacity to inhibit the uptake of LDL. However, since IgG anti-PC has anti-inflammatory properties, different subclasses could also play an important role through this mechanism in CVD.

Natural or induced antibodies reacting with PC might dynamically orchestrate protective host responses in infection, autoimmunity and atherosclerosis.

I believe, that hopefully, immunization, passive or active, inducing raised levels of anti-PC will be of value in the treatment of diseases such as atherosclerosis on humans.

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