

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
(Ph.D.)

THE ROLE OF ANTI-OXLDL AUTOANTIBODY-
MEDIATED PROCESSES IN ASSOCIATION WITH
IMMUNE MECHANISMS OF ATHEROTHROMBOTIC
DISEASES

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INTRODUCTION

Atherosclerosis is a pathological process characterized by atherosclerotic plaques in the arterial wall. It has a high prevalence with life-threatening complications. Immune-mediated inflammatory processes play an important role not only in the development of the plaques, but even in their progression, which can eventuate in plaque instability which is a possible source of severe atherothrombotic complications. In these days, immune-mediated inflammatory mechanisms within the plaque are highly investigated. Mapping of the key autoantigens, immune cells, mediators and their functions can reveal new therapeutic targets for the medical practice.

1. Immune-mediated inflammatory mechanisms in the unstable plaque

Based on recent research, it is well known that a chronic low-grade inflammation takes place within unstable plaques which has been visualised recently by FDG-PET in several preclinical animal experiments and human clinical investigations.

Immunologically, the macrophage has a key role in those inflammatory mechanisms which leads to the development of plaque vulnerability. The present, active macrophages produce pro-inflammatory cytokines, chemokines, extracellular matrix metalloproteinases, tissue factor, reactive oxygen species, prostanoids and complement factors. It has been detected that intense macrophage infiltration correlates well with plaque progression and its rupture tendency. Most of the T cells are in active stage, while the majority of T cell subpopulations are CD8 positive. Moreover, in the plaque B cells, antibody-secreting plasma cells and natural killer (NK) cells are present. Close to T cells, activated HLA-DR positive smooth muscle cells can be found. Between these two cell types active interaction has been described. In these processes, the TH1 shift (macrophage activator IFN- γ , IL-1, TNF- α) of the T-helper1/T-helper2 balance has been described, also the B cell activation of Th2-type cytokines are crucial. The cellular collaboration is driven mainly through the CD40-CD40L interaction, which also leads to macrophage activation.

Autoregulative T cells also present in the plaques. The role of these cells in the development of atherosclerosis is not completely clear yet, however, protective effect is suspected on the basis of a few preclinical investigations. IL-17 producing Th17 cells are also involved in the pathomechanism of atherosclerosis. They affect the migration of T cells via VCAM-1 and the adhesion of monocytes through CXCL-1, although their pro- or antiatherogenic effect is still under debate in the disease.

Autoantibody-mediated processes also present in the pathomechanism. Currently we can determine three significant autoantigens, which play an important role in the development and the progression of the plaque. The autoantigens are: oxidized-LDL, β 2-glycoprotein I and heat-shock protein of 60 kDa. On the one hand, these act as autoantigens in the autoantibody-dependent pathomechanisms, on the other hand, they induce autoantibody-mediated processes which have individual pathogenic role.

2. Oxidized LDL

An immunogenic macromolecule, the oxidized LDL is an oxidatively modified form of LDL particle, which has an essential function in the development of the plaque. The preliminary step of atherosclerosis is the fact that circulating LDL molecules reach the subendothelial space of the arterial wall through the endothelium. It is well known from the literature, that both the endothelial permeability and the LDL transport towards the intimal layer are highly elevated mainly in the bifurcations and sharp curvatures of the vessels. In this process, the glycocalix layer which covers the luminal side of endothelial cells, has an atheroprotective function and regulatory role as it can reduce the amount of the penetrating LDL. Shear-stress induced NO-release protects against LDL penetration, as well. However, chronic low shear-stress at certain places with the harmful effect of some noxas can reduce the synthesis of glycocalix which becomes thinner with changes in its structure, therefore, LDL can stream freely to the intima.

The LDL molecule, trapped in the extracellular matrix of the subendothelial space, is oxidized during a multistep process.

3. The process of LDL oxidation. Proatherogenic properties of oxidized LDL

On LDL oxidation its lipids, phospholipids and the apolipoprotein B-100 undergo chemical changes and modifications.

In consideration of its several biological effects, oxidized LDL is presumably a proatherogenic particle due to its oxidized phospholipids in the outer layer.

The biological characteristics of oxidized cholesteryl esters: they have cytotoxic effect on endothelial cell cultures in serum-free medium, in addition, they also induce the expression of M-CSF, VCAM-1, MCP-1 on endothelial cells, which promote the chemotaxis of monocytes towards the intima and inhibit the migration of tissue macrophages. In cell cultures, the oxidized LDL inhibits the lipopolysaccharide-induced NF κ B expression and apoptosis, although enhances the collagen synthesis of smooth muscle cells. It can cause vasospasm (via inhibiting the function/release of NO), thrombosis (via escalating the activity of tissue factor from endothelial cells) and inflammation (via inducing the secretion of many proinflammatory cytokines through macrophages). Oxidized LDL is also immunogenic, autoantibodies are produced against oxidation-specific epitopes. Clinically, the effect of oxidized LDL can be described well, for example, in several autoimmune disorders, acute coronary syndrome or stable coronary artery disease in which conditions it is highly pathogenic.

According to our knowledge, the majority of LDL oxidation does not take place in the circulation, because the strong antioxidant, protective mechanisms and the alpha-tocopherol (Vitamin E), the transport molecule of LDL which can inhibit the oxidation there. *In vivo*, LDL is oxidized mainly in the subendothelial space which has mainly two steps: in the first phase, mostly the lipids of LDL are undergone oxidation, the apoB100 is not or only barely involved („minimally oxidized LDL”), while in the second phase, lipids and apoB100 are further oxidized and modified which result in the oxidized LDL. The minimally oxidized LDL induces anti-apoptotic signal pathways and the secretion of proinflammatory chemokines and cytokines. Both the LDL and the minimally oxidized LDL,

which have been uptaken by macrophages via LDL receptors, are not able to accumulate in macrophages, because increasing amount of intracellular cholesterol downregulates LDL receptors through the SREBP. However, the fully oxidized LDL can bind with high affinity to some specific plasma membrane receptors (scavenger receptor A, scavenger receptor B /CD36/, LOX-1) of macrophages. Since these receptors are not downregulated parallelly with the increase of cholesterol content in macrophages, this process results in the progressive accumulation of cholesterol which can lead to the formation of foam cells.

LDL can be oxidized in non-enzymatic or enzymatic ways (e.g. metal-ions, hem, peroxy-nitrate, other catalysts or enzymes: lipoxygenase, myeloperoxidase, glycosylated enzymes) in the arterial wall.

In vitro, the LDL oxidation mechanism include the generation of reactive aldehydes, lipo-PC and oxidized phospholipids (oxPLs). These molecules, (lots of their epitope variations) have important role in the immunogenicity of oxLDL, which are recognized by a wide-range of autoantibodies. There are several studies which describe the association between the antibodies to oxLDL and the degree of atherosclerosis.

4. The effect of anti-oxLDL antibodies on the activation of foam cells

OxLDL particles are phagocytosed by macrophages in the plaque. Incorporation of oxLDL can occur through the classical, non-specific pathway of scavenger receptors, the oxLDL/anti-oxLDL complexes are taken up via specific Fcγ receptors – this is a way, how the esterified cholesterol-loaded foam cells are produced. Foam cells show permanent activity and produce the aforementioned inflammatory factors by which they contribute to the maintenance of the inflammation cascade which leads to the progression and the later instability or rupture of the plaque.

Anti-oxLDL antibodies accelerate the uptake of oxLDL molecules and increase the activation of macrophages. In addition, IgG-type anti-oxLDL/oxLDL immune complexes activate the Akt-dependent survival signal pathway of monocytes by crosslinking the

Fc γ RI receptors. This mechanism is also important in foam-cell formation. CD4⁺ T-cells can recognize oxLDL in a HLA-DR-dependent manner – thus, the oxLDL can initiate not only humoral, but cellular immune responses, as well.

The complex-forming tendency of oxLDL with β 2GPI is well known, in addition, these complexes function as target autoantigens. IgG-type anti- β 2GPI antibodies bind to oxLDL/ β 2GPI complexes which are incorporated rapidly by macrophages through Fc γ R I receptors. The previously mentioned process is much faster than the classical scavenger receptor-mediated one, and can activate macrophages, as well, so it is easy to accept that the formation of foam-cells highly increases, which clinically manifests in the progression of atherosclerosis. OxLDL/ β 2GPI complexes and autoantibodies against them present frequently in the serum of patients with several autoimmune disorders (SLE, APS) which can be in connection with the acceleration of atherosclerosis.

Beside oxLDL/ β 2GPI particles, CRP/oxLDL/ β 2GPI aggregates can be generated, as well. In oxLDL/ β 2GPI complexes, the C-reactive protein binds to the phosphoril-cholin chains of the oxLDL component, thus, it can opsonize these complexes. In this way, the CRP can enhance further the clearance of immune complexes via Fc γ receptors. Originally, the CRP is essential in innate immunity and it activates the classical complement pathway which causes the increase of phagocytosis.

5. The clinical role of anti-oxLDL antibodies. Are they pathogenic or protective?

OxLDL stimulates the anti-oxLDL antibody-production of B cells. In the literature, there are numerous publications related to the protective or pathogenic effect of anti-oxLDL autoantibodies. Initially, articles related to the immunization of rabbits and mice by oxLDL were in favour of the protective role of anti-oxLDL antibodies. In these cases, the reduction of atherosclerosis were correlated with the level of IgG-type anti-oxLDL antibodies.

The human anti-oxLDL autoantibodies play crucial role in the regulation of oxidized LDL-levels. These antibodies present in children, healthy adults and, for example, in patients with coronary

artery disease. It was found that the serum level of autoantibodies was significantly elevated in children compared to adults. Based on this fact, it seems to be possible that high antibody-levels can modulate the oxLDL antigen in children, therefore, they provide protection against atherosclerosis and cardiovascular diseases. Another study with the participation of 130 healthy individuals demonstrated that the plasma concentrations of oxLDL correlated negatively with the levels of anti-oxLDL antibodies. Moreover, anti-oxLDL antibodies were correlated negatively with the intima-media thickness of the carotid arteries. These findings suggest that in healthy individuals, anti-oxLDL autoantibodies have a protective function against the development of atherosclerosis and cardiovascular disease.

In spite of the aforementioned issues, studies associated with the pathogenic role of anti-oxLDL antibodies have come to light in the last few years. In atherosclerotic lesions, immunoglobulins which are specific for oxLDL are identified. Anti-oxLDL and/or anti-malondialdehyde-LDL antibodies present in the sera of patients with coronary artery disease and reach higher titers than in healthy controls, therefore, it is suggested that the detected antibodies may have a diagnostic or a prognostic value in coronary artery disease. These autoantibodies can indicate the extent of atherosclerosis in the coronary vessels. Their presence can be connected to the high risk of restenosis observed in coronary arteries after PTCA. Elevated antibody-levels also correlated with the extent of atherosclerosis in patients with peripheral artery disease. In a further publication, elevated levels of IgG-type autoantibodies to the aldehyde-modified apoB-100 peptide 210 showed strong association with the incidence of vulnerable plaques rich in lipids and poor in fibres, while IgM-type autoantibodies against aldehyde-modified apoB-100 peptide 210 and 240 correlated well with the incidence of lipid-poor, stable plaques rich in fibers. Tsimikas et al revealed a positive linear correlation between the IgG-type anti-oxLDL antibodies and coronary artery disease confirmed by angiography, while a negative correlation is proven in regard to IgM-type anti-oxLDL antibodies, although neither antibodies were independent predictors of future

cardiovascular events. Human anti-oxLDL antibodies have different classes and subclasses, the followings are already identified: IgA, IgG1, IgG2, IgG3 and IgM.

In the literature, there are controversial data about the pathogenic or protective role of anti-oxLDL autoantibodies in the pathogenesis of atherosclerosis. A potential interpretation of this controversy has been described in different studies by Shoenfeld et al and Tsimikas et al, in which the different roles of these various autoantibody-isotypes have been confirmed in atherosclerosis. According to these publications, the IgM-type antibodies has rather a protective function, while the IgG-type antibodies has an unequivocal proatherogenic effect.

Another experimental study revealed that low-dose IgG-type anti-oxLDL antibodies facilitate the uptake of oxLDL into macrophages through both of CD32A (Fc γ RIIA) and CD36 (scavenger) receptors, however, larger doses inhibit the uptake of oxLDL via CD36 receptors, while facilitate it through CD32A receptors.

6. Atherogenic effect of the immune response against oxLDL/ β 2GPI in antiphospholipid syndrome.

In an experimental study, mouse model of antiphospholipid syndrome (APS) has been used to determine the effect of oxLDL compared to native LDL. It has been found that a more serious disease developed in mice which were immunized with oxLDL than in those immunized with native LDL. Several clinical studies deal with accelerated atherosclerosis (increased intima-media thickness in carotid arteries, peripheral artery disease, accelerated atherosclerosis of the aorta) observed in patients with APS and its connection with anti-oxLDL-, and antiphospholipid antibodies against the oxLDL/ β 2GPI complexes.

It becomes known, that there is a mutual connection between oxLDL, β 2-glycoprotein I and the humoral mechanisms directed against them: oxLDL promotes the conformational change of β 2GPI molecules in order to facilitate the binding of antiphospholipid autoantibodies. Anti- β 2GPI autoantibodies enhance the uptake of the oxLDL/ β 2GPI complexes into the macrophages through CD64

(FcγRI) receptors, in this way, they increase the accumulation of oxLDL and the formation of proatherogenic foam cells.

In antiphospholipid syndrome, there is a connection between the IgG-type anti-oxLDL/β2GPI antibodies and the arterial thromboembolic events. It is accepted that the Th2-type cytokines and humoral immune mechanisms are dominant in the pathogenesis of APS, however, we have to take into consideration those studies, in which chronic *in vivo* stimulation of CD4+ and CD8+ T cells and secretion of Th1-type cytokines are found in primary APS. On the basis of these facts, anti-oxLDL antibodies can worsen the clinical manifestations of APS, in addition, antiphospholipid autoantibodies to oxLDL complexes may contribute to the pathomechanism of accelerated atherosclerosis in APS. The oxLDL - foam cell - inflammatory cascade increases the activation of the endothelial cells, which leads to further recruitment and migration of leukocytes. The inflammatory cells of the plaque are considered to be a consequence of the immune-inflammatory response in the arterial wall, therefore, the atherosclerosis can progress even further.

7. C-reactive protein

C-reactive protein is proven to be a strong, independent risk factor of cardiovascular diseases. Increased level of CRP has a prognostic importance. Data from the literature have pointed out, that the level of CRP is proven to be elevated in patients with unstable angina or acute myocardial infarction compared to healthy controls and increased level of CRP is associated with worse clinical outcome in patients with myocardial infarction. However, the clinical use of CRP for the assessment of cardiovascular risk is limited, because its level can increase in several diseases of inflammatory pathogenesis.

8. Pleiotropic effect and role of statins in plaque stabilization

Statins are the basic drugs with complex effects for the treatment of elevated cholesterol. Statins are inhibitors of the 3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) reductase enzyme, which is a member of the mevalonate pathway of cholesterol synthesis in the liver. Statins stimulate the clearance of LDL particles via up-regulation of LDL-receptors. In addition, they

exert pleiotropic effects including the depletion and stabilization of the lipid core, strengthening of the fibrous cap, inhibition of platelet aggregation and thrombus formation. Statins also appear to alter various immune processes independently of their lipid-lowering effect. Statins reduce the number of macrophages within atherosclerotic plaques, suppress the release of proteases involved in fibrous cap rupture and downregulate the expression of cellular adhesion molecules involved in the monocytes rolling and attaching to the endothelium. Recent data also suggest that statins may decelerate the development of diabetes mellitus.

AIMS

I. In the experimental study, our aim was to investigate the effect of stimulation by oxidized LDL on lymphocytes from patients with antiphospholipid syndrome and healthy individuals.

In vitro proliferation and cytokine secretion of lymphocytes derived from patients with antiphospholipid syndrome and healthy controls were assessed in the presence of oxLDL autoantigen. We tried to find the answer how these lymphocytes respond to oxLDL autoantigen stimulus in the mentioned disease.

During the study we investigated the followings:

1. How does the proliferation of separated lymphocytes change in the presence of different concentrations (5 and 20 µg/ml) of oxLDL? Moreover, how does the 20 µg/ml oxLDL alter the cytokine secretion of lymphocytes? Is there any statistically significant difference between the stimulated lymphocyte activation of APS patients and controls?

2. Which ones of the secreted cytokines are induced by oxLDL in APS? Is there any association between the Th1-, or Th2-mediated immune responses and the secreted cytokines?

3. Is there any connection between the arterial or venous thromboembolic events and the mentioned proliferation and cytokine secretion in APS?

II. In the retrospective study, our aim was to investigate the level of anti-oxLDL autoantibodies in patients with acute coronary syndrome, stable coronary artery disease and in age, sex and risk factor matched healthy controls.

We had the following questions:

1. Are there any statistically significant difference in the levels of anti-oxLDL antibodies between patients with acute coronary syndrome and stable coronary artery disease compared to controls?

2. Are there any relations between anti-oxLDL antibody-titers and clinical events observed during hospitalization?

3. Is there any correlation between the CRP (which is a well-known inflammatory marker with strong predictive value in acute coronary syndrome) and the anti-oxLDL autoantibodies?

III. Based on the results of our previous study, we planned a prospective clinical investigation in this topic. We analyzed the relationships of IgG-type anti-oxLDL antibodies, acute clinical events during hospitalization and statin therapy among a larger cohort of patients with ACS.

Our questions were the followings:

1. Are there any associations between the level of IgG-type anti-oxLDL autoantibodies (measured at the time of admission) and complicated/uncomplicated courses of acute coronary syndrome? Do these antibodies have a predictive value for the clinical outcome of ACS?

2. Are there any relations between the cholesterol-lowering statin therapy and the titers of IgG-type anti-oxLDL antibodies in patients with ACS?

PATIENTS AND METHODS

I./1. Characteristic features of patients included in the experimental study

Thirteen patients with primary APS who visited regularly the Cardiovascular Outpatient Department of Institute of Internal Medicine, University of Debrecen were enrolled in this study. Patients fulfilled the modified Sapporo classification criteria for APS. The mean age of the patients was 47.3 ± 12.1 years (range: 27–67 years); 1 man and 12 women. APLA antibodies were present in the patients' sera, principally anti- $\beta 2$ GPI autoantibodies of IgG isotype, anti-cardiolipin antibodies of IgG isotype, and antiphosphatidil-serine antibodies of IgG isotype. Four patients were positive for only one of these autoantibodies, while nine were double or triple positive for different combinations of these antibodies. Inflammatory parameters (white blood cell count, erythrocyte sedimentation rate, CRP, and serum IL-1) were within the normal range. We determined subgroups of patients with APS, depending on the dominant arterial- or venous-type thromboembolic events during the progression of the disease. In the arterial APS group ($n=5$), patients had dominant arterial thromboses, which could manifest as transient ischemic attack, stroke, epilepsy, acute coronary syndrome, or thrombosis of the central retinal artery. In the venous group ($n=8$), patients had thromboembolic events affecting the venous vessels, manifested clinically in deep vein thrombosis, or pulmonary embolism. Nine healthy (serum APLA negative) individuals served as controls (49.6 ± 7.3 years, 32–56 years); two men and seven women. Peripheral blood samples were simultaneously obtained and immediately processed, while serum samples were frozen and stored at -70°C until further analysis.

I./2. Separation of peripheral mononuclear cells

Human peripheral mononuclear cells were isolated from native venous blood samples using Ficoll-Histopaque 1077 gradient centrifugation. Mononuclear cells were washed with PBS, then they

were resuspended in RPMI 1640 medium (37 °C; 86 % humidity; 5 % CO₂) at a concentration of 2×10^6 cells/ml in 96-well microplates.

I./3. In vitro lymphocyte proliferation upon stimulation

PBMCs were stimulated by the purified autoantigen, oxLDL (Intracel, Frederick, MD, USA). Different dilutions of oxLDL (at 5 and 20 µg/ml concentrations) were added to the wells. Cell proliferation was evaluated by ELISA (Roche Diagnostics GmbH, Penzberg, Germany), according to the manufacturer's instruction. Briefly, the test is a colorimetric immunoassay for the quantification of cell proliferation based on the measurement of BrdU incorporation during DNA synthesis. After 84 h incubation at 37°C, BrdU was added to each well and cells were incubated for an additional 12 h at 37°C. We used two negative controls, such as medium alone or cells without mitogens added. For positive control, a non-specific mitogen, phytohemagglutinin was added at 1 and 10mg/ml concentrations. Plates were analyzed by using automated multichannel photometer Multiskan MS (Labsystems, Helsinki, Finland) at 450 nm.

I./4. In vitro stimulated cytokine secretion

PBMCs were cultured at 37°C in the presence of immunogenic oxLDL at 20 µg/ml concentrations. For negative control, cells alone (without added antigens) were used. After 26h, microplates were centrifuged and supernatants were analyzed. Cytokines (IL-2, IL-4, IL-10, TNF- α , and IFN- γ) were assessed by ELISA (BD Opt EIA kite, BD Biosciences, San Diego, CA, USA) at 450 nm, according to the manufacturer's instructions.

I./5. Indices of cell proliferation and cytokine secretion

In order to assess changes in proliferation capacity as a response to antigens, we used a ratio, denoted as proliferation index (PI), determined as follows:

PI = proliferation with given antigen / proliferation without antigen

To compare and standardize the different levels of cytokine secretion upon activation by specific antigens, a ratio denoted as cytokine secretion index (CSI) was used, determined as follows:

CSI = cytokine secretion in the presence of antigen / cytokine secretion without antigen X 100

II. Patients enrolled in the retrospective clinical study

Patients with acute coronary syndrome (ACS), stable coronary artery disease (stable CAD) and healthy individuals were enrolled in the retrospective clinical study.

The mean age of the thirty-four patients (fourteen males and twenty females) with ACS (treated at the Intensive Care Unit, 3rd Department of Internal Medicine, University of Debrecen) was 72.3 ± 9.4 years (range: 47-87 years). The diagnoses were as follows: 10 unstable angina, 5 myocardial infarction without ST-segment elevation (NSTEMI) and 19 myocardial infarction with ST-segment elevation (STEMI).

Patients with ACS were divided into two groups, such as high-risk and low-risk groups based on their cardiac complications in order to analyze the predictive values of oxLDL.

1. „High-risk” group: those patients who developed *malignant ventricular arrhythmias, recurrent ischaemic pain, circulatory failure or cardiac death* in the hospitalization period.

2. „Low-risk” group: those patients whose *unstable angina or myocardial infarction* processed without the mentioned complications.

Sixty-two patients with stable CAD attending the Cardiovascular Outpatient Department of 3rd Department of Internal Medicine, University of Debrecen were enrolled. Their mean age was 66.8 ± 11.2 years (range: 31-84 years): 43 men and 19 women. In the stable CAD group 27 patients had had a myocardial infarction in the past, three had coronary artery disease diagnosed by coronarography and 32 patients had a positive stress test along with angina pectoris.

Fifty healthy individuals served as age-, sex- and risk factor matched controls.

III./1. Patients enrolled in the prospective clinical study

In total, 54 patients with ACS admitted to our institution were enrolled in this study. The mean age of these patients was 69.8 ± 11.1 years (range: 30–77 years). We included 28 men and 26 women. The diagnoses included unstable angina in 18 patients, myocardial infarction without ST segment elevation (NSTEMI) in 11 patients and myocardial infarction with ST segment elevation (STEMI) in 25 patients. During the hospitalization period, the medical treatment and the developing clinical complications of these patients were regularly recorded in the electronic patient charts. Patients with connective tissue diseases, infections, malignancies, liver or renal failure and those who took corticosteroids at any time in the past were excluded from the study. Patients in the statin-user group had started statin treatment at least 6 months before admission to the hospital. Patients receiving statins in the past but not currently were also excluded from the study. Patients with ACS were divided into two subgroups as high-risk and low-risk groups based on cardiac complications.

The high-risk group included 33 patients (17 men, 16 women; mean age: 67.2 ± 10.4 years), who developed circulatory insufficiency, malignant arrhythmias, recurring ischaemic pain or died in sudden cardiac death during hospitalization. In this group, five patients (15%) received statins.

The low-risk group included 21 patients (11 men, 10 women; mean age: 64.4 ± 9.8 years) who did not develop any of these complications. In this group, six patients (28.5%) received statin therapy.

The two groups did not differ significantly with respect to Framingham risk factors. In addition, 41 age- and sex matched healthy individuals served as controls.

III./2. Measurement of anti-oxLDL antibodies and C-reactive protein

In both clinical studies peripheral blood samples were obtained from patients and controls by routine venipuncture at the time of admission to our unit before any medical treatment was initiated. These serum samples were processed immediately in the

Regional Immune Laboratory. IgG anti-oxLDL antibody levels were determined by Immulisa oxLDL antibody ELISA kits (IMMCO, Buffalo, NY, USA) according to the manufacturer's instructions.

CRP levels in the retrospective study were determined by turbidimetry (Integra-400; Hoffmann-La Roche Ltd.; Basel, Switzerland).

IV. Statistical analysis

Statistical analysis was performed by using Statistica for Windows (Version 7.0) software. Statistical differences were determined by two-tailed Student's t-test if data exerted normal distribution, otherwise the Mann–Whitney test was performed; p values less than 0.05 were considered statistically significant. Correlations are detected with Pearson correlation test in case of normal distribution and Spearman test in case of asymmetric distribution.

RESULTS

I./1. *In vitro* lymphocyte proliferation

Comparison of lymphocyte proliferation in APS and controls.

Upon 5 µg/ml oxLDL stimulation, the mean proliferation indices were lower than 1.0 both in patients with APS and controls. Namely, the full lymphocyte cell count after stimulation compared to the initial one decreased in both groups, although the difference between them was not so significant (PI_{APS} : 0.95 ± 0.28 and $PI_{control}$: 0.79 ± 0.10 ; $p = ns$).

We found significantly elevated PI in patients with APS compared to healthy individuals in the presence of oxLDL at a concentration of 20 µg/ml (PI_{APS} : 1.76 ± 1.8 and $PI_{control}$: 0.56 ± 0.42 ; $p = 0.032$).

Additionally, APS patients were sub-categorized as follows: (1) APS patients with arterial thromboembolic events (arterial APS; $n=5$) and (2) APS patients with venous thromboembolic events (venous APS; $n=8$). When the proliferation capacity of PBMCs from patients with arterial APS and venous APS in the presence of oxLDL at the concentration of 20 µg/ml was compared, we found increased proliferation (PI) in patients with venous APS (venous APS: 2.1 ± 2.28 , arterial APS: 0.98 ± 0.28 ; $p=0.03$). Furthermore, when PBMCs from patients with venous APS were compared to healthy individuals in the presence of oxLDL at the concentration of 20 µg/ml, we found significantly elevated PI in the patient group (venous APS: 2.1 ± 2.28 , control: 0.56 ± 0.42 ; $p=0.002$).

I./2. *In vitro* cytokine secretion

Comparison of cytokine secretion by PBMCs from patients with APS and healthy individuals.

Upon oxLDL stimulus, the secretion of IL-2 reached a significant twofold increase when compared to controls (CSI_{APS} median: 118.8, interkvartile range_{25-75%}: 84.9-175.2 and $CSI_{control}$ median: 63.3, interkvartile range_{25-75%}: 44.9-80.5; $p = 0.018$). Increased IFN- γ secretion was detected in APS patients compared to

controls (CSI_{APS}: 163.2 ± 98.5 and CSI_{control}: 77.4 ± 46.6 ; $p = 0.025$). Concerning TNF- α , IL-4 and IL-10, no significant differences were detected between the APS and control groups.

In the patient subsets, when PBMCs were incubated in the presence of oxLDL, in the venous APS group, IL-2 secretion was detected to be elevated compared to healthy individuals, yet it did not reach a significant level, while in arterial APS, we found moderately increased IL-2 and IFN- γ secretion compared to controls, while TNF- α , IL-4 and IL-10 secretion were consistently at a low level in both APS groups.

II./1. Evaluation of antibodies to oxLDL of IgG isotype

In the retrospective clinical study, antibodies to oxLDL, both in patients with ACS (21.6 ± 26.45 EU/ml) and in stable CAD (15.25 ± 16.64 EU/ml) were elevated compared to healthy individuals (5.95 ± 6.21 EU/ml) ($p=0.0002$ and $p=0.0016$, respectively). When we compared the levels of anti-oxLDL between patients with ACS and stable CAD we found significantly higher levels in the ACS group compared to patients with stable CAD ($p=0.0288$).

After having reviewed the data and taking the large standard deviation into consideration, we decided to interpret the data with group analysis. We divided patients with ACS into two groups based on the clinical manifestations during observation. We compared the clinical data with anti-oxLDL levels and we found close association between elevated levels of anti-oxLDL antibodies and high-risk complications (e.g. malignant ventricular arrhythmia, recurrent ischemic pain, need for urgent coronary intervention, circulatory failure and sudden cardiac death) during hospitalization of patients with ACS. In low-risk ACS group, the anti-oxLDL levels were significantly lower compared to those with severe coronary complications (10.87 ± 9.26 EU/ml vs. 32.98 ± 31.72 EU/ml $p=0.0018$).

II./2. C-reactive protein values

CRP levels in patients with ACS (43.5 ± 66.06 mg/l) were significantly higher than in those with stable CAD (15.25 ± 9.13

mg/l; $p=0.0027$) compared to healthy controls (2.8 ± 0.36 mg/l; $p=0.011$).

Interestingly, we found that patients with APS who died during their disease course during hospitalization had significantly elevated CRP levels compared to those who did not suffer a fatal outcome ($p=0.000075$). Therefore, we propose that initial CRP levels are good predictor for mortality in ACS.

When the correlation between the level of CRP and antibodies to oxLDL was evaluated, we found linear correlation both in patients with acute and stable CAD. Interestingly, this correlation was strong in patients with ACS ($R = 0.73$; $p<0.0001$), while in patients with stable CAD the correlation was only medium-strong ($R=0.43$; $p<0.0001$).

III. Investigation of IgG type anti-oxLDL antibodies

In our second, prospective clinical investigation higher IgG anti-oxLDL levels were found in patients with ACS versus controls (22.8 ± 23.3 vs. 7.5 ± 5.27 EU/ml; $p<0.0001$)

We divided patients with ACS into two groups. In the high-risk group which included patients with unstable clinical complications, the levels of circulating IgG anti-oxLDL antibodies were 21.5 EU/ml (interquartile range_{25-75%}: 15.0–29.5 EU/ml). In the low-risk group, which included ACS patients without the aforementioned complications, the levels of IgG anti-oxLDL were 11.4 EU/ml (interquartile range_{25-75%}: 6.3–14.0 EU/ml). Thus, in the low-risk group, anti-oxLDL levels were significantly decreased compared to those suffering from severe coronary complications ($p<0.001$).

With respect to pre-hospitalization statin treatment, out of the 12 patients receiving statins prior to admittance, only four patients developed high-risk complications during hospitalization for ACS. When levels of anti-oxLDL antibodies, LDL and total cholesterol were compared in patients on statin therapy and in patients without this treatment, levels of total cholesterol (4.9 ± 1.1 vs. 5.3 ± 1.3 , respectively) and LDL (2.2 ± 0.8 vs. 3.2 ± 1.1 , respectively) did not differ significantly between the two groups. In contrast, IgG anti-

oxLDL antibody levels were significantly lower in patients with previous statin therapy (11.4 ± 3.9 EU/ml) in comparison to those patients without statin therapy (25.8 ± 25.2 EU/ml) ($p < 0.03$).

DISCUSSION

Atherosclerosis is the chronic inflammatory disease of the arterial wall in which the immune mediated inflammatory processes are highly pathogenic. Within these immune mechanisms the essential, plaque-associated target antigen is the oxidized LDL, which presents not only in atherosclerosis, but in other autoimmune conditions (e.g. antiphospholipid syndrome, systemic lupus erythematosus), as well. Autoantibodies to oxidized LDL facilitate the accumulation of oxLDL in macrophages, moreover, they activate the complement-associated and cellular pathways which contribute to the further increase of the inflammation in the arterial wall, therefore, atherosclerosis can progress rapidly.

In one part of our project, in an *in vitro* investigation, we evaluated the oxLDL- stimulated proliferation and cytokine secretion of lymphocytes from patients with antiphospholipid syndrome. It is well known from the literature, that oxLDL forms complexes with β 2GPI in antiphospholipid syndrome and autoantibodies against them promote the generation of foam cells, thus, these processes lead to atherogenesis. Clinically, several evidences exist on accelerated atherosclerosis in patients with APS.

Based on these facts we decided to investigate the effect of oxidized LDL on lymphocytes separated from patients with APS and we compared these data with the clinical manifestations. It is known, that the level of circulating oxLDL is low (1-5 μ g/ml) in healthy adults which supports the use of 5 μ g/ml concentration (of oxLDL) in our project. However, this concentration can reach a 4- or 5-fold, moreover, even a 70-fold higher level in atherosclerotic lesions of patients, hence, in the experiment we also used another, larger concentration (20 μ g/ml) of oxLDL.

The 20 μ g/ml oxLDL stimulus caused a definite, significant increase in the proliferation of lymphocytes in the APS group, but not in controls. Interestingly, at 5 μ g/ml oxLDL stimulus we did not find any significant proliferation, on the contrary, the mean proliferation indices decreased both in APS and control groups ($PI < 1.0$). Xavier et al. published previously similar results, that the

level of circulating oxLDL has a dose-dependent proliferation inhibiting (possible cytotoxic) effect at low concentrations (1–8 µg/ml) on endothelial cell cultures of human coronary arteries. Cytotoxic effect of oxLDL has been proven not only on endothelial cell cultures, but smooth muscle cell-, and lymphoblast cultures, as well. The special, intensified lymphocyte proliferation-response in APS in the presence of 20 µg/ml oxLDL raises the possibility that oxLDL has a proliferation-inducing effect in the disease and might have a role in the pathogenesis of accelerated atherosclerosis observed during the progression of the disease.

We classified APS patients into two subsets with the domination of arterial or venous thromboembolic events in their history. These groups differ from each other not only in the vascular localization of the clinical events, but in their presumed pathogenesis, too. In the background of APS with venous manifestations, B cell-driven mechanisms present, characterized by a predominant Th2 response and high levels of antiphospholipid antibodies. In our cohort of venous APS patients at the time of the study, no manifest thromboembolic events presented, although pathological autoantibodies were found in each patient. Opposed to these, in APS patients with arterial manifestations, the overall clinical picture was characterized by a mixed etiology, where APS features were present along with atherosclerotic processes. In these patients, accelerated atherosclerotic processes can be found in the presence of antiphospholipid antibodies, while APS-related processes intensify the damage in the plaques. Since anti-β2GPI autoantibodies have pathogenic role in APS, hyper/dyslipidemia can also be found in these patients and these autoantibodies can facilitate the migration of oxLDL into the atherosclerotic plaques, we assume that these autoantibodies contribute to the acceleration of atherothrombotic events.

In APS, the 20 µg/ml oxLDL increased significantly the production of IL-2 and IFN-γ by lymphocytes in comparison with controls, however, their interpretation is different in arterial and venous APS. We hypothesize that oxLDL in venous APS also alters the clinical picture by maintaining immune activation. Opposed to

these, in APS with arterial thromboembolism mainly Th1-driven processes are dominant and oxLDL can perpetuate principally the plaque-associated inflammatory processes. In summary, we could identify that oxLDLs have a direct role in steering these processes, characterized by induced proliferation and proinflammatory cytokine secretion. Besides the diagnostic possibilities, we believe that by targeting and silencing these immune responses, novel therapeutical approaches can be achieved in APS.

In the other part of our project, we studied the serum levels of anti-oxLDL autoantibodies in patients with acute coronary syndrome and stable coronary artery disease. The pathological background of acute and stable coronary artery disease is the atherosclerotic plaque of the coronary arteries. Macrophages and lymphocytes present even in the very early stage of atherosclerosis (in early fatty streaks). The majority of monocytes and macrophages turn to foam cells. The macrophages secrete proinflammatory cytokines, chemokines, extracellular matrix metalloproteinases, tissue factor, reactive oxygen species, prostanoids and complement factors, while the majority of T cells are CD8 positive in the plaque. B lymphocytes, natural killer cells and HLA-DR positive smooth muscle cells are also detected in the plaque. Inside the plaque, the shift of the T-helper1/T-helper2 balance towards Th1 is crucial (especially the effect of the macrophage activator IFN- γ and IL-1), while the role of the B-lymphocyte activator Th2-cytokines is also important.

In the background of unstable coronary diseases (unstable angina, acute myocardial infarction) the injury of the atherosclerotic plaque is a well known event which leads to atherothrombotic complications. The complex atheroma obstructs the lumen, the amount of inflammatory factors produced by activated macrophages increases significantly. In parallel with these, there is a remarkable smooth muscle cell proliferation, as well. The injury of the plaque is essential in the progression of atheroma and in the development of clinical symptoms. Superficial lesions of the atheroma, in situ thrombosis on the plaque, microbleeding inside the plaque and the rupture of the fibrous cap of the plaque are responsible for approximately 75% of the cases with acute myocardial infarction.

Currently, we can determine three key autoantigens which play an important role in the development and progression of the plaque. These are the oxidized-LDL, β 2-glycoprotein I and a heat-shock protein of 60 kDa.

The oxidized LDL is a plaque-specific component which is highly immunogenic and it induces not only cellular but humoral immune responses, mainly the secretion of anti-oxLDL autoantibodies. These autoantibodies can be categorized into different serotype-classes (IgA, IgG1, IgG2, IgG3 and IgM), although there are pros and cons to their role in atherosclerosis.

A certain number of studies supports the protective role of anti-oxLDL autoantibodies. In some animal experiments, the reduction of atherosclerosis correlated well with the level of IgG type anti-oxLDL antibodies, moreover, these antibodies have remarkable impact on the regulation of circulating oxLDL. These autoantibodies also present in children, healthy adults and patients with coronary artery disease. These antibodies are determined to be elevated among children in comparison with adults which can be interpreted by the fact that they can provide protection against atherosclerosis and cardiovascular disease through the neutralization of oxLDL. Recently, many articles have been published on the pathogenic role of anti-oxLDL antibodies, where the elevated IgG anti-oxLDL antibody concentrations were connected to the development of cardiovascular and peripheral artery diseases. The presence of these antibodies indicates a high risk for restenosis of coronary arteries after coronary angioplasty.

At the moment, we do not know any sensitive, plaque-specific biomarkers with which we could monitor the progression of atherosclerosis or predict the rupture of the plaque, despite the fact, that the highly sensitive CRP is a strong, independent risk factor of myocardial infarction. The CRP is not plaque-specific although a very sensitive acute phase protein. Its level can rise not only in case of low-grade inflammation in the plaque, but in several infective diseases, autoimmune conditions and malignancies, as well. A plaque-specific, sensitive biomarker is extremely required, which would correlate well with the inflammatory processes in the plaque.

In the dissertation, we assessed the role of anti-oxLDL antibodies in the clinical course of ACS. A strong association was observed between anti-oxLDL antibodies and severe, life-threatening clinical events in patients with acute coronary syndrome during the hospitalization period. These complications were the followings: malignant ventricular arrhythmias, recurrent angina, need for subsequent, urgent coronary intervention, circulatory failure or sudden cardiac death. We managed to confirm the findings of the retrospective study in a second, prospective clinical investigation with the involvement of larger cohort of patients with ACS. In ACS patients with statin therapy, the levels of anti-oxLDL antibodies were significantly lower compared to those who did not receive statin. Interestingly, this result is independent from the lipid-lowering effect of statins, it can rather be interpreted by its pleiotropic (anti-inflammatory) action. Hence, this serves as indirect evidence for the close association of anti-oxLDL antibodies and inflammatory processes of the plaque.

In the literature, the aforementioned studies support our results and the hypothesis of the pathogenic role of IgG-type anti-oxLDL antibodies. Opposed to these, there are other articles such as the study by Santos et al, which deny the elevation of IgG-type anti-oxLDL antibodies in acute coronary syndrome and stand up for their protective role. According to our opinion, these contradictions can be arisen from the fact that there are numerous immunogenic epitopes on the surface of oxLDL, and every epitope can be a target for the antibodies. On experiments, the method of LDL oxidation varies and the oxLDL epitops used in the studies are different according to the biomedical companies, therefore it is hard to standardize the measurements, or compare them if they had been applied different types of oxLDL epitops.

In acute coronary syndrome the level of IgG anti-oxLDL may predict the severe, unstable clinical complications (for example: the development of myocardial reinfarctions, unstable angina, circulatory failure, sudden cardiac death).

In summary, we conclude that IgG type anti-oxLDL antibody presumably is a plaque-specific, novel biomarker of the clinical

progression of atherosclerosis, however further evaluations on a large cohort of patients are warranted.

NEW RESULTS

We investigated immune processes induced by oxidized LDL with the involvement of mononuclear cells separated from patients with antiphospholipid syndrome (APS), in addition, we analyzed the clinical relations of antibodies to oxLDL in patients with acute coronary syndrome and stable coronary artery disease.

1. *In vitro*, we could confirm that high concentrations of oxidized LDL increase proliferation and IL-2, IFN- γ secretion of mononuclear cells in antiphospholipid syndrome.

2. It has been proven that this phenomenon is typical for patients with APS, it can not be observed in lymphocytes separated from healthy individuals.

3. Based on the vascular localization of thromboembolic events, patients with APS were divided into arterious and venous subgroups. The oxLDL stimulus induced Th1-cytokine secretion and lymphocyte proliferation in both subgroups.

4. In consideration of the etiology of venous APS, we suggest that the Th1-derived immune processes can promote persistence of the pathological immune response and worsen the clinical outcome. At the same time, oxLDL triggered Th1-derived immune mechanisms (with dyslipidaemia and anti- β 2GPI antibodies) can have an emphasized role in the etiopathogenesis of accelerated atherosclerosis which develops in parallel with arterial thromboses.

5. It has been detected that the level of IgG-type anti-oxLDL antibodies were elevated both in patients with acute coronary syndrome (ACS) and stable coronary artery disease compared to healthy controls, moreover, these antibodies were present with much larger rate in acute coronary syndrome.

6. It has been confirmed that in acute coronary syndrome elevated initial anti-oxLDL antibody-titers correlate well with unstable clinical events (complications such as circulatory failure, sudden cardiac death, malignant arrhythmias, recurrent angina) during hospitalization.

7. It is revealed that ACS patients with statin therapy has significantly lower levels of IgG anti-oxLDL antibodies compared

to those without statin therapy, which can be construed by pleiotropic (anti-inflammatory) effect of statins. This phenomenon could give an indirect evidence of close connection of these antibodies and inflammatory processes in the plaque.

8. In ACS, the strong correlation of IgG-type anti-oxLDL antibodies and CRP can also support the important role of these antibodies in unstable progression of atherosclerotic plaque.

9. In summarizing these results, we conclude that oxidized LDL can stimulate lymphocyte proliferation and secretion of Th1-type cytokines in antiphospholipid syndrome, which results in accelerated atherosclerosis. Moreover, the circulating IgG anti-oxLDL antibodies participate in unstable plaque-associated immune processes and their initial elevation has a predictive value in the unstable outcome of acute coronary syndrome.

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List of publications related to the dissertation

1. Soltész, P., Veres, K., **Laczik, R.**, Dér, H., Csipő, I., Tímár, O., Szomják, E., Szegedi, G., Szodoray, P.: Evaluation of antibodies to oxidized low-density lipoprotein and assessment of C-reactive protein in acute coronary syndrome and stable coronary artery disease.
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