ANTI-CHOLESTEROL ANTIBODIES (ACHA):
CHARACTERISTICS, CELLULAR APPLICATIONS AND
CLINICAL APPROACHES

PhD Thesis
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INTRODUCTION

The existence of anti-cholesterol antibodies (ACHA) was suggested early in the 1920s, but it was proved just after long years by Alving and his coworkers. Antibodies reacting with cholesterol got much interest because of their proposed role in the regulation of lipid metabolism and later with the spread of the lipid-raft theory, their binding to cell membranes came also to the focus.

The role of lipoproteins and cholesterol in the pathogenesis of atherosclerosis has been extensively studied. Atherosclerosis is characterized by the additional formation of foam cells, i.e. macrophages and smooth muscle cells that have taken up chemically modified (oxidized) low density lipoproteins (oxLDL), leading to overloading of these cells with lipids and the deposition of extracellular cholesterol crystals. It is also well known that modified LDL activates the complement system. Several studies have addressed complement activation by the different LDL forms in human sera, but under these conditions the role of different influencing factors is hard to describe.

Anti-cholesterol antibodies were found to bind to circulating lipoprotein fractions and upon animal experiments their role in regulating lipid metabolism and in preventing atherosclerosis was suggested. Our previous results showed that high ACHA levels are protective against the development of stroke.

Cell membrane lipid rafts are receptor organizing structures, which are enriched in cholesterol and sphingolipids. These dynamic assemblies are involved in sorting and distributing lipids and proteins to the cell surface, where they play an important role in signal transduction and in generating cell surface polarity.

As cholesterol is one of the major structural lipids in the cell membrane, it was tempting to speculate whether naturally occurring antibodies against cholesterol could bind to the cell surface. There have been mostly suppositions according to this question, and it was shown that cholesterol microdomains could not be visualized on normal cells with monoclonal antibodies against cholesterol.
A number of pathogens have evolved to utilize these microdomains to gain control of the host cells or to exert their pathogenic effects. HIV-1 (Human Immunodeficiency Virus) has evolved to rely on the host cell lipid rafts to passage across a new host’s mucosa, viral entry into immune cells, signaling of changes in host cell functions as well as viral exit from cells, and dispersion through the host’s vascular system. HIV-1 budding occurs through lipid rafts, thereby accounting for the cholesterol-rich, sphingolipid-rich virus membrane.

Our previous results showed that anti-cholesterol antibodies levels were significantly higher in HIV positive patients than in HIV seronegative controls, that can be due to the increase of immunogenicity of cholesterol because of its presence in the envelope of HIV virions.

Experiments summarized in this work are focused on the characteristics of antibodies reacting with cholesterol, their interactions with cells and their role in virus infections and atherosclerosis.

**AIMS**

1. **Serum levels of anti-cholesterol antibodies in patients with severe carotid atherosclerosis**

Recently we have measured significant lower ACHA levels in patients with ischaemic stroke compared to the healthy control subjects. We were curious whether this alteration in anti-cholesterol antibody levels is also found in patients with severe carotid stenosis, who have a high risk to develop stroke. These patients undergo carotid endarterectomy to prevent stroke. We also aimed to follow anti-cholesterol antibody levels in patients after the removal of atherosclerotic plaque.

2. **Serum anti-cholesterol antibodies in chronic hepatitis-C patients during IFN-? -2b treatment**
In our group Anna Horváth followed ACHA levels in HIV infected patients during HAART. We aimed to establish whether high ACHA levels are specific for HIV infection or this phenomenon is also observed in other chronic viral infection. We measured ACHA titers in a viral infection that is characterized with polyclonal B-cell activation and alterations in lipid metabolism similarly to HIV infection. We measured anti-cholesterol antibody titers parallel with total cholesterol level, complement activation products and HCV-RNA in sera of patients at the onset of IFN-?,-2b treatment and after 3, 6 and 12 months treatment.

3. Production and characterization of monoclonal antibodies against cholesterol

Our aim was to produce IgG isotype of monoclonal ACHA, which has not only practical benefits but also theoretical significance. Furthermore, our aim was to characterize IgG antibodies against cholesterol, and thereafter to specify their function and their role in the lipid metabolism and viral infections, and their affects on cell signaling.

4. Complement activation by lipoproteins

Complement activation by modified lipoproteins was found to be important in the pathogenesis of atherosclerosis. Our aim was to test the ability of native lipoproteins and the modified forms of LDL to trigger direct activation of the C1 complex and control the effect of influencing factors in an in vitro system. Furthermore we aimed to study the effect of CRP on the activation process, and the binding of C1q and CRP to native and modified LDL using surface plasmon resonance spectroscopy.
METHODS

Study population
70 consecutive patients (47 males, 23 females; mean age (±SD) 66.7 ± 9.1 years) who underwent eversion endarterectomy at the Department of Cardiovascular Surgery, Semmelweis University, Budapest were included in the study. Serum samples from 66 healthy volunteers (23 males, 43 females, mean age (±SD) 45.0 ± 9.2 years) served as controls.

39 patients with CHC (20 males, 19 females; mean age (±SD) 45.6 ± 7.3, range 31-58) participated in this study, who were treated with IFN-?-2b (Schering-Plough) 5 MU daily for 6 weeks, than 5 MU TIW. The patients were divided in two groups according to their response to the treatment: Responder (R) patients: >50% decrease of the pretreatment HCV RNA concentration during the 3 months period. Non-responder (NR) patients: < 50% decrease, no change or increase of the pretreatment viral load. Fifty-two age- and sex-matched healthy subjects (28 males, 24 females; mean age (±SD) 47.6.2. range 33-59) without any evidence of viral hepatitis served as control.

Methods
Anti-cholesterol antibody levels were determined by solid phase ELISA method. Serum concentrations of total cholesterol and triglycerides, HDL-cholesterol were measured by enzymatic methods and LDL-cholesterol levels were determined by Friedewald-formule. C-reactive protein serum concentrations were measured by particle enhanced immunoturbidimetric assay, fibrinogen concentrations were determined by using the method of Clauss. Production of monoclonal anti-cholesterol antibodies (mACHA) was performed by immunizing mice with 71% cholesterol containing liposomes. We used ELISA method for the characterization of mACHA, and flow cytometry analysis and confocal leser scanning microscopy to investigate mACHA binding to intact and papain treated cells. After oxidative and enzymatic modification
we tested LDL induced C1 activation by an SDS-PAGE and Western Blot based method. C1q and CRP binding to native and modified LDL were tested by surface plasmon resonance spectroscopy.

**RESULTS**

**Serum levels of anti-cholesterol antibodies in patients with severe carotid atherosclerosis**

Serum ACHA concentrations (median (interquartile range) were found to be significantly (p<0.0001) lower in the sera of the patients with carotid atherosclerosis (13.5 (8.4-21.3) AU/ml) than in the healthy subjects (26.1 (20.9-33.2) AU/ml) (Mann-Whitney test). No correlation was found between serum ACHA concentrations and total cholesterol (p=0.168), triglycerides (p=0.578), and HDL cholesterol (p=0.296) levels. We found, however, a strong negative correlation (r=-0.367; p=0.0018) between ACHA and LDL-cholesterol concentrations. ACHA levels measured in the same patients before, 6 weeks and 14 months post-surgery were compared by using the non-parametric Friedman test followed by the post hoc Dunn test. Serum ACHA concentrations significantly (p<0.001) increased from the values measured before operation ((13.5 (8.4-21.3) AU/ml) to 27.1 (19.9-34.7) AU/ml measured at the end of the follow-up. Six weeks postsurgery levels (16.2 (9.1-29.3) were higher but did not significantly differ from the preoperative levels. ACHA concentrations measured at the end of follow up did not significantly differ (p=0.933) from those present in the sera of healthy controls as calculated by the Mann-Whitney test.

**Serum anti-cholesterol antibodies in chronic hepatitis-C patients during IFN-? -2b treatment**

The concentration of ACHA was significantly (p=0.0062) higher in sera of CHC patients (median 39.68 (25th-75th percentile 23.54-69.25 AU/ml)) than in the 52
healthy controls tested (median 26.09 (25th-75th percentile 19.65-35.40 AU/ml)). We found significant differences between ACHA level of patients with low (<4.0mmol/l) and with normal (?4.0mmol/l) cholesterol concentrations (p=0.0422, Mann Whitney). The ACHA levels were almost twice higher in patients with low (44.87 AU/ml) than in patients with normal cholesterol concentrations (25.78 AU/ml). At 3 months of the treatment a significant decrease in serum ACHA concentrations was observed falling to the healthy control level (p=0.0107, Wilcoxon test). In responders ACHA levels significantly (p=0.0008) decreased to a value (28.6 (18.7-54.3) AU/ml) comparable to values measured in sera of healthy controls. By contrast, in non-responders ACHA level did not significantly change (p=0.9097) after 3 months of treatment (33.9 (26.2-52.4 AU/ml).

Production of monoclonal antibodies against cholesterol
We have efficiently generated mouse monoclonal IgG (IgG3) antibodies (MAbs), which bind dose-dependently to cholesterol (K_D=11.5±1.3 nM). The response did not vary between 0.1 µg/well and 10 µg/well cholesterol. The reactivity decreased significantly when the concentration of cholesterol dropped below 0.1 µg/well. The ELISA results showed that mACHA give a higher response with ergosterol and with 7-ketocholesterol (3?-OH containing sterols similarly to cholesterol) than with the other sterols (coprostane, cholesteryl-oleate, 25-hydroxy -cholesterol). To examine whether anti-cholesterol antibodies would interact with the human lipoproteins VLDL, LDL and HDL, purified monoclonal IgG was mixed with different concentrations of lipoprotein preparations and incubated at 37°C for 60 minutes. We found that IgG binding to cholesterol coated plates could be blocked by human lipoproteins (VLDL, LDL and HDL) in a dose-dependent manner. When the results were normalized to surface area, HDL expressed lower inhibition than VLDL and LDL.
Monoclonal ACHA displayed a weak spontaneous binding to intact human and mouse lymphoid and myeloid cell types. Papain-digestion of the cell surface strongly enhanced the binding of IgG type ACHAs to all cells, proportionally with the papain dose. This suggests that a limited digestion by papain (10 U/ml, 5 min.) of the cells can serve as an ‘epitope exposition tool’. The mACHA could also bind intracellularly in T helper hybridoma cells, after saponin-permeabilization/formaldehyde fixation, the intracellular staining may be attributed to small cholesterol-containing lipid vesicles. The mACHA and cholera toxin B (raft marker) showed a high degree of colocalization on Concanavalin-A activated mouse T-helper cells, suggesting a co-redistribution of their binding sites, i.e. GM₁ gangliosides and membrane cholesterol.

**C1 activation by native and modified lipoproteins**

As observed for the unmodified LDL preparation, oxLDL did not induce significant C1 activation in the absence of CRP. In contrast, incubation of oxLDL (1µM) with C1 and C1 inhibitor in the presence of increasing concentrations of CRP resulted in increased C1 activation, up to an extent of about 32% at a CRP concentration of 100 µg/ml. The ability of LDL to induce C1 activation in the presence of CRP was clearly correlated with the oxidation time, and hence probably with the extent of oxidation. To investigate the ability of enzymatically modified LDL to mediate C1 activation, LDL was initially treated sequentially with trypsin, cholesterol esterase and then neuraminidase. This triple enzyme treatment conferred LDL the ability to trigger direct C1 activation in the absence of CRP. C1 activation was dose-dependent and reached a maximum value of about 60% at E-LDL concentrations of 0.1-1 µM. In order to investigate whether one or more particular steps of the enzymatic modification procedure was essential to yield a C1 activating derivative of LDL, the unmodified LDL preparation was submitted to single-enzyme treatments using either different proteases or cholesterol esterase, and double- and triple-
enzyme treatments. Treatment with trypsin, plasmin, or proteinase K alone only slightly increased the ability of LDL to activate C1. Treatment with cholesterol esterase alone was slightly more efficient, but resulted in C1 activation values below 20%. In contrast, sequential treatments with either trypsin, plasmin or proteinase K, and then with cholesterol esterase strongly increased the C1 activating ability of LDL, with values close to 57% in the case of trypsin. Subsequent treatment of LDL with neuraminidase did not increase, or even slightly decreased, LDL ability to activate C1. The ability of C1q and CRP to bind to LDL, oxLDL and E-LDL was studied by surface plasmon resonance spectroscopy, using the lipoproteins as immobilized ligands and C1q or CRP as the soluble analyte. C1q readily bound to both oxLDL and E-LDL, but did not show significant binding to unmodified LDL, however CRP bound to oxLDL, E-LDL, as well as to unmodified LDL.

THE MOST IMPORTANT FINDINGS, CONCLUSIONS

The results summarized in my PhD thesis give new data to the characterization of anti-cholesterol antibodies and to the description of their clinical significance. Our main results are the following:

- In patients with severe carotid atherosclerosis ACHA levels were significantly lower than in the healthy controls. Surgical removal of the plaques resulted in increase in the ACHA levels, which reached the mean of those measured in the healthy controls.

- Serum concentration of ACHA was significantly higher in HCV infected patients before the treatment, than in the control population. ACHA levels decreased significantly, close to the normal level on the course of IFN therapy in responder patients.

- We found in both clinical studies reverse correlation between serum LDL-cholesterol or total cholesterol levels and ACHA concentrations.
We succeeded in producing IgG isotype cholesterol specific monoclonal antibodies. During characterization of the antibody we found that monoclonal ACHA binds to cholesterol-similar, 3?-OH group containing sterols and to all human lipoprotein fractions. The mAbs also bound to the surface of human and murine cell types of different origin, with low avidity that could be enhanced by a preceding papain cleavage of the long or sizeable extracellular protein domains. The membrane-bound ACHA colocalized and co-redistributed upon T cell activation with choleratoxin B, a known lipid-raft marker, suggesting that ACHA can also bind to and mark raft-associated cholesterol at the cell surface.

During the investigation of lipoproteins induced complement activation, we managed to justify the modified lipoproteins induced classical complement pathway activating effect with a method, that could further help us to study the effect of individual components and mACHA on this activation process.

Summarizing our results obtained in clinical and basic research we can conclude, that cholesterol binding antibodies present in all individual can bind to circulating lipoproteins and could play a role in their homeostasis. However it is still not clear, whether Alving’s theory, that ACHA could opsonize LDL and play a role in its removal from the circulation and thereby it can exert a protective effect against “bad” cholesterol, is true or not. We think that ACHA has a more complex role, than that, and due to the binding to human lipoproteins it could activate the complement system. To study this we established the C1 activation assay, a method for investigating the initiation of the classical complement pathway. We would like to further investigate the mACHA’s effect on the modified lipoproteins induced C1 complex activation. Whereas it is incontestable, that some clinical results – the strong negative correlation between LDL-cholesterol and ACHA in the patients with severe carotid stenosis, and the high ACHA level in virus infected patients with low total...
cholesterol level – could confirm Alving’s hypothesis. Our previous results, high ACHA levels in patients with ischeamic heart disease, and the lack of correlation between LDL and ACHA in atherosclerotic patients, are still against Alving’s assumption.

Viral infections are pathologic states, in which cholesterol is a potential activator of the humoral immune response. The immunogenecity of cholesterol could be increased by the fact that this molecule is crucial in viral entry to the host cell and in the progression of the infection. Both in HIV and HCV infected patients we found significant correlation between the viral load and the immune response against cholesterol and during effective therapy the decrease of the viral load was followed by normalization of ACHA titer. There are investigations in course to study the effect of monoclonal anti-cholesterol IgG on the progress of HIV infection in the Institute of Medical Microbiology, University of Debrecen. We hope that these experiments will contribute to the more precise description of anti-cholesterol antibodies’ role in viral infections.

Upon the binding of monoclonal ACHA to cell membranes, we suppose that this antibody could bind to cell surface only when the exposition of cholesterol is changed. During in vitro experiments we found significant binding to the cell membranes only when cholesterol became accessible due protease digestion, and conceivably also in vivo there is no binding to intact cell membranes, but various effects (for example during viral infections the increased lipid raft exposition, the altered cell membrane composition of apoptotic cells) could enhance anti-cholesterol antibodies’ binding. We plan further experiments to describe more precisely these cellular applications with the Immunology Department of the Eötvös Loránd University.
SCIENTIFIC PUBLICATIONS

Publications summerized in the dissertation:


Publications corresponding to the topic of the dissertation:


Other publications: