ROMANIAN ACADEMY

INSTITUTE OF CELLULAR BIOLOGY AND PATHOLOGY

"NICOLAE SIMIONESCU"

PhD THESIS SUMMARY

Synthetic lipoproteins with therapeutic potential in atherosclerosis

Scientific coordinator: Acad. MAYA SIMIONESCU

PhD student: Florina-Irina Tudorache

BUCHAREST

2023

CONTENTS:

SUMMARY

Keywords: Atherosclerosis, Lipoproteins, Apolipoproteins, Nanotechnology, Gene regulation, Nanoparticles, ApoE, ApoA-I,

Total number of pages – 128

Number of figures in Part I – 14

Number of Tables in Part I – 2

Number of figures in Part II – 37

Number of tables in Part II – 6

Bibliographic references – 327

Works published in ISI indexed journals during the doctoral internship – 4 (2 main author)

Posters presented at international scientific events – 6 (main author)

Oral communications given at international scientific events – 2

Patents – 1

Courses and specializations - 1

Scholarship obtained during the doctoral program – 1 (doctoral scholarship from the Romanian Academy)

Participation in national research projects related to the topic of the PhD thesis – 5

INTRODUCTION AND OBJECTIVES

Atherosclerosis is one of the main causes of mortality in developed countries. Data from the literature support that atherosclerosis is a multifactorial disease in which inflammation and dyslipidemia intersect and that regardless of the initiator, both participate from the early stages to the final fate of the atheromatous plaque. One of the major risk factors in atherosclerosis is alteration of plasma homeostasis, such as hypercholesterolemia. This initially affects the endothelial cells, which they activate and as a result, they begin to express new adhesion molecules and chemotactic factors that lead to the initiation of an inflammatory process. Inflammation involves the recruitment of circulating immune cells that aggravate and accelerate the development of atheroma [1].

The initial inflammatory response can be considered as a defense reaction mechanism, but its subsequent amplification accelerates atherosclerosis [2, 3]. Also, components of plasma lipoproteins, various microbial structures or heat shock proteins induce an inflammatory process

that, by itself, can generate the formation of atherosclerotic plaque. Approximately half of patients develop atherosclerosis in the absence of systemic hypercholesterolemia [4].

Although the entire vascular system is exposed to risk factors, atherosclerosis preferentially develops in certain areas such as the branching points, the outer wall of the bifurcations, the inner wall of the curves or heart valves, areas characterized by variations in flow stress or flow disorders [5, 6]. Atheroma development is a continuous process. The progressive involvement of vascular resident and non-resident cells and their secretory products define a sequence of consecutive steps leading from the formation of lipid striae to fibro-lipid plaque and finally to plaque rupture and atherothrombosis [2].

Lipoproteins are structures made up mainly of lipids and proteins. They fulfill the function of transporting cholesterol, triglycerides and phospholipids in the plasma. Lipoproteins have two components: a hydrophilic outer shell containing apolipoproteins, phospholipids and non-esterified cholesterol and the hydrophobic central core, made up of esterified cholesterol and triglycerides. Apolipoproteins ensure the solubility of lipoproteins, but they are also enzyme cofactors or ligands for specific cell receptors. There are four major classes of apolipoproteins: A, B, C and E. Apolipoprotein E (apoE) is a component of plasma lipoproteins, which participates in the removal of atherogenic lipoproteins from circulation. In vitro and in vivo studies have shown that apoE mutations are associated with elevated cholesterol levels and premature atherosclerosis in both experimental animals and humans [7]. The physiological level of apoE in plasma is associated with a decrease in the risk of cardiovascular diseases, by reducing the level of circulating atherogenic lipoproteins, by cholesterol efflux, but also by inhibiting the expression of adhesion molecules at the endothelial surface [8].

Recent advances in cardiovascular therapies have led to the development of new agents that target specific apolipoproteins to reduce the risk of atherosclerosis [9, 10].

Nanoparticle science is rapidly changing the outlook of various scientific fields and defining new technology platforms. This is probably even more evident in the field of nanomedicine where nanoparticles have been used as a tool for the treatment and diagnosis of many diseases [11]. However, despite the immense benefit conferred, there are also restrictions of this technology aimed at short- and long-term effects on the human body.

The aim of this doctoral thesis was to determine some mechanisms of gene regulation that lead to the decrease of the expression of some apolipoproteins and to produce fullerenol-based

nanoparticles coupled with apoE-ε3 (Full-apoE) that mimic lipoprotein particles and that could be used to compensate for the low expression of apoE.

Our **hypothesis** states that fullerenol-based nanoparticles with apoE-ε3 (Full-apoE) molecules bound to the surface mimic lipoproteins rich in apoE, offering significant anti-atherosclerotic benefits, without side effects.

STRUCTURE OF THE PhD THESIS

In the first part of the thesis, data from the literature targeting the specialized functions of lipoproteins and associated apolipoproteins are presented, together with their roles in plasma lipid circulation and the development of atherosclerosis (**Chapter 1**) as well as new promising therapies targeting specific apolipoproteins that could ultimately reduces the risk of atherosclerosis.

In **Chapter 2**, customized nanotechnologies for targeting cells involved in the development of atherosclerotic plaques and the main classes of nanoparticles used in the diagnosis or treatment of atherosclerosis are synthesized. We also highlighted the synthesis methods, stability and main antioxidant characteristics of the fullerenol molecule.

In the second part of the thesis, the original results obtained on the gene regulation of some important apolipoproteins in the atherogenesis process and the synthesis of nanoparticles with anti-atherosclerotic potential are presented. **Chapter 3** presents the results obtained in vivo and in vitro on the regulation of some apolipoproteins involved in the development of atherosclerotic plaques by some aromatic polyhydrocarbons present in the environment: Bisphenol A (BPA) and Benzo(a)pyrene (BaP). Both BPA and BaP activate transcription factors that bind to specific sites in the promoters of apolipoproteins apoA-I and/or apoC-I and contribute to the development of atherosclerotic lesions, through mechanisms involving NF-κB or AHR activation.

The methods used and presented in **chapter 4** aimed to obtain by biotechnology the apoE-ε3 protein marked and secreted by mammalian cells, in order to build nanosystems with the apoE-ε3 isoform.

Chapter 5 presents the methods for obtaining nanoparticles based on fullerenol, coupled with apoE and testing the anti-atherosclerotic effect in vivo on the line of apoE deficient mice. The obtained fullerenol-based nanoparticles carrying apoE-ε3 combine the anti-hyperlipidemic and anti-oxidative properties of fullerenol with the anti-atherosclerotic and anti-inflammatory properties of apoE-ε3 and may represent a promising strategy for atherosclerosis.

GENERAL CONCLUSIONS

Although clinical outcomes in coronary heart disease have improved considerably over the past several decades, the incidence of myocardial infarction and stroke continues to remain high. Nanotechnology offers a unique approach by developing cell-specific multifunctional nanoparticles. This will lead to new frontiers in diagnosis and therapy by targeting the molecules involved in the processes leading to atherosclerosis, thus maintaining the potential for continuous improvement in cardiovascular outcomes.

In this work, we analyzed the regulation of some important apolipoproteins in the atherogenesis process by the substances present in the environment: bisphenol A (BPA) and benzo[a]pyrene (BaP). Starting from the hypothesis that the pro-atherogenic effects of BPA are mediated, at least in part, by the downregulation of the APOA-I gene, thus leading to a low amount of anti-atherosclerotic HDL; we investigated whether BPA modulates APOA-I gene expression and the molecular mechanisms involved in this regulation. The results obtained on LDLR-/- mice treated with BPA showed that (i) BPA increased the area of atherosclerotic lesions in the aortas of LDLR-/- mice; (ii) decreased HDL and apoA-I levels and increased plasma cholesterol and triglyceride concentrations; (iii) the mechanism of this process involves the activation of NF-κB pathways and consequently, the reduction of apoA-I expression in the liver. Our data demonstrated that the APOA-I promoter contains functional NF-κB binding sites that are responsible for the regulation of BPA-induced APOA-I gene expression.

We also investigated the modulatory potential of BaP on the expression of some key apolipoproteins in lipid metabolism: apoA-I, apoC-I and apoE, and pursued a possible mechanism by which BaP modulates the expression of these apolipoproteins, through in vitro studies, in hepatocytes. The results of this study show that BaP induces the upregulation of apoA-I and apoC-I in hepatocytes through an AHR-dependent mechanism. AHR binds to the active sites in the apoA-I and apoC-I promoters and leads to a decrease in apoA-I expression, respectively an increase in apoC-I expression, in hepatocytes; which determines a potential risk for the development and support of atherosclerotic plaques. A possible regulation mechanism of these apolipoproteins based on the results obtained and what is known from the literature could be both directly through AHR and indirectly through the dimerization of other transcription factors.

At the end of the paper I addressed nanotechnological strategies for the regression of atherosclerotic plaques. For this, we deepened the genetic engineering techniques for obtaining

plasmids that express recombinant apoE. With these plasmids, we stably transfected mammalian cells that synthesized apoE in the culture medium, from where we could isolate the protein of interest, by affinity chromatography. We used this protein in order to obtain nanoparticles with therapeutic potential in atherosclerosis.

Nanoparticles with apoE have been used to treat apoE deficient mice and we have observed some significant anti-atherosclerotic benefits. The results obtained following the administration of derivatized Full-apoE nanoparticles to atherosclerotic apoE-/- mice showed that Full-apoE NPs (i) were included in the HDL and VLDL fractions; (ii) were distributed mainly in the liver, but also in other tissues (such as brain, lung and kidney); (iii) caused an increased hepatic expression of important molecules involved in lipid metabolism (such as apoA-I, ABCA1, SR-B1 and sortilin); (iv) caused a decrease in plasma cholesterol levels and (v) inhibited the development of atherosclerotic plaques. Fullerenol-based nanoparticles carrying apoE-ε3 combine the anti-hyperlipidemic and anti-oxidative properties of fullerenol with the anti-atherosclerotic and anti-inflammatory properties of apoE-ε3 and may represent a promising strategy for atherosclerosis.

The **main original contributions** of this paper are:

(i) the gene regulation mechanism for APOA1 in response to BPA activity, by activating NF-kB and identifying the sites responsible for binding transcription factors on the APOA1 gene promoter, resulting in a decrease in APOA1 gene expression.

(ii) gene regulation of APOA1 and APOC1 in response to BaP through AHR activation and translocation to the nucleus, where it binds to the promoters of these genes inducing a decrease in APOA1 and an increase in APOC1, which indicates a pro-atherosclerotic mechanism.

(iii) obtaining plasmid vectors encoding recombinant apoE and stably transfected RAW 293.7 and bEnd.3 cells expressing the glycosylated apoE protein in the cell growth medium.

(iv) Design and characterization of apoE-coupled fullerene-based nanoparticles and in vivo validation of the anti-atherosclerotic effects of Full-apoE.

RECOVERY THE RESULTS

The results obtained during the doctoral program at the "Nicolae Simionescu" Institute of Cellular Biology and Pathology of the Romanian Academy were:

- published in 4 papers in ISI indexed journals: 1 as first author, 1 as co-author and 2 reviews;

● presented at national and international conferences in the form of 2 oral presentations and 6 posters.

ACTIVITIES WITHIN THE DOCTORAL INTERNSHIP

Works published in ISI rated international journals:

1. Apolipoprotein E - A Multifunctional Protein with Implications in Various Pathologies as a Result of Its Structural Features. (Review) *Tudorache IF, *Trusca VG, Gafencu AV. Comput Struct Biotechnol J. 2017 Jun 6;15:359-365. two: 10.1016/j.csbj.2017.05.003. eCollection 2017. PMID: 28660014

2. The Mechanism of Bisphenol A Atherogenicity Involves Apolipoprotein A-I Downregulation through NF-κB Activation. Trusca VG, Dumitrescu M, Fenyo IM, Tudorache IF, Simionescu M, Gafencu AV. Int J Mol Sci. 2019 Dec 12;20(24):6281. doi: 10.3390/ijms20246281. PMID: 31842455

3. Synthetic lipoproteins based on apolipoprotein E coupled to fullerenol have anti-atherosclerotic properties. Tudorache IF, Bivol VG, Dumitrescu M, Fenyo IM, Simionescu M, Gafencu AV. Pharmacol Rep. 2022 Aug;74(4):684-695. two: 10.1007/s43440-022-00379-8. Epub 2022 Jul 5. PMID: 35790693.

4. Apolipoprotein A-II, a Player in Multiple Processes and Diseases. (Review) *Florea G, *Tudorache IF, Fuior EV, Ionita R, Dumitrescu M, Fenyo IM, Bivol VG, Gafencu AV. Biomedicines. 2022 Jul 2;10(7):1578. doi: 10.3390/biomedicines10071578. Free PMC article. PMID: 35884883

Oral communications at national and international scientific events:

1. Biotechnological approaches for labeling of apolipoprotein E secreted by mammalian cells, SRBC 2016

2. Apolipoprotein E gene regulation by ligand-activated AHR, Advanced Course on Crossroads of Atherosclerosis, Immunology & Lipids (AIL); EAS 2016

Posters at national and international scientific events:

1. Apolipoprotein E gene regulation by omeprazole activated AHR/ARNT complex,

Irina Grosu, Violeta Trusca, Anca Gafencu, SRBC 2015.

2. Biotechnological approaches to prepare synthetic lipoproteins with therapeutic potential in atherosclerosis; Irina F. Tudorache, Violeta G. Trusca, Elena V. Fuior, Anca V. Gafencu, EAS 2017.

3. Regulation of apolipoproteins by ligand-activated aryl hydrocarbon receptor; Irina F. Tudorache, Violeta G. Trusca, Anca V. Gafencu, BIT 2017

4. Benzo(a)pyrene-activated AHR regulates apolipoproteins expression in hepatocytes; Irina F. Tudorache, Violeta G. Trusca, Anca V. Gafencu, EAS 2018

5. Fullerenol – based nanoparticles carrying apolipoprotein E; Irina. F. Tudorache, M. Dumitrescu, V.G. Trusca, M.I. Fenyo, M. Simionescu, A.V. Gafencu, MMT 2021

6. Anti-atherosclerotic properties of fullerenol-based nanoparticles carrying apoE3; Irina. F. Tudorache, M. Dumitrescu, V.G. Trusca, M.I. Fenyo, M. Simionescu, A.V. Gafencu, SRBC 2021

Specializations and courses completed:

1. EAS Advanced Course on Crossroads of Atherosclerosis, Immunology & Lipids (AIL) Amsterdam, The Netherlands, March 22 - March 25, 2016.

Participation in research projects:

1. Combined hormonal treatment-induced gene transactivation of anti-atherosclerotic proteins as an innovative therapeutic approach for atherosclerosis.

2. Genetically engineered apolipoproteins immobilized on nanoparticles: a Molecular Trojan horse targeting atherosclerotic plaque.

3. Intelligent therapies for non-communicable diseases based on controlled release of pharmacological compounds from encapsulated engineered cells and targeted bionanoparticles.

4. Demonstration of the enhanced anti-atherosclerotic potential of targeted apolipoprotein E to the activated endothelium by fusion with VCAM-1 binding peptide.

5. Apolipoprotein A-II derived peptides with anti-atherosclerotic potential.

SCHOLARSHIP OBTAINED DURING THE DOCTORAL PROGRAM

Doctoral scholarship: Romanian Academy (SCOSAAR): 2015-2018

FUNDING OF RESEARCH:

PN-II-RU-TE-2014-4-2660. Transactivation of genes for anti-atherosclerotic proteins by combined hormonal treatments, with therapeutic approach in atherosclerosis.

PN-II-RU-TE-2014-4-2143. Genetically engineered apolipoproteins immobilized on nanoparticles: a molecular Trojan horse for targeting atherosclerotic plaque

Romanian Academy SCOSAAR

Selective Bibliography:

1. Libby, P., et al., *Inflammation in atherosclerosis: from pathophysiology to practice.* J Am Coll Cardiol, 2009. 54(23): p. 2129-38.
2. Manduteanu, I. and M. Simionescu, *Inflammation in atherosclerosis: a cause or a result of vascular disorders?* J Cell Mol Med, 2012. 16(9): p. 1978-90.
3. Horejsi, B. and R. Ceska, *Apolipoproteins and atherosclerosis. Apolipoprotein E and apolipoprotein(a) as candidate genes of premature development of atherosclerosis.* Physiol Res, 2000. 49 Suppl 1: p. S63-9.
4. Millonig, G., G.T. Malcom, and G. Wick, *Early inflammatory-immunological lesions in juvenile atherosclerosis from the Pathobiological Determinants of Atherosclerosis in Youth (PDAY)-study.* Atherosclerosis, 2002. 160(2): p. 441-8.
5. VanderLaan, P.A., C.A. Reardon, and G.S. Getz, *Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators.* Arterioscler Thromb Vasc Biol, 2004. 24(1): p. 12-22.
6. Simionescu, N., et al., *Prelesional events in atherogenesis. Accumulation of extracellular cholesterol-rich liposomes in the arterial intima and cardiac valves of the hyperlipidemic rabbit.* Am J Pathol, 1986. 123(1): p. 109-25.
7. Zannis, V.I., et al. *8 Lipoproteins and atherogenesis*. 2004.
8. Gaudreault, N., et al., *Macrophage-specific apoE gene repair reduces diet-induced hyperlipidemia and atherosclerosis in hypomorphic Apoe mice.* PLoS One, 2012. 7(5): p. e35816.
9. Schoenhagen, P. and J.L. Conyers, *Nanotechnology and atherosclerosis imaging: emerging diagnostic and therapeutic applications.* Recent Pat Cardiovasc Drug Discov, 2008. 3(2): p. 98-104.
10. Wickline, S.A., et al., *Applications of nanotechnology to atherosclerosis, thrombosis, and vascular biology.* Arterioscler Thromb Vasc Biol, 2006. 26(3): p. 435-41.
11. Foroozandeh, P. and A.A. Aziz, *Insight into Cellular Uptake and Intracellular Trafficking of Nanoparticles.* Nanoscale Res Lett, 2018. **13**(1): p. 339.