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„NICOLAE SIMIONESCU”

MOLECULAR INTERVENTIONS ON THE EXPRESSION OF PRO AND ANTI-ATHEROGEN PROTEINS FOR DIMINISHING ATHEROSCLEROSIS

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ABSTRACT

Atherosclerosis is a complex disease characterized by the accumulation of lipid deposits in the arterial wall, which in time turn into advanced plaques, with complex cellular and molecular architecture and the composition, that disrupt the normal flow of blood and that, following erosion or rupture, may lead to acute coronary syndromes such as myocardial infarction and stroke.

Over time, many theories have been proposed to explain the formation of atherosclerotic plaques. Today, the causal relationship between hypercholesterolemia, development of atherosclerotic lesions and cardiovascular diseases is universally proven and accepted. However, the so-called "cholesterol hypothesis" can not exclusively illustrate the complexity of the pathologic process in atherosclerosis, which comprises, among others, the activation and the dysfunction of endothelial cells (EC), as well as the inflammatory process which takes a major role in all lesional phases ("response to injury hypothesis"), the retention of atherogenic lipoproteins in the subendothelial space ("response to retention hypothesis") and their oxidative modifications ("oxidative modifications hypothesis").

Moreover, the multitude of scientific data accumulated so far shows that atherosclerosis is a multifactorial disease in which participates a number of other risk factors such as hypertension, hyperglycemia and diabetes mellitus, obesity, smoking and family history.

The approaches currently in use for the treatment of atherosclerosis are focused mainly on regulating cholesterol metabolism and lipoprotein metabolism, on reducing the inflammatory process or on preventing the thrombotic complications. Nevertheless, these therapies do not work in all patients. Therefore, in order to increase the efficiency of the treatment it is necessary to find alternative therapeutic methods.

The different therapeutic strategies used so far to counteract oxidative stress have focused mainly on the use of antioxidant supplements and vitamins or finding selective inhibitors of various enzymatic sources of reactive oxygen species (ROS). Although promising in the beginning, the antioxidant therapy has not produced the expected results in several randomized clinical trials because these compounds act only

as ROS scavengers and not as modulators of the different intracellular redox signaling processes that occur in the vascular wall.

Because of their major contribution to the generation of ROS and their involvement in mediating signals induced by various cardiovascular risk factors, the pharmacological inhibition of vascular NADPH oxidases (Nox) is considered an attractive and potentially effective strategy to combat oxidative stress associated with various pathological conditions.

As an alternative to the direct inhibition of Nox, one can take into account other efficient options, such as the pleiotropic effect of some conventional drugs used to treat cardiovascular disorders (for example, statins, inhibitors of angiotensin converting enzyme, angiotensin II receptor blockers, thiazolidinediones), or *the targeting of different specific signaling pathways involved in regulating the expression and activity of NADPH oxidases*.

Therefore, the first objective of this thesis is "*Reducing oxidative stress in atherosclerosis by inhibiting NADPH oxidase activity*".

On the other hand, another process deeply affected in atherosclerosis is the efflux of lipoprotein particles from the subendothelial space and from the foam cells accumulated in the atherosclerotic lesions. A possible approach in order to improve this issue is *to increase the expression of different proteins involved in the process and which have a beneficial action in atherosclerosis, such as apolipoprotein E*.

Therefore, the second objective of this thesis is "*Developing strategies to increase the expression of apolipoprotein E in cells involved in the atherosclerotic plaque*".

The present paper is structured into two main parts and a chapter of general conclusions.

Part I - The current state of knowledge, is a review of the literature concerning the two proteins studied and their involvement in atherosclerosis. Part I consists of two chapters.

Chapter 1 – NADPH oxidases - promoters of oxidative stress in atherosclerosis, presents published data relating to: the modifications induced by oxidative stress in atherosclerosis; the sources of ROS in the vascular system; the structural and functional characterization of vascular NADPH oxidases; the distribution of NADPH oxidases in the

vascular wall; the regulation of vascular NADPH oxidases activity and expression, and methods of inhibiting NADPH oxidases activity in order to reduce the oxidative stress.

Chapter 2 – Apolipoprotein E – role and involvement in atherosclerosis, presents published data relating to: the structural and functional characterization of apolipoprotein E gene and protein, apoE isoforms and apoE receptors; the role of apoE in lipid metabolism; the role of apoE in atherosclerosis; features of apoE expression and secretion characteristic to macrophages, and murine experimental models for the study of apoE.

PART II - Original contributions, summarizes the experimental data gathered to achieve two main objectives.

Objective I - Reducing oxidative stress in atherosclerosis by inhibiting NADPH oxidase activity, aimed to investigate the potential of two pharmacological inhibitors of Jak2 signaling pathway, compounds AG490 and WP1066, have to diminish the NADPH oxidases dependent ROS generation and to reduce the development of atherosclerotic lesions in hypercholesterolemic ApoE-deficient mice (apoE$^{-/-}$).

1. Evaluation of oxidative stress and inflammation in the vascular wall of hypercholesterolemic mice. The experiments have shown that hypercholesterolemia is associated with increased oxidative stress and inflammation in the aortas of hypercholesterolemic mice. This is demonstrated by a significant increase in gene and protein expression levels of all Nox isoforms, a feature characterizing both early and more advanced stages of atherosclerosis. This growth translates into a sustained production of ROS in the vascular wall.

2. The effect of tyrphostin AG490 treatment on the expression and activity of NAPDH oxidase in the aortas of hypercholesterolemic ApoE$^{-/-}$ mice. The experiments have shown that treatment of ApoE$^{-/-}$ mice fed a high cholesterol diet with tyrphostin AG490 has reduced significantly Nox activity and decreased the mRNA and protein expression levels of each Nox isoform. In addition, the administration of AG490 in hypercholesterolemic ApoE$^{-/-}$ mice induced a significant reduction of macrophage infiltration in the aortic wall as compared to vehicle-injected animals. Consistent with this observation, the morphometric analysis of the lesions showed a significant reduction of early lesions in total aortas and aortic arches in animals treated with AG490 compared to the ApoE$^{-/-}$ mice fed a high cholesterol diet.

3. The effect of WP1066 treatment on the expression and activity of NAPDH oxidase in the aortas of hypercholesterolemic ApoE-/- mice. The results showed that the treatment with WP1066 significantly reduces NOX activity and the mRNA expression levels of Nox1 and Nox4 isoforms. Moreover, the pharmacological inhibition of Jak2 by WP1066 slightly reduced atherosclerotic lesion area expansion, but not in a significant way.

The results obtained in the experiments made under Objective 1 represent the first study to demonstrate that AG490 has an anti-atherosclerotic and anti-oxidant effect *in vivo* by regulating Nox, highlighting a new mechanism of tyrphostin action in the cardiovascular system. Taken together, these results suggest that the modulation of signaling pathways activated by Jak may represent a new pharmacological strategy to counteract the harmful effects of oxidative stress in atherosclerosis.

Objective 2 - Developing strategies to increase the expression of apolipoprotein E in cells involved in the atherosclerotic plaque, was to find ways to increase or induce the expression of apoE in the arterial wall. For this, the experiments have focused on two cell types involved in the development and progression of atherosclerotic lesions, namely macrophages and EC.

1. The study of retinoid induced modulation of apolipoprotein E gene expression in macrophages. The selective expression of apoE in macrophages has potential for the reduction of the cardiovascular risk. Targeting processes such as the apoE expression and secretion in macrophages allows, on one hand, to investigate the role played by the apoE secreted by these cells in the economy of the plaque, and on the other hand, may represent a therapeutic strategy in atherosclerosis . This study aimed to find out whether there is a direct effect of retinoids on apoE gene expression in macrophages and to investigate the mechanism involved. The results demonstrate that retinoids induce the increase of endogenous apoE gene expression in macrophages, both *in vitro* and *in vivo*. Thus, 9-cis retinoic acid induced the increase of apoE mRNA level in RAW 264.7 murine macrophages and vitamin A increases the apoE gene expression in mouse peritoneal macrophages. 9-cis retinoic acid does not act directly on the gene promoter but it exerts its effect on the multienhancer ME.2, by means of which it induces a significant increase in the promoter activity, an increase demonstrated in transfection experiments by the increase in luciferase activity . The analysis of a series of deletion

mutants of ME.2 indicated the existence of a binding site for RXR in the region 405-420 of ME.2. The increase in apoE expression in macrophages is a new potential beneficial effect of retinoids, suggesting that these compounds may have therapeutic use for the regulation of lipid metabolism and in the prevention and/or amelioration of atherosclerosis.

2. Generation of a murine conditional transgenic model to induce in a controlled manner the expression of human apolipoprotein E isoform E3 specifically in the vascular endothelium. EC do not expresse apoE but they express receptors from the LDLR family that recognize and bind apoE. Moreover, they are capable to do transcytosis, transporting macromolecules between blood and the subendothelial space. In view of these functional characteristics of EC, the induction of apoE gene expression in the endothelium may contribute, on one hand, to the rise of plasmatic levels of apoE, increasing its beneficial effects at systemic level, and on the other hand, to the increase of apoE concentration in the plaque, where it could actively participate in the efflux of lipoproteins from the subendothelial space.

The mechanistic principle at the base of the generation of this model was the principle transcriptional transactivation dependent of tetracycline. In the experiments reported in the present paper, we used the binary gene expression system Tet-On 3G (Clontech) to produce two transgenes:

(i) the transgene Tek/Tie2promoter-Tet3G-Tek/Tie2enhancer (referred to as Tek Tet3G) (5200pb) containing the transactivator protein Tet-On3G gene under control of the endothelial specific promoter Tek/Tie2 which ensures the expression specific to the endothelium. The transgene also contains the specific endothelial enhancer Tek/Tie2 that regulates the promoter activity ensuring sustained expression.

(ii) the transgene TRE-hapoE3 (5523pb) containing the gene of interest for human ApoE3 isoform under the control of the P_{TRE3G} inducible promoter.

In the presence of inducer doxycycline (Dox), a synthetic derivative of tetracycline, Tet-On 3G specifically binds to P_{TRE3G} and activates the transcription of the gene of interest. P_{TRE3G} does not contain any binding sites for endogenous mammalian transcription factors, so it is essentially inactive in the absence of the inducer Dox.

Each of the two transgenes was used to create a transgenic animal: a mouse that carries the Tek-Tet3G transgene, and a mouse that carries the TRE-hapoE3transgene.

By pairing these two animals, a double transgenic mouse will be produced, carrying both transgenes, and which will express the human ApoE3 isoform only when Dox should be administered.

To ensure the human ApoE3 isoform expression in the absence of endogenous murine apoE gene, this double transgenic mouse will be mated with mice ApoE$^{-/-}$ mice, deficient in apoE.

In this paper, we present the first three stages undertaken to generate this model, namely:

A. The construction of the transgenes – in order to obtain the constructs for transgenesis, we have used classical molecular cloning techniques (bacterial transformation, restriction digestion, dephosphorylation, and ligation). In the present study, the transgenes required to generate a conditional transgenic murine model in which the expression of the human ApoE3 isoform can be induced in a controlled manner, in the absence of endogenous murine apoE gene, were successfully obtained. The functionality of the constructs has been demonstrated by the results obtained after transfecting several cell types with plasmids containing the transgenes, followed by cultivation of the cells in medium supplemented with or without Dox. The functionality and the specificity of the endothelial Tek/Tie2 promoter has also been demonstrated by measuring its ability to activate the transcription of the luciferase gene specifically in the endothelial cells.

B. The transgenesis procedure – the transfer of transgenes into the animals. The method used in this study to generate transgenic animals was the classical method of transgenesis, namely the pronuclear DNA microinjection.

For the transgenesis procedure, we used four types of animals: fertilized eggs donor females; fertile males used to obtain fertilized eggs; pseudo-pregnant females used as surrogate mothers, and vasectomised males used to obtain pseudo-pregnant females. The transgenesis procedure involved: the collection of embryos after prior induction of superovulation by the use of placental hormones in fertilized eggs donor FVB females; the transfer of DNA (the two transgene linearized and purified) made by microinjection into the male pronucleus of a fertilized egg. The volume of DNA solution injected per egg was of the order of picolitres, and the concentration of DNA used varied

from 1 to 5ng/µl; the re-implantation of viable eggs into pseud-pregnant B6CBAF1 females. B6CBA males were used to generate vasectomized males.

In the present thesis, three rounds of transgenesis were performed - two for the transgene TRE-hapoE3 and one for transgene Tek-Teton.

C. Identification of the founders – the PCR genotyping of the offspring resulted in the first generation and the identification of animals positive for the transgenes. Following the transgenesis experiments, we managed to obtain one founder to be used for starting a line of transgenic mice carrying the TRE-hapoE3 transgene, line that is to be further used to obtain a double transgenic mouse in which the expression of the human ApoE3 can be induced by Dox administration.

GENERAL CONCLUSIONS. This thesis addresses an actual topic in the field of biomedical sciences, namely finding new alternative methods for preventing and slowing down the atherosclerotic process.

This paper proposes a new strategy to target the NADPH oxidase activity in order to improve the vascular production of ROS under conditions of hypercholesterolemia. The results demonstrated that such an approach, in this case targeting signaling pathways that control the function of NADPH oxidases, provides a valid perspective for therapy. Specifically, the modulation of the signaling pathways activated by Jak may represent a new pharmacological strategy to counteract the harmful effects of oxidative stress in atherosclerosis.

The paper also proposes two strategies with respect to the way by which the efflux of lipoprotein particles accumulated in foam cells and in the subendothelial space may be modulated by increasing the expression of apoE, a protein with well-known anti-atherogenic functions. The first strategy identifies vitamin A and retinoids as modulators of apolipoprotein E gene expression in macrophages, and demonstrates that these compounds may induce an increased expression of endogenous apoE gene in macrophages both *in vitro* and *in vivo*. The results suggest that these compounds may have a therapeutic use for the regulation of lipid metabolism and in the prevention and/or the amelioration of atherosclerosis.

The second strategy refers to the induction of apoE gene expression in endothelial cells, and proposes to generate a conditional transgenic murine model in which the expression of the human apoE3 isoform can be induced in a controlled

manner, in the absence of endogenous murine apoE gene. In the present study, the first three stages of the development of such a model were achieved, first by obtaining and successfully testing the necessary transgenes. The transgenesis experiments generated one founder that will further be used to set up a strain of mice transgenic for TRE - hapoE3 to be used to obtain the double transgenic mice in which the expression of human apoE3 can be induced by Dox administration. The creation of this model will enable further investigation regarding the apoE function in atherosclerosis and how an increased concentration of this protein in the plaque, as well as in the circulation, can improve the pathological process.