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Intracellular Cholesterol Retention—New Target for Direct Anti-Atherosclerotic Therapy[*]

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ABSTRACT

Accumulation of cholesterol in arterial cells, intracellular cholesterol retention, may be responsible for all major manifestations of atherosclerosis on a cellular level. Previously we have shown that intracellular cholesterol retention is the principal event in the genesis of atherosclerotic lesions. This allows us to consider cellular retention of cholesterol as a novel target for anti-atherosclerotic therapy. In this case the target is not the level of blood cholesterol but the level of cholesterol in vascular cells. This review describes our approach based on the use of cultured human arterial cells for the development of direct anti-atherosclerotic therapy. We use natural products as the basis of promising drugs for anti-atherosclerotic therapy. Using natural products, we have developed an approach to prevent intracellular cholesterol retention in cultured cells. Our knowledge of the mechanisms of atherosclerosis is the foundation on which we have developed drugs that have a direct anti-atherosclerotic effect, namely Allicor on the basis of garlic powder, anti-inflammatory drug Inflaminat (calendula, elder, and violet) possessing anti-cytokine activity and phytoestrogen-rich drug Karinat (garlic powder, extract of grape seeds, green tea leaves, hop cones, β-carotene, α-tocopherol, and ascorbic acid). Treatment with allicor or inflaminat has a direct anti-atherosclerotic effect on carotid atherosclerosis in asymptomatic men. Karinat prevents the development of carotid atherosclerosis in postmenopausal women. Thus, the main findings of our basic research have been successfully translated into clinical practice. As a result, this translation, a novel approach to the development of anti-atherosclerotic therapy, has been established. Our clinical trials have confirmed the suitability of innovative approach and the efficacy of novel drugs developed on the basis our methodology.

Keywords: Allicor; Anti-Atherosclerotic Therapy; Atherosclerosis; Cell Culture; Drugs; Imaging; Natural Products

1. Introduction

The only hypothesis that has received confirmation in the clinic is the cholesterol hypothesis. This hypothesis was proposed more than 100 years ago by Nikolai Anitschkow. The Anitschkow's hypothesis linked atherosclerosis with high levels of total cholesterol in the blood. The modern paradigm only explains some aspects of this hypothesis, in particular, as atherosclerosis is not associated with the total level of cholesterol but with atherogenic low-density lipoprotein (LDL) cholesterol and anti-atherogenic high-density lipoprotein (HDL) cholesterol [1-3]. Furthermore, the role of different molecules involved in lipoprotein metabolism is discussed.

Retention of intracellular lipids or lipidosis that is the accumulation of cholesterol and other lipids in the arterial cells is the most prominent manifestation of atherosclerosis at the arterial cell level. Retention of intracellular cholesterol is accompanied by increased proliferative activity of vascular cells and increased synthesis of extracellular matrix [4,5]. Both proliferation and fibrosis are characteristic features of atherosclerosis at the arterial cell level, too. Thus, intracellular cholesterol retention, may be responsible for all major manifestations of atherosclerosis on a cellular level. This allows us to consider cellular retention of cholesterol as a novel target for anti-atherosclerotic therapy. In this case the target is not the level of blood cholesterol but the level of cholesterol in vascular cells.

This review summarizes the mechanisms of intracellular retention of cholesterol. We describe our cellular models to search for anti-atherosclerotic agents, and demonstrate our successful attempt to use these models to develop effective anti-atherosclerotic drugs.

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2. Mechanisms of Intracellular Cholesterol Retention

Intracellular retention of cholesterol can be induced by LDL. However, native lipoprotein usually does not increase the cholesterol content of the cell but the incubation of cell cultures with chemically modified LDL results in a massive accumulation of cholesterol in the cells [6]. Thus, modified, but not native, LDL is the source of lipids that accumulate in arterial cells. Cells that populate atherosclerotic lesions are overloaded with lipids, and their cytoplasms are almost completely filled with lipid inclusions [7]. These cells are referred to as foam cells because of foamy appearance under microscope.

In the blood plasma of patients with coronary atherosclerosis we have discovered modified desialylated LDL [8-11]. This naturally occurring LDL induces cholesterol retention in cultured arterial cells [8-11]. Circulating modified LDL is multiple modified lipoprotein possessing lower sialic acid, triglyceride and cholesterol contents; smaller particle size; greater density and negative charge; higher aggregative activity; and some other specific features [12]. In patients' blood we have discovered an enzyme, trans-sialydase, that is responsible for the desialylation of LDL particles in the blood [13].

In addition to desialylated LDL, more electronegative LDL and small dense LDL have been discover in human blood by other researchers [14,15]. A cooperative comparative study showed that the more electronegative LDL isolated by ion-exchange chromatography is desialylated LDL [16]. On the other hand, desialylated LDL isolated from patient blood [8-11] is more electronegative. Desialylated LDL particle is smaller and denser than that of native LDL [17]. On the other hand, small dense LDL isolated from patients has a low content of sialic acid, *i.e.*, it is desialylated [18]. Glycosylation is a type of *in vivo* LDL modification in the blood of patients with diabetes mellitus [19]. This LDL is also atherogenic causing intracellular cholesterol retention [20]. Oxidation is likely a type of atherogenic modification of LDL, however, there is no direct evidence of the presence of oxidized LDL in blood [21].

Modified LDL stimulates anti-LDL auto-antibodies production [22-24]. The interaction of anti-LDL auto-antibodies with modified LDL results in the formation of LDL-containing circulating immune complexes [25]. Multiple modified LDL, which enters the cells as a component of immune complexes, possesses a higher atherogenic potential compared with free modified lipoprotein; that is, it induces a more intense intracellular cholesterol retention [25,26]. We have found LDL-containing circulating immune complexes and anti-LDL auto-antibodies in the blood of most atherosclerotic patients [27-29]. We have demonstrated a positive correlation between the le-

vels of LDL-containing immune complexes and the severity of atherosclerosis [27-31]. LDL is able to form complexes with cellular debris, collagen, elastin, and proteoglycans of the human aortic intima [32-37]. These LDL-containing complexes stimulate intracellular cholesterol retention causing increased uptake and decreased intracellular degradation of lipoproteins in complexes [36]. Naturally occurring multiple modified LDL forms self-associates under cell culture conditions, while native LDL does not associate [35]. We have found a positive correlation between the atherogenic activity of modified LDL and the degree of LDL self-association [35,36]. LDL-associates isolated by gel filtration were shown to induce a dramatic intracellular cholesterol retention. The removal of LDL-associates from the incubation medium by filtration through filters with a pore diameter of 0.1 μm completely prevented intracellular cholesterol retention [36]. We can conclude that the formation of large LDL containing complexes (self-associates, immune complexes, and complexes with connective tissue matrix) is a necessary and sufficient condition for intracellular cholesterol retention.

Our knowledge of mechanisms of intracellular cholesterol retention allowed us to identify possible targets for anti-atherosclerotic therapy. Target 1 is atherogenic modification (desialylation) of the LDL particle in blood. Target 2 is selective removal of modified LDL from blood. Target 3 is the prevention of modified LDL accumulation in arterial cells. Target 4 is the removal of excess lipids from foam cells. We have used all four approaches to anti-atherosclerotic therapy. All of them were effective, however, the most suitable approach is the third, namely, the prevention of modified LDL accumulation in arterial cells. *i.e.* the initial step in intracellular cholesterol retention. We use this approach for the development of anti-atherosclerotic therapy.

3. Development of Anti-Atherosclerotic Drugs Preventing Intracellular Cholesterol Retention Using Cellular Models

We use primary culture of human aortic cells for the screening of potential drugs, the investigation of their mechanisms of action, and the optimization of anti-atherosclerotic drug therapy.

Cells are isolated from the subendothelial part of the human aortic intima between the endothelial lining and the media [38]. Using collagenase and elastase, viable cells are isolated from the subendothelial layer of the intima [39-41]. Isolated cells can be classified as the mixture of smooth muscle cells, pericyte-like cells, and macrophages [38-42]. The culture on which our experiments are performed is represented by this mixed population

[38].

Cells isolated from atherosclerotic lesions retain all major characteristics of atherosclerotic cells when cultured. They are capable of synthesizing collagen, proteoglycans and other components of the extracellular matrix [4]. Cell cultured from fatty lesions have an enhanced proliferative activity [43], higher than that of cells cultured from unaffected intima [43,44]. Considerable part of cells cultured from atherosclerotic lesions are foam cells, which contain numerous inclusions, likely lipid droplets, that fill the entirety of the cytoplasm [40]. Excess lipids in foam cells are mainly free cholesterol and cholesteryl esters [40]. It is important that the content and composition of lipids in cultured cells within the first 10 - 12 days in culture remain unchanged and correspond to the respective indices of freshly isolated cells [40,44]. Thus, our investigations are carried out directly on exactly those cells that require a therapeutic action *in vivo*. Using this model, we have examined the effects of different drugs and chemicals.

The prevention of intracellular cholesterol retention may be regarded as anti-atherosclerotic effect. In terms of arterial cells, any drug effect that does not directly prevent intracellular cholesterol retention is regarded as an indirect anti-atherosclerotic action. Only a drug that exhibits its preventive activity at the arterial level is a direct anti-atherosclerotic drug.

Naturally, the question arises whether the anti-atherosclerotic effects revealed in *in vitro* cellular model can be manifested *in vivo*. To answer this question, an *ex vivo* model was developed. In the *ex vivo* model, instead of agents, blood sera taken from patients after oral drug administration is added to cultured cells.

4. Natural Products

Ex vivo cellular model can be used to test foodstuffs. We have investigated prevention of intracellular cholesterol retention caused by certain mushroom species and sea products.

Extracts from 20 Korean mushroom species exhibit intracellular cholesterol retention revealed by cell culture test [45]. Among sea products, mollusk and krill meat were investigated. Two hours after a single dietary load with canned meat of a mollusk belonging to the genus Buccinum, the patient's blood serum acquired marked anti-atherosclerotic properties (**Table 1**). Incubation of this serum with cultured atherosclerotic cells led to a fall in intracellular cholesterol retention. Patients of another group received a single dietary dose of Antarctic krill meat. Two hours later, the retention of cellular cholesterol induced by blood sera decreased, and four hours later, it was practically absent (**Table 1**).

To develop a dietary therapy based on the krill meat,

Table 1. Effects of sea products on atherosclerosis-related properties of patients' plasma.

Product	Intracellular cholesterol content, μg/mg cell protein			
	Control	0 hours	2 hours	4 hours
Buccinum	196 + 17	204 + 16	181 + 19	150 + 16*
Krill	37 + 5	86 + 4	71 + 7	49 + 4*

*Significant difference from 0 hr ($p < 0.05$). From [46].

the effective dose and proper regimen have been established. The anti-atherosclerotic activity of krill meat was evaluated by the ability to reduce intracellular cholesterol retention. The dose-effect dependence was revealed by comparing the efficacy of the two doses, and we found that krill meat possesses anti-atherosclerotic effects at a dose of 10 - 20 g, half-maximum effect was reached at a dose of 30 g, and the maximum effect was achieved at a dose of 50 g. We believe that this approach will be useful in the development and optimization of anti-aterhosclerotic dietary therapies.

4.1. Botanicals

The anti-atherogenic effects of dietary products promote the development of anti-atherosclerotic therapies based on natural products. Atherosclerosis develops over many years, so anti-atherosclerotic therapies should be long-term or even lifelong. For such long-term therapies, conventional medicine will not work. Drugs based on natural products can be a good alternative.

We have tested numerous natural products' extracts to reveal their effects on blood atherogenicity or their capacity to prevent intracellular cholesterol accumulation caused by atherogenic blood sera from patients. **Table 2** presents only the effective natural products. Naturally, the tested agents included anti-atherogenic, pro-atherogenic, and neutral products. Among the anti-atherogenic natural products, the most effective was garlic.

We investigated the *in vitro* effect of garlic extract on lipids of cultured human aortic cells. Garlic prevented the serum-induced accumulation of free cholesterol and reduced the accumulation of cholesteryl esters [47]. The effect of garlic on the enzymes responsible for the intracellular metabolism of cholesteryl esters was studied. We have shown that garlic inhibits acyl-CoA: cholesterol acyltransferase, which participates in cholesteryl ester formation, and stimulates cholesteryl ester hydrolase, which degrades cholesteryl esters [47].

Further investigations *ex vivo* and *in vivo* confirmed the *in vitro* effects of garlic [48]. These data stimulated us to develop a drug based on garlic powder and carried out a clinical study of the effects of this drug on atherosclerosis regression.

Table 2. Anti-atherosclerotic effects of natural products.

Product	Intracellular cholesterol retention decrease, %
Salsola collina	11
Allium cepa	21
Beta vulgaris	31
Spirulina platensis	51
Pinus sylvestris	52
Triticum vulgaris	70
Glycyrrhiza glabra	55
Allium sativum	**77**

From [46].

4.2. Allicor (Garlic Tablets)

We have developed the time-released garlic powder tablets Allicor, registered and produced by INAT-Farma, Ltd. (Russia). The AMAR study (Atherosclerosis Monitoring and Atherogenicity Reduction) was designed to estimate the effect of two-year treatment with Allicor on the progression of carotid atherosclerosis in asymptomatic men in a double-blinded, placebo-controlled randomized clinical trial (ClinicalTrials.gov Identifier, NCT01734707). The primary outcome was the rate of atherosclerosis progression, measured by high-resolution B-mode ultrasonography as the increase in carotid intima-media thickness (CIMT) of the far wall of common carotid arteries [49].

The overall dynamic of changes in prevention of intracellular cholesterol retention (decrease of blood serum atherogenicity) is presented in **Figure 1**. At the baseline, blood serum taken from patients induced 1.56-fold increase in intracellular cholesterol content in cell culture test. In the placebo group, the mean level of serum induced intracellular cholesterol retention did not change significantly during two years. On the opposite, in Allicor-treated patients the mean value for intracellulare cholesterol retention was significantly lowered already after first 3 months of study, and this effect was maintained during the study. We found statistically significant difference in the dynamic of changes in intracellular cholesterol retention between Allicor-treated and placebo groups.

Allicor significantly reduced CIMT compared to baseline and the placebo group. While spontaneous atherosclerosis progression prevailed in the placebo group, allicor beneficially affected early carotid atherosclerosis, significantly increasing lesion regression and reducing the net number of progressive lesions. The CIMT measures were significantly different from the baseline measures and from the placebo group after the first 12 months of treatment [49]. After two years the difference between the placebo and allicor recipients increased and remained

statistically significant. The overall lesion progression was clearly different in the treated and untreated patients (**Figure 2**).

Our data are generally consistent with the results of a double-blinded, placebo-controlled randomized study by Koscielny *et al*. [50]. That study has been demonstrated that 4-year treatment with the garlic-based drug Kwai inhibited the increase in volume of atherosclerotic plaques in carotid and femoral arteries by 5% - 18%.

Table 3 demonstrated that the decrease in CIMT that was achieved during the AMAR study is comparable with the results of most successful trials with other compounds [51-57]. Those studies employed potent lipid-lowering agents or calcium antagonists, whose beneficial effects of treatment were attributed to reduction in LDL
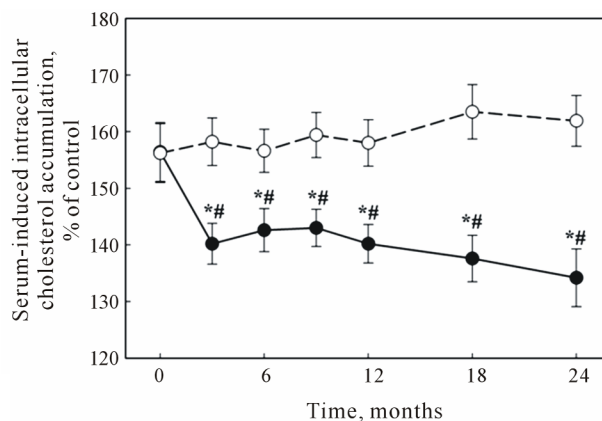
Figure 1. The dynamics of serum atherogenicity changes. Solid circles, Allicor-treated patients; open circles, placebo patients. *significant IMT change as compared to baseline, P < 0.05; #significant difference from placebo group, P < 0.05. From [49].
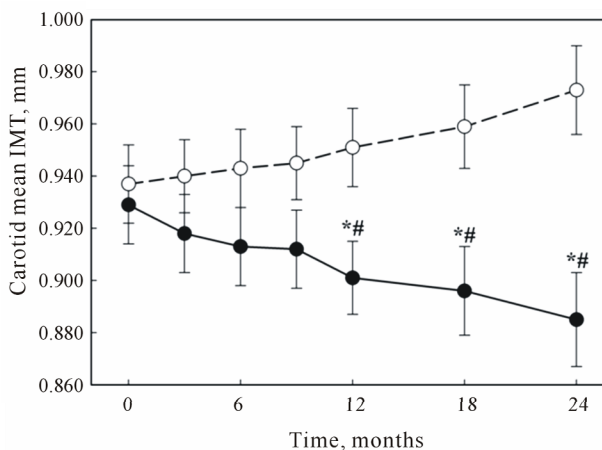
Figure 2. Anti-atherosclerotic effects of allicor. The dynamics of IMT changes. Solid circles, Allicor-treated patients; open circles, placebo patients. *significant IMT change as compared to baseline, P < 0.05; #significant difference from placebo group, P < 0.05. From [49].

Table 3. The comparative data from clinical trials on carotid atherosclerosis regression.

Trial		Mean annual IMT change, mm		Reference
	Medication	Placebo	Treatment	
PLAC II	Pravastatin	0.068	0.059	[51]
KAPS	Pravastatin	0.029	0.010	[52]
ASAP	Simvastatin	-	−0.009	[53]
PREVENT	Amlodipine	0.011	−0.015	[54]
ASAP	Atorvastatin	-	−0.020	[53]
CLAS	Cholestipol, Niacin	0.010	−0.020	[55,56]
MARS	Lovastatin	0.015	−0.028	[56,57]
VHAS	Verapamil	-	−0.028	[58]
AMAR	**Allicor**	**0.015**	**−0.022**	**[49]**

From [46].

cholesterol, the major risk factor for atherosclerosis development, or arterial wall stress.

The results of our study demonstrate that long-term treatment with the time-released, garlic-based drug allicor provides a direct anti-atherosclerotic effect on carotid atherosclerosis. As a natural drug, allicor is safe with respect to adverse effects and allows even perpetual administration, which may be quite necessary for the prevention and treatment of subclinical atherosclerosis. Effects of allicor promoted the start of clinical trials of two other drugs based on natural products: Inflaminat, which possesses anti-cytokine activity, and the phytoestrogen-rich drug Karinat, which is designed for postmenopausal women.

4.3. Inflaminat (Calendula, Elder, Violet)

Atherosclerosis is accompanied with elements of local aseptic inflammation, and inflammatory cytokines play a role at every stage of atherosclerosis development [59-61]. Thus, anti-inflammatory drugs may be effective for the prevention of atherosclerosis. Natural drugs are suitable for the early prevention of atherosclerosis because they have almost no side effects and exert regulatory effects at physiological limits, allowing longer, almost life-long, medication. We investigated the atherosclerosis regression effect of the natural drug "Inflaminat", which is based on calendula, elder and violet. Our laboratory investigation demonstrated that these botanicals suppress secretion of pro-inflammatory cytokines. We have carried out a pilot study (ClinicalTrials.gov Identifier, NCT01743404) with inflaminat using a protocol similar to that of the AMAR study. Inflaminat demonstrated atherosclerosis regression effects and a statistically significant difference from the baseline as well as from placebo group [49].

Table 4 demonstrates the anti-atherosclerotic effect of inflaminat in asymptomatic men.

4.4. Karinat (Phytoestrogen-Rich Combination)

Modern medicine does not provide an effective approach to atherosclerosis prevention in postmenopausal women. Hormone replacement therapy is not acceptable as a tool for atherosclerosis prevention in women due to the negative results of clinical studies, including WHI, PEPI, and HERS [62-67]. Phytoestrogens may be regarded as a possible alternative to hormone replacement therapy, but practically nothing is known about their effects on atherosclerosis.

We selected phytoestrogen-rich botanicals on the basis of their ability to prevent intracellular cholesterol retention using the above described *ex vivo* test system. The following botanicals were chosen: garlic powder, extract of grape seeds, green tea leaves, and hop cones, all of them produced significant anti-atherogenic effects. This combination of botanicals was used for development of novel isoflavonoid-rich dietary supplement "Karinat". Karinat prevents intracellular cholesterol retention and is characterized by an improved phytoestrogen profile, providing additional amounts of biologically active polyphenols, including resveratrol, genisteine, and daidzeine, which are claimed to exert effects on atherosclerosis development. In addition, karinat contains additional amounts of β-carotene, α-tocopherol and ascorbic acid to provide the necessary daily intake of antioxidants.

We have carried out a randomized, double-blinded, placebo-controlled pilot clinical study on the anti-atherosclerotic effects of karinat in healthy postmenopausal women to characterize the risks and benefits of phytoestrogen therapy in relation to atherosclerosis progression (ClinicalTrials.gov Identifiers, NCT01741974 and NCT01742000). The annual rate of changes in common carotid artery intima-media thickening was monitored. The effects of karinat treatment on the dynamics of carotid atherosclerosis in postmenopausal women are demonstrated in **Table 5**. The rate atherosclerosis in postmenopausal women was extremely high: the average increase in IMT was 13% per year, and the growth of athe-

Table 4. CIMT changes in 1-year inflaminat pilot study.

	Inflaminat	Placebo	p[**]
Number of participants	81	77	-
IMT change, μm	−62 ± 48[*] (−91; −32) p = 0.002	42 ± 75 (−9; 93) p = 0.109	0.002

[*]significant differences, p < 0.05, Wilcoxon's signed ranked test; [**]statistical significance of differences was estimated by Mann-Whitney U-test. From [49].

Table 5. CIMT changes in 1-year karinat pilot study on postmenopausal women.

	Karinat	P	Placebo	P
Number of participants	80	-	77	-
IMT change, μm	+6 (85)	NS	+111 (91)	P < 0.02
Plaques, scores	+0.21 (0.59)	0.009	+0.31 (0.55)	<0.001

From [49].

rosclerotic plaques was 40% per year. In the karinat group the average CIMT was not changed (statistically insignificant increase of 6 μm per year, less than 1%). The progression of existing plaques was slower by 32% per year.

Thus, the use of the phytoestrogen-rich combination in postmenopausal women almost completely suppresses the formation of new atherosclerotic lesions, and it slows the progression of existing lesions [49].

5. Conclusion

Our basic studies have shown that intracellular cholesterol retention is the principal event in the genesis of atherosclerotic lesions. On the basis of the results of our basic research we developed cellular models and an approach to prevent intracellular cholesterol retention. Prevention of intracellular cholesterol retention led to the prevention of atherosclerosis progression and/or the regression in patients. We can conclude that our basic findings were successfully translated into clinical practice.

Two-year treatment with allicor (garlic powder) has a direct anti-atherosclerotic effect on carotid atherosclerosis. Anti-cytokine combination inflaminat (calendula, elder and violet) caused the regression of carotid atherosclerosis in a 1-year treatment of asymptomatic men. The phytoestrogen-rich combination karinat (garlic powder, extract of grape seeds, green tea leaves, hop cones, β-carotene, α-tocopherol and ascorbic acid) prevented the development of carotid atherosclerosis in postmenopausal women.

Unfortunately, natural products with anti-atherosclerotic therapeutic potential are not prescribed by medical practitioners as anti-atherosclerotic agents. However, our data allow us to consider botanicals as mainline additional supplements or prescriptions [68].

6. Acknowledgements

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