Chapter 2

Intracellular Cholesterol Lowering as Novel Target for Anti-Atherosclerotic Therapy

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Additional information is available at the end of the chapter

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Abstract

Atherosclerosis and disorders associated with cardiovascular system remain the major problem of modern medicine and the leading cause of mortality in developed countries. According to the current knowledge, atherosclerosis development can begin early in life. Clinically silent early-stage lesions can be detected in a large population of young adults. Despite substantial progress in the recent years, therapy of atherosclerosis mostly remains limited to plasma lipid profile correction. Moreover, no therapy is currently available for the treatment of asymptomatic early stages of the disease. The existing synthetic drugs could not be used for this purpose, because of the unfavourable risk/benefit ratio and high cost of treatment, which has to be long-lasting. In this regard, medications based on natural agents with anti-atherosclerotic activity may offer interesting possibilities. Current research should focus on detection and evaluation of such agents. One of the important tools for anti-atherosclerotic drug evaluation is a cell-based model, which allows measurement of intracellular lipid accumulation. Anti-atherosclerotic activity of various substances can therefore be evaluated by the decrease of intracellular lipid storage. In this chapter, we will discuss the development and application of cellular models based on primary culture of human arterial wall cells that are suitable for detection and measurement of anti-atherosclerotic activity of various substances. Using these models, several natural agents have been successfully evaluated, which led to the development of pharmaceutical products with anti-atherosclerotic activity based on botanicals.

Keywords: atherosclerosis, arteries, cholesterol accumulation, cellular models, anti-atherosclerotic drugs

1. Introduction

Atherosclerosis remains one of the most challenging problems of modern medicine. Epidemiological data on atherosclerosis and cardiovascular diseases are frequently updated and demonstrate an increase in overall mortality, partly because of the ageing of human population, especially in favourable economic conditions [1]. In developed countries, cardiovascular diseases remain the primary cause of overall morbidity and mortality [2]. Atherosclerotic lesions develop in the walls of large arteries and cause occlusion of blood vessels as a result of either arterial wall thickening or thrombus formation on the surface of unstable plaques. This latter condition is especially dangerous, since it can lead to a sudden and often fatal thromboembolia, which represents the first clinical manifestation of atherosclerosis in many patients. By contrast, early stages of the disease usually pass unnoticed. Recent studies have demonstrated that asymptomatic atherosclerosis is, in fact, a widespread condition among young adults [2–5]. In this cohort of subjects, the incidence of atherosclerotic lesions reaches 100%, although no clinical manifestations can be observed [3–5].

The development of atherosclerosis is a complex process, which, despite the significant progress made during the last decade, still remains to be fully understood. Atherosclerosis and related cardiovascular disorders are associated with several known risk factors, including elevated plasma cholesterol level, diabetes, tobacco smoking and others [6, 7].

Modern atherosclerosis prevention strategies are largely based on elimination or attenuation of relevant risk factors, which slows down the atherosclerotic plaque progression in an indirect way [8]. For instance, statins are commonly used for plasma cholesterol reduction and attenuation of atherosclerosis progression. However, limited indications and serious side effects make statins unsuitable for preventive therapy of atherosclerosis, which has to be long-term. Currently, there exists no widespread "direct" anti-atherosclerotic therapy that could be suitable for treatment of the early, subclinical stages of the disease. Such therapy should target the molecular and cellular mechanisms of atherogenesis at the level of blood vessel wall and should result in prevention of *de novo* lesion formation or regression of existing plaques [8–10]. Natural agents appear to be attractive candidates for preventive anti-atherosclerosis therapy because of their favourable safety profile and low cost. Because of their complex composition, biologically active substances of botanical origin and their combinations may have a wider range of effects than synthetic drugs, targeting several atherosclerosis risk factors simultaneously. It is therefore possible that the botanical substances can possess both direct and indirect anti-atherosclerotic effects, such as protective activity at the cellular level combined with cholesterol lowering and hypotensive activity. Current knowledge of cardioprotective effects of natural agents and nutraceuticals is rather limited, although they have been actively studied by several groups during the recent years [11–17]. It is important to establish novel anti-atherosclerotic preventive therapies based on natural products and confirm their effectiveness by clinical studies.

The search for potential anti-atherosclerotic agents and evaluation of their activity requires adequate test models. Lipid accumulation is one of the most prominent features of atherosclerotic lesions. Lipid uptake and storage are performed by several cell types of the arterial

wall. Both resident cells and inflammatory cells that are recruited to the lesion site can participate in the process. Increased lipid content can be observed already at the earliest stages of the plaque development. The main source of cholesterol deposit in the arterial wall is low-density lipoprotein (LDL), especially its modified, atherogenic forms. The risk of atherosclerosis development has been demonstrated to be associated with unfavourable plasma lipid profile and the increased contents of atherogenic LDL types, such as small dense LDL [18]. The ability of the blood plasma to cause lipid accumulation in the arterial wall cells is referred to as blood serum atherogenicity [19]. Anti-atherosclerotic effect of a substance can be evaluated by its ability to prevent lipid accumulation in cultured arterial wall cells induced by the exposure to atherogenic LDL. Importantly, lipid profile in cells with or without treatment can precisely be measured to quantitatively evaluate anti-atherosclerotic potential.

In this chapter, we will give an overview of current knowledge on atherosclerotic lesion progression and discuss the development and application of models based on primary culture of human arterial wall cells.

2. Atherosclerotic plaque development

According to the classic lipid theory of atherogenesis, atherosclerotic lesion development is caused by extracellular and intracellular lipid accumulation in the intimal layer of the arterial wall [20, 21]. It has been shown that the major source of lipid accumulation in the intimal cells is circulating LDL, especially its atherogenic forms, such as chemically modified and aggregated LDL. Chemical modification of lipoprotein particles appears to be necessary for the atherogenic effect, since native (non-modified) LDL added to cultured cells could not induce significant lipid accumulation. Atherogenic modifications of LDL in the bloodstream include desialylation, acquisition of negative charge and increase of the particle hydrated density (small dense LDL formation). All these modifications can be accompanied by oxidation [22–25]. Study of the atherogenic LDL modification in the bloodstream currently remains challenging. Different laboratory methods of LDL isolation, quantification and analysis deliver different results, which hinders direct comparison of studies employing different methods and protocols. For instance, analysing LDL size and density by ultracentrifugation in different buffers will give slightly different outcome. Moreover, no consensus has been reached so far on the classification of LDL subfractions [22]. It is likely that LDL particles undergo multiple atherogenic modification in human plasma, but the resulting products are differently evaluated by different methods from several laboratories [26–28]. One of the earliest atherogenic modifications demonstrated to occur in human bloodstream is desialylation. The removal of sialic acid residues from the carbohydrate components of LDL particles is performed by trans-sialidase, which is active in the bloodstream. Increased level of circulating modified LDL leads to aggregation of the particles, which is facilitated by increased surface charge. The resulting large complexes have especially high atherogenic potential. Moreover, modified forms of LDL can induce formation of autoantibodies triggering inflammatory response and giving rise to circulating immune complexes. Another feature that can significantly increase atherogenic potential of modified LDL is its ability to associate with the components of extracellular matrix

proteins in the subendothelial space of the arterial wall, which prolongs its residence time and facilitates lipid accumulation. Unlike native LDL, which is internalized by cells via receptor-mediated uptake, modified LDL complexes enter the cells through uncontrolled phagocytosis and follow a distinct metabolic pathway [29]. This can explain the rapid accumulation of atherogenic modified LDL in cellular cytoplasm, mostly in the form of lipid droplets. Cells containing large amounts of lipid inclusions in the cytoplasm are called "foam cells" because of their microscopic appearance. Such cells commonly occur in atherosclerotic lesions.

Figure 1 shows the development of atherosclerotic lesions and the main stages of the atherogenesis [30]. According to the current knowledge, atherosclerotic lesion initiation is dependent on two conditions: the presence of modified atherogenic LDL in the bloodstream in sufficient quantities and the internalization of LDL by the arterial wall cells. The latter is usually triggered by local disturbance of endothelial function that causes increased permeability of the endothelial lining allowing modified LDL to penetrate into the intimal layer of the arterial wall. Atherogenic modification of LDL may also occur in the intimal layer, after the particles have crossed the endothelial barrier. Local disturbances of endothelial function frequently take place in certain parts of the vascular system, such as branching points and bends, where laminar blood flow is altered [31]. Sites of the arterial wall that are especially vulnerable are marked by altered morphology of endothelial cells and presence of enlarged multinucleated cells. The pre-existent mosaicism of the endothelial lining may explain the focal development of atherosclerotic lesions. However, more studies are needed to determine the mechanisms of endothelial dysfunction leading to atherosclerosis.

Focal lipid infiltration into the arterial wall intima marks the early stages of atherosclerotic lesion development. Apparently, several cell types of the arterial wall participate in lipid accumulation. Cells populating the intimal layer can be either resident mesenchymal cells,

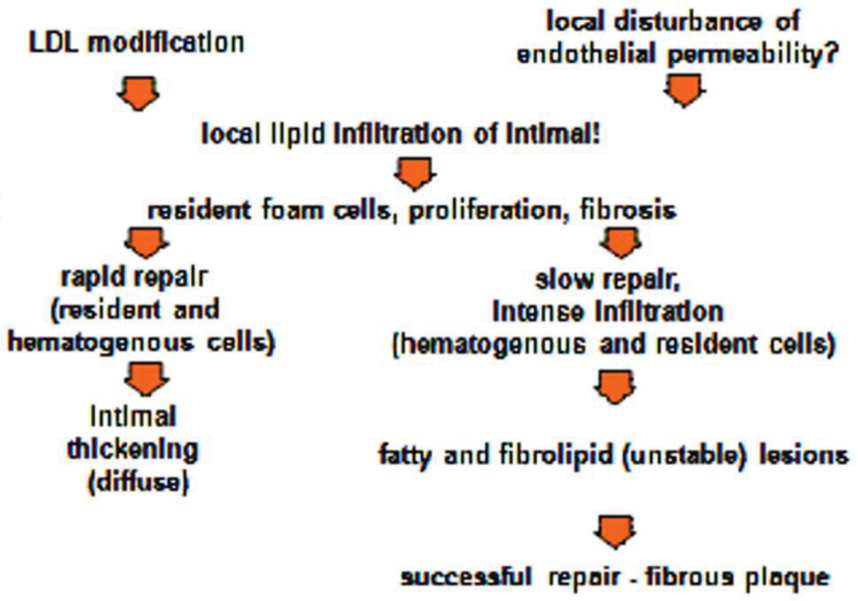

Figure 1. Scheme showing the consecutive events in the development of atherosclerotic lesions. Reproduced with permission from [30].

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such as smooth muscle cells, or inflammatory cells, such as monocytes/macrophages, that can be recruited from the bloodstream in large numbers by a local inflammatory response. Along with macrophages, smooth muscular cells also take part in lipid uptake and can be transformed into foam cells. While native LDL particles are metabolized by intimal cells through a well-developed and controlled receptor-mediated endocytosis, it is likely that the LDL associations are recognized by macrophages as pathogens that have to be cleared by phagocytosis [32]. Such clearance is accompanied by secretion of signalling molecules that attract immune cells to the developing lesion site and therefore initiation of the inflammatory process [33]. Phagocytosis-mediated lipid accumulation in atherosclerosis can therefore be regarded as a variation of innate immune response. Enhanced phagocytosis followed by lipid accumulation and foam cell formation contributes to lesion development. Lipid accumulation affects intercellular contacts that are essential for proper function of intimal wall resident cells [34]. On the other hand, lipid accumulation also triggers processes that are typical for the reparative phase of inflammation, such as proliferation and extracellular matrix synthesis leading to the fibrosis. In favourable conditions, these reparation processes rapidly lead to formation of areas with increased cellularity and extracellular matrix deposition. Gradual development of such focal lesion areas leads to a diffuse intimal thickening, which is frequently observed in adult arteries. However, the inflammatory response can become chronic, with continuous local lipid infiltration, increased cellularity due to the proliferation of cells in the lesion site and enhanced fibrosis.

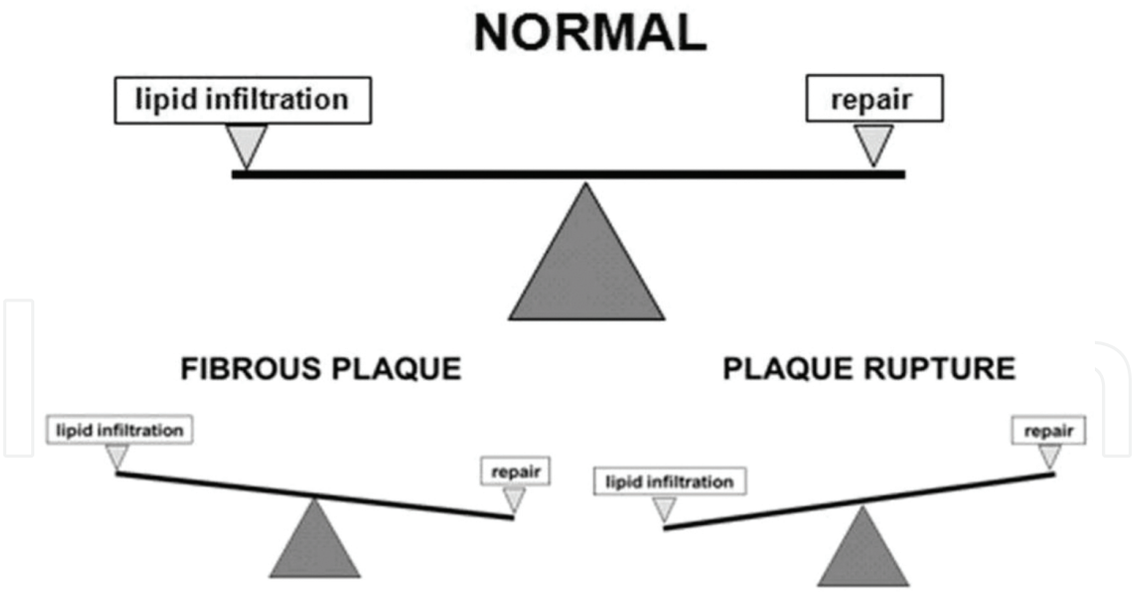

Figure 2. Scheme showing the delicate balance between infiltrative and reparative phases in fatty atherosclerotic lesion. Reproduced with permission from [30].

Atherosclerotic plaques can be protected from the bloodstream by formation of a fibrous cap, which serves as a barrier for lipoproteins and inflammatory cells. Such isolation of the local inflammatory site has a protective role, suppressing the inflammatory response and restoring

the tissue functions. On the other hand, formation of fibrolipid plaques predisposed to rupture (unstable plaques) can have fatal consequences because of thrombus formation.

In fibrolipid plaques, two opposing processes are likely to take place: infiltration and reparation that exist in a state of unstable equilibrium (**Figure 2**). Shifting the balance towards reparation leads to the formation of fibrous plaques, which is a favourable outcome from the clinical point of view. Inefficient reparation and continuous lipid infiltration cause plaque rupture with possible thrombus formation. Lipidosis plays therefore a crucial role in atherosclerotic lesion development at cellular and tissue levels and represents an important target for the development of anti-atherosclerotic therapy.

3. Evaluation of substances' anti-atherosclerotic activity using cellular models

Preventive anti-atherosclerotic therapy should be aimed at reduction of intracellular lipid accumulation [35]. Such reduction can be achieved by different approaches [36]. First, the therapy may decrease the level of circulating modified LDL. Second, it can target atherogenic modification of LDL in the bloodstream. Third, it can reduce lipid uptake and storage by the arterial wall cells. Finally, the therapy can be aimed at depletion of the existing intracellular lipid stores. All these approaches can be evaluated by measuring the reduction of intracellular lipid accumulation and the decrease of the intracellular pool of cholesterol esters [9, 37, 38]. A number of available medications can be used to decrease blood serum atherogenicity [9, 36, 38, 39], which is defined as the ability of blood serum to induce cholesterol accumulation in cultured cells. Blood serum from patients with coronary atherosclerosis usually has high atherogenicity [19]. Changes of blood serum atherogenicity reflect lipid accumulation in the arterial wall and are therefore relevant for the development of preventive therapy. Such changes can be detected using cultured cells as models of early stages of human atherogenesis [9, 38, 40]. Cellular models can be used for evaluation of anti-atherosclerotic potential of different drugs and active substances, for screening of potential anti-atherosclerotic agents and for evaluation of potential clinical efficacy of various molecules.

4. *In vitro* model

In vitro model based on primary culture of human aortic wall cells was developed for screening of potential anti-atherosclerotic substances. Cells were isolated from the subendothelial layer of healthy human aortic intima, the layer of the arterial wall, which is most severely affected in atherosclerosis [41]. The process of cell isolation from autopsy material using collagenase and elastase treatment has been described previously [9, 42–44]. The obtained cell population has been characterized using immunocytochemistry methods and was found to be heterogeneous and containing smooth muscle cells (20–50%), pericytes (30–70%) and inflammatory cells and tissue macrophages (10%) (**Table 1**) [9, 43, 44].

Smooth muscle α-actin$^+$	3G5$^+$	2A7$^+$	CD45$^+$	CD68$^+$
89.6 ± 6.7%	45.8 ± 10.9%	24.1 ± 9.9%	3.6 ± 0.4%	5.2 ± 1.3%

Table 1. Proportion of cell types in primary culture cells isolated from human aortic subendothelial intima (% of positive cells for each marker).

Substance	References
Anti-atherosclerotic	
Cyclic AMP	[9, 44, 46–49]
Prostacyclin	[9, 50–54]
Prostaglandin E_2	[9, 52, 55]
Artificial HDL*	[56]
Antioxidants	[9]
Calcium antagonists	[9, 51, 57–59]
Trapidil and its derivatives	[60, 61]
Lipoxygenase inhibitors	[55]
Lipostabil	[9]
Mushroom extracts	[62]
Pro-atherogenic	
Beta blockers	[58, 63]
Thromboxane A_2	[51, 55]
Phenothiazine	[58]
Indifferent	
Nitrates	[58]
Cholestyramine	[58]
Sulfonylureas	[64]

[a] HDL, high-density lipoprotein.

Table 2. Substances that have been tested *in vitro* cell model.

Smooth muscle cells and pericytes were positive for smooth muscle α-actin. Pericytes had a distinct stellate shape and were identified using antibodies to 3G5 and 2A7 that are expressed by resting and activated pericytes, respectively. Together, smooth muscle cells and pericytes represented the majority of cell population in the obtained primary cultures. A smaller population consisted of the inflammatory cells that could be detected using antibodies to leukocyte-specific marker CD45 and macrophage marker CD68 [45]. Cellular lipid accumula-

tion was induced by incubation of cells with atherogenic serum obtained from patients with confirmed atherosclerosis. The increase of cellular cholesterol content reached as high as two folds after a 24-h incubation with atherogenic serum.

Potential anti-atherogenic substances were evaluated by concomitant incubation of cells with atherogenic serum and aqueous solutions of tested substances. Anti-atherosclerotic effect was measured as a decrease in the levels of intracellular cholesterol in the cells with test substances compared to the control cells (treated with atherogenic serum only). The described model allowed evaluating a number of different drugs and substances and detecting several novel active molecules with anti-atherosclerotic potential. Some substances were demonstrated to possess a pro-atherogenic effect, enhancing intracellular cholesterol accumulation induced by atherogenic serum (**Table 2**).

5. *Ex vivo* model

Ex vivo model is based on primary culture of cells from unaffected human aortic intima that are incubated with blood serum from patients treated with the substance of interest. Therefore, potential anti-atherogenic properties of substances are evaluated based on their pharmacodynamic properties, or the influence on blood serum atherogenicity after digestion and possible metabolic modifications in patient's body. Blood samples are drawn before and after administration of single doses of tested substances, and serum obtained from the samples is added to cultured primary cells. *Ex vivo* model can be used for testing drugs with known safety profiles, as well as various natural products.

Several studies have demonstrated successful application of this model for evaluation of anti-atherogenic properties of botanicals. Screening studies were performed on volunteers (groups of 4–8 men and women 45–60 years old) with high blood serum atherogenicity. One of the tested natural products with anti-atherosclerotic properties was encapsulated onion (*Allium cepa*) bulb powder (300 mg) (**Figure 3**). Administration of a single dose of the product resulted in a moderate decrease of blood serum atherogenicity by 12, 28, and 24% from the baseline after 2, 4, and 6 h, respectively. Another tested natural product with anti-atherosclerotic properties was preparation of wheat seedlings (*Triticum aestivum*). Administration of a single dose of 300 mg of the preparation resulted in a pronounced reduction of blood serum atherogenicity after 4 h (**Figure 4**). Moderate but prolonged anti-atherosclerotic effect was registered for dry beet (*Beta vulgaris*) juice (encapsulated preparation of 300 mg) (**Figure 5**). Garlic (*Allium sativum*) powder possessed a strong and prolonged effect (**Figure 6**). Blood serum atherogenicity was completely suppressed 4 h after administration of a single dose of 300 mg of the preparation. Several other natural products were screened for potential anti-atherosclerotic activity using the *ex vivo* model (**Table 3**). The highest activity after a single dose administration was detected for garlic powder and wheat seedlings, with garlic powder providing the strongest effect. Importantly, anti-atherosclerotic effects of garlic have been reported by several independent groups during the recent years [65–67].

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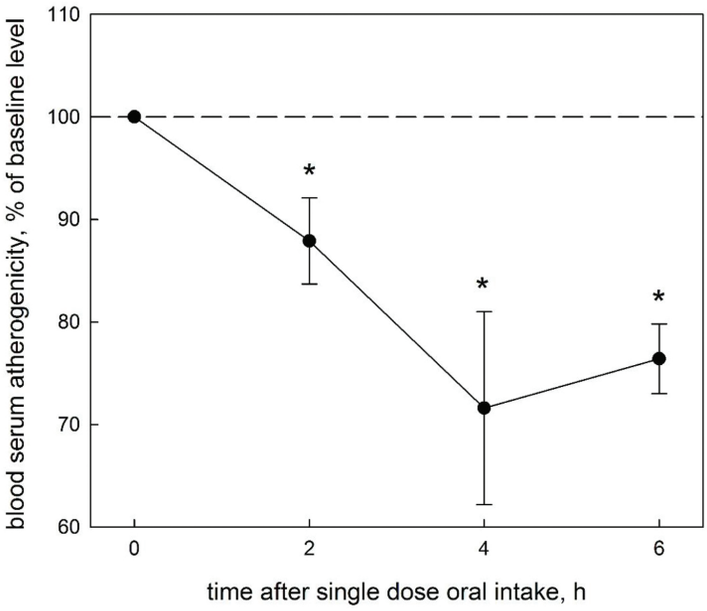

Figure 3. Anti-atherosclerotic effect of onion in *ex vivo* model.The study involved four volunteers (three males, one female, mean age 57 ± 5 years) whose blood serum induced 1.3–1.5-fold increase in cholesterol content of cells cultured from unaffected human aortic intima (the average level of serum atherogenicity was 141 ± 4%). Intracellular cholesterol in control cultures was 38.4 ± 1.1 mg/mg cell protein. Baseline serum atherogenicity was taken as 100%. The average values of changes of serum atherogenicity with indication of standard errors are presented. Reproduced with permission from [30].

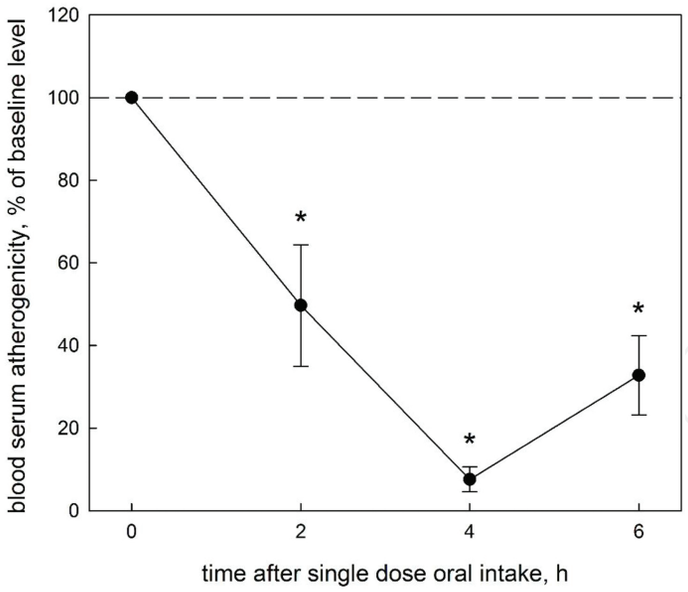

Figure 4. Anti-atherosclerotic effect of wheat seedlings in *ex vivo* model.The study involved eight volunteers (five males, three females, mean age 51 ± 2 years) whose blood serum induced 1.7–2.3-fold increase in cholesterol content of cells cultured from unaffected human aortic intima (the average level of serum atherogenicity was 199 ± 6%). Intracellular cholesterol in control cultures was 28.0 ± 1.2 mg/mg cell protein. Baseline serum atherogenicity was taken as 100%. The average values of changes of serum atherogenicity with indication of standard errors are presented. *, Significant decrease of serum atherogenicity, $p < 0.05$. Reproduced with permission from [30].

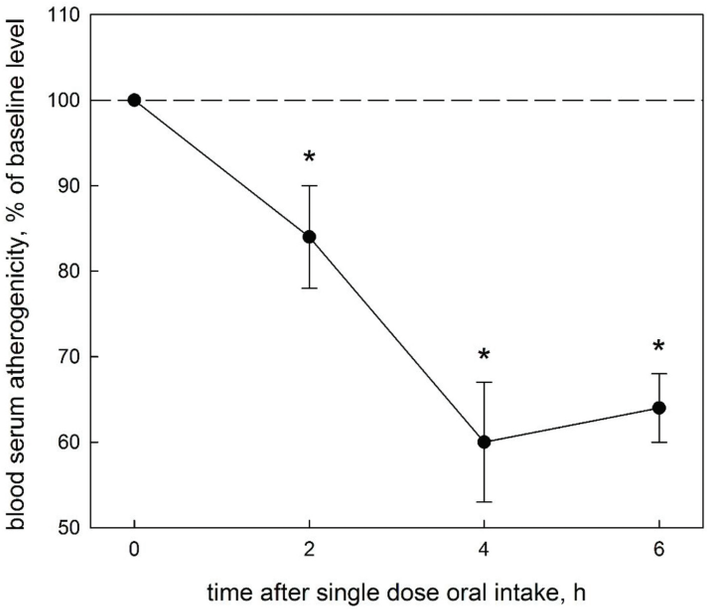

Figure 5. Anti-atherosclerotic effect of beet juice in *ex vivo* model.The study involved eight volunteers (six males, two females, mean age 53 ± 5 years) whose blood serum induced 1.3–2.2-fold increase in cholesterol content of cells cultured from unaffected human aortic intima (the average level of serum atherogenicity was 161 ± 8%). Intracellular cholesterol in control cultures was 37.0 ± 3.6 mg/mg cell protein. Baseline serum atherogenicity was taken as 100%. The average values of changes of serum atherogenicity with indication of standard errors are presented. *, Significant decrease of serum atherogenicity, $p < 0.05$. Reproduced with permission from [30].

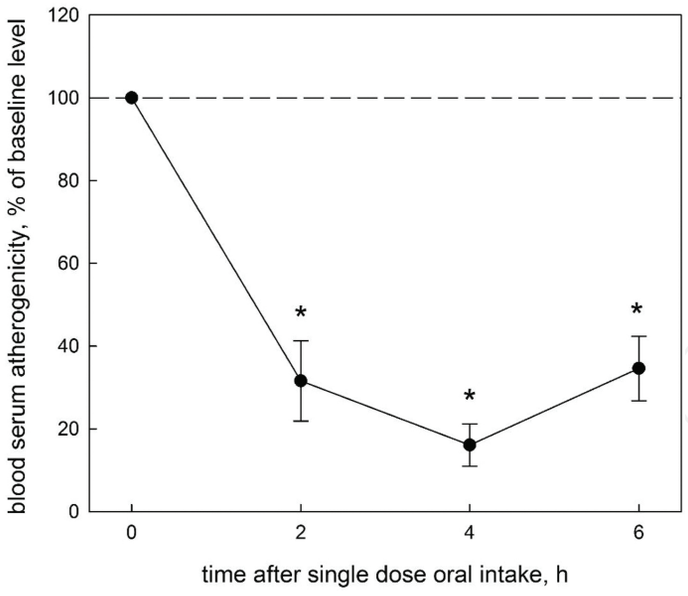

Figure 6. Anti-atherosclerotic effect of garlic powder in the *ex vivo* model.The study involved eight volunteers (six males, two females, mean age 53 ± 5 years) whose blood serum induced 1.3–2.7-fold increase in cholesterol content of cells cultured from unaffected human aortic intima (the average level of serum atherogenicity was 164 ± 9%). Intracellular cholesterol in control cultures was 39.0 ± 4.2 mg/mg cell protein. Baseline serum atherogenicity was taken as 100%. The average values of changes of serum atherogenicity with indication of standard errors are presented. *, Significant decrease of serum atherogenicity, $p < 0.05$. Reproduced with permission from [30].

Botanical and its source	The mean efficiency of atherogenic reduction (%)	Maximum effect (%)
Spirulina platensis powder	50.7	61
Onion (*Allium cepa*) bulb powder	21.4	28
Beet (*Beta vulgaris*) juice powder	30.7	40
Wheat (*Triticum vulgaris*) seedlings powder	70.0	100
Licorice (*Glycyrrhiza glabra*) root powder	54.6	32
Salsola collina leaf powder	10.9	28
Garlic (*Allium sativum*) bulbs powder	76.6	100
Pine (*Pinus sylvestris*) needles extract	52.1	62

*The integrated effect was calculated as a mean reduction in serum atherogenicity for 6 h after a single oral dose.

Table 3. Integral estimation of anti-atherogenic actions of natural products*.

The described *ex vivo* model could be used for establishing the effective dose and posology of the potential anti-atherosclerotic natural products. For this purpose, blood samples were drawn before and after (2 and 4 h) administration of a single dose to patients with high blood serum atherogenicity. Dose dependency was tested by comparison of the effect of two different doses. Each dose was evaluated on at least six different study participants. It was demonstrated that the anti-atherosclerotic effect of garlic powder was present in the dose range from 50 to 300 mg with half-maximal effect observed at a dose of 100 mg, and maximal effect—at 150 mg. Therefore, natural products of botanical origin can be regarded as an important source of agents with anti-atherosclerotic activity that can be used for the development of direct anti-atherosclerotic therapy. Based on the obtained results, several dietary supplements were registered and further evaluated in clinical studies presented below.

As any model, cellular models for studying atherosclerosis development have their limitations [68–71]. Limitations of the experimental models used for atherosclerosis research have been discussed in a number of comprehensive reviews [72–77]. However, the described test system allows performing the initial screening for anti-atherosclerotic activity that can be further studied and confirmed in pre-clinical and clinical studies.

6. Clinical studies

Tests on cellular models demonstrated that garlic powder preparations possessed a pronounced anti-atherosclerotic activity. Based on the obtained results, a garlic-based dietary supplement (Allicor, INAT-Farma, Russia) was developed. The effect of the supplement on carotid intima-media thickness (cIMT) was evaluated in an open-label prospective pilot study conducted on 28 men (46–58 years old, mean age 52.0, SD = 9.0). The study participants had no signs of coronary heart disease, no chronic diseases requiring treatment with vasoactive drugs,

diuretics, lipid-lowering or antidiabetic drugs and were normolipidemic or mildly hyperlipidemic. Study subjects were analysed for presence of diffuse intimal thickening by ultrasound imaging of common carotid arteries [65]. The cut-off cIMT value of 0.7 mm in the distal segment of at least one common carotid artery was set up to diagnose diffuse intimal thickening. The mean cIMT value at the baseline was 0.832 ± 0.024 mm. Study participants were divided into two groups. Subjects from Allicor group (n = 16) received 600 mg of Allicor daily, and subjects from the control group (n = 12) received no treatment. The total duration of the study was 12 months, with interviews and ultrasound assessment of cIMT every 3 months. No adverse effects were observed during the follow-up period, and the product was demonstrated to have good tolerability. The results of cIMT assessments are presented on **Figure 7**.

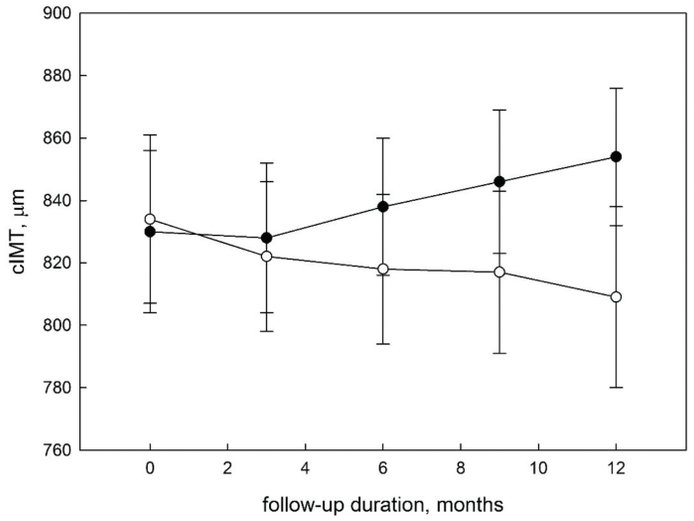

Figure 7. The effects of garlic-based drug Allicor on atherosclerosis determined by cIMT. Open circles, Allicor recipients; solid circles, control subjects. Presented are mean values ±S.E.M. Reproduced from [30].

No statistically significant changes of cIMT were observed after 12 months, and the value was not significantly different between the two groups. However, regression analysis revealed a significant difference between the trends of cIMT dynamics ($p < 0.05$). In the control group, a tendency to cIMT increase was detected, which was significantly different from that of null hypothesis of no change (F-test, 31.72; $p = 0.011$). In the Allicor-treated group, the tendency to cIMT decrease was revealed, which was also significantly different from that of null hypothesis (F-test, 28.81; $p = 0.013$). These results indicate that treatment with Allicor may potentially halter the development and induce the regression of subclinical atherosclerosis. The statistical power of this pilot study was insufficient to avoid type 2 error. Therefore, the pilot study was followed by a larger prospective clinical study, in which a number of clinical and biochemical parameters associated with atherogenesis were taken into account. The dynamic of serum atherogenicity was also assessed. This double-blind placebo-controlled clinical study evaluated the effect of garlic powder tablets Allicor on the progression of cIMT in 211 men (40–74 years old) with no symptoms of atherosclerosis (ClinicalTrials.gov identifier, NCT01734707). The primary outcome was the progression of subclinical atherosclerosis evaluated by B-mode

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ultrasonography as the increase of cIMT. By the end of the first 12-month follow-up period, a decrease of cIMT by 0.028 ± 0.008 mm was observed in the Allicor group. At the same time, moderate increase of 0.014 ± 0.009 mm was observed in the placebo group ($p = 0.002$). Serum atherogenicity was decreased in the Allicor group by 45% from the baseline and remained unaltered in the placebo group. Therefore, long-term treatment with Allicor had a direct anti-atherosclerotic effect in patients with subclinical atherosclerosis associated with decreased serum atherogenicity [78]. By the end of the 24-month follow-up period, the mean rate of cIMT was decreased in the Allicor group by 0.022 ± 0.007 mm per year, which was significantly different ($p = 0.002$) from the placebo group, in which there was a moderate but statistically significant progression of 0.015 ± 0.008 mm at the overall mean baseline cIMT of 0.931 ± 0.009 mm [37, 39]. A significant reduction of cIMT was observed in 47.3% of study subjects from the Allicor group vs 30.1% in the placebo group ($p < 0.05$). Further significant increase of cIMT was registered in 32.2% study participants in Allicor-treated group vs 47.3% in placebo group ($p < 0.05$). Study of blood serum atherogenicity demonstrated a 1.56-fold increase of intracellular cholesterol accumulation in the cellular test at the baseline. Study participants from Allicor group had an average 30% decrease of blood serum atherogenicity, while in the placebo group, this parameter remained unaltered during the study. A significant correlation was observed between changes of blood serum atherogenicity and intima-media thickness of common carotid arteries ($r = 0.144$, $p = 0.045$) (**Figures 8** and **9**). Therefore, it was demonstrated that garlic-based food supplement Allicor possessed a direct anti-atherosclerotic effect at the subclinical stage of the disease, which could be attributed to the decrease of blood serum atherogenicity [37, 39].

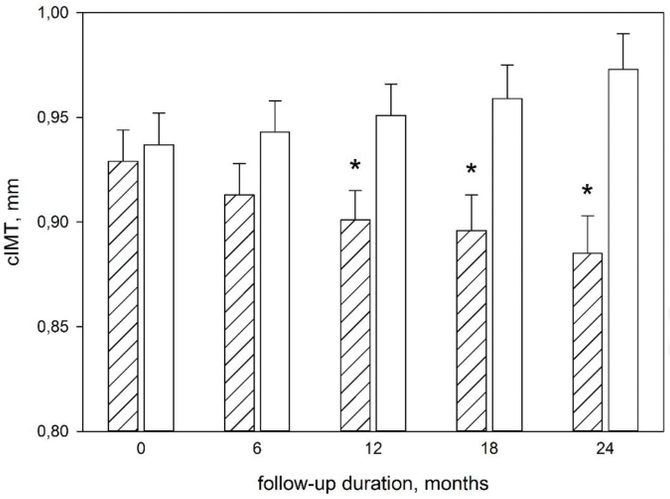

Figure 8. The dynamics of cIMT in double-blind placebo-controlled study on anti-atherosclerotic effects of garlic-based drug Allicor. Hatched bars, Allicor recipients; open bars, placebo recipients. Presented are mean values ±S.E.M. *, significant difference between groups, $p < 0.05$. Reproduced with permission from [30].

Another clinical study was focused on the evaluation of potential anti-atherosclerotic activity of herbal products with anti–inflammatory effects. Atherosclerosis is tightly associated with the inflammatory process at all stages of the disease development [79, 80]. Substances with

systemic anti-inflammatory properties can therefore be regarded as potential therapeutic agents for treatment and prevention of atherosclerosis.

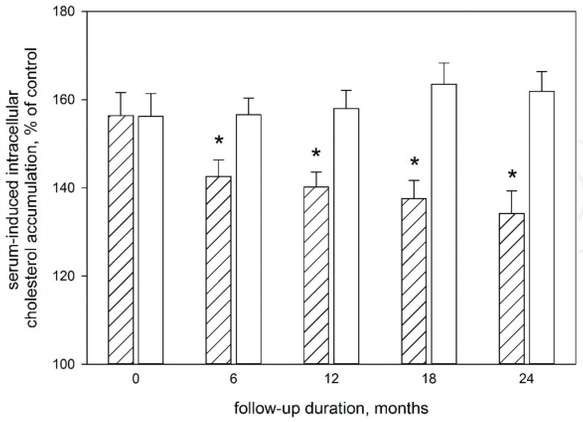

Figure 9. The dynamics of serum atherogenicity in double-blind placebo-controlled study on anti-atherosclerotic effects of garlic-based drug Allicor. Hatched bars, Allicor recipients; open bars, placebo recipients. Presented are mean values ±S.E.M. *, significant difference between groups, $p < 0.05$. Reproduced with permission from [30].

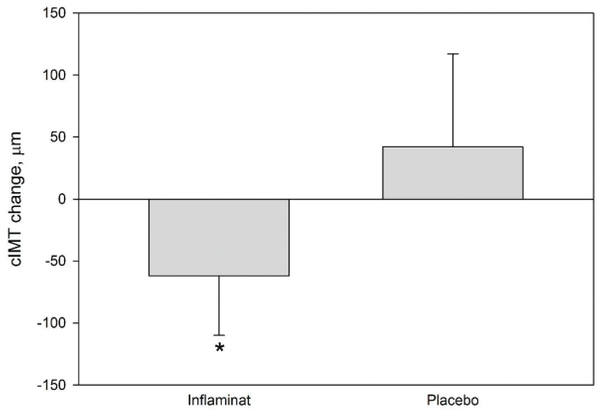

Figure 10. The changes of cIMT in double-masked placebo-controlled study on anti-atherosclerotic effects of Inflaminat. Presented are mean values ±S.E.M. *, significant difference between groups, $p < 0.05$. Reproduced with permission from [30].

Several natural compounds, such as calendula (*Calendula officinalis*), elder (*Sambucus nigra*) and violet (*Viola* sp.), were demonstrated to possess not only anti-inflammatory, but also anti-atherosclerotic effects [81–83]. The combination of these herbs was used for the development of a novel dietary supplement (Inflaminat, INAT-Farma, Russia) [84]. The effect of Inflaminat on cIMT dynamics was evaluated in a pilot placebo-controlled double-blinded study performed on 67 asymptomatic men (ClinicalTrials.gov Identifier, NCT01743404) [39, 85]. The protocol of the 12-month study was similar to that described for Allicor food supplement. Administration of Inflaminat induced cIMT regression in subclinical atherosclerosis, with statistically significant difference between the baseline as the placebo group (**Figure 10**). Therefore, Inflaminat was demonstrated to possess anti-inflammatory and anti-atherosclerotic

effects at the cellular level and to induce regression of subclinical atherosclerotic lesions in asymptomatic men.

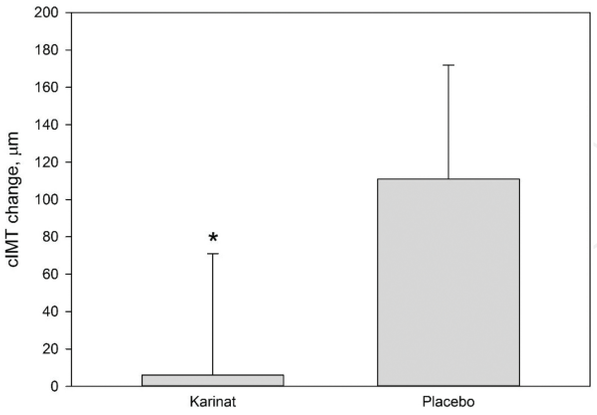

Figure 11. The changes of cIMT in double-masked placebo-controlled study on anti-atherosclerotic effects of Karinat. The data are presented in the terms of means and S.D. *, significant difference between groups, $p < 0.05$. Reproduced with permission from [30].

Finally, several phytoestrogen-rich natural substances were evaluated for potential anti-atherosclerotic activity using the described *in vitro* and an *ex vivo* models [86–88]. The most promising of these compounds were garlic powder, extract of grape seeds, green tea leaf and hop cones. All these substances possessed a significant anti-atherogenic effect. A combination of these compounds was used for development of a novel isoflavonoid-rich dietary supplement (Karinat, INAT-Farma, Russia). The resulting supplement is a source of biologically active polyphenols, including resveratrol, genisteine and daidzeine that are claimed to produce beneficial effects on atherosclerosis development. The efficiency of Karinat was evaluated in a randomized double-blind placebo-controlled 12-month clinical study conducted on 157 asymptomatic postmenopausal women (ClinicalTrials.gov Identifier, NCT01742000) [89, 90]. The primary endpoint was the annual rate of cIMT change. The protocol of the study was similar to that reported above. An annual increase of mean cIMT of more than 100 μm (13% per year) was observed in the placebo group, indicative of a high rate of cIMT progression in postmenopausal women. Growth of atherosclerotic plaques was estimated to be 40% per year. In the Karinat group, mean cIMT value remained unaltered, with a statistically insignificant increase of 6 μm per year, *that is* <1% (**Figure 11**). Therefore, phytoestrogen-rich substances were proven to possess beneficial effects on the dynamics of subclinical atherosclerosis progression in postmenopausal women [39, 91].

7. Conclusions

Introduction of the concept of blood serum atherogenicity allowed creating cell model suitable for screening of substances with potential anti-atherosclerotic activity. Such models helped revealing several novel compounds of botanical origin that could be used for the development

of dietary supplements for treatment of subclinical (asymptomatic) atherosclerosis. The effect of "direct" anti-atherosclerotic therapy can be observed at the level of the arterial wall cells by a decrease of intracellular lipid accumulation. Therapy of patients with established atherosclerosis should induce regression of the existing plaques or hinder the progression of novel lesions. Introduction of food supplements from botanicals with anti-atherosclerotic properties and suitable for long-term consumption is an important step toward the improvement of the preventive treatment of atherosclerosis. Further studies will help revealing natural products with anti-atherogenic and anti-atherosclerotic effects that can be used for the development of novel cardiovascular drugs possessing mechanistic mode of action. Despite the unavoidable limitations of the described models, the obtained results have demonstrated that cultured arterial wall cells offer a suitable instrument for initial analysis of drug effects. The discovery of anti-atherosclerotic activity of natural products opens great opportunities for prevention and treatment of atherosclerotic disease, reducing cardiovascular morbidity and mortality.

Disclosure statement

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