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Chapter 8

Cell Composition of the Subendothelial Aortic Intima and the Role of Alpha-Smooth Muscle Actin Expressing Pericyte-Like Cells and Smooth Muscle Cells in the Development of Atherosclerosis

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Additional information is available at the end of the chapter

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Abstract

The cell composition of the human arterial intima has been intensely studied but is still not well understood. The majority of cell population in normal and atherosclerotic intima is represented by cells expressing smooth muscle α-actin, which are thought to be smooth muscle cells. Some antigens, which are absent in medial smooth muscle cells, were detected in intimal smooth muscle cells. In particular, using 3G5 antipericyte antibody, presence of stellate-shaped pericyte-like resident cells in normal and atherosclerotic human aortic intima has been found. In all analyzed aortic tissue specimens, 3G5+ cells were found to account for more than 30% of the total intimal cell population of undiseased intima. In the atherosclerotic lesions, the number of 3G5+ cells becomes notably lower than that in undiseased intima. The use of 2A7 antibody that identifies activated pericytes revealed the presence of 2A7+ cells in atherosclerotic plaques, while no 2A7+ cells were detected in normal intima. The strongest correlation was established between the number of pericyte-like cells and the content of intimal lipids. The correlation coefficients between the number of pericyte-like cells and collagen content and intimal thickness were greater than the correlation coefficients for smooth muscle cells. On the basis of these findings, pericyte-like cells but not smooth muscle cells or other cell types have been declared to be the key cellular element driving the formation of atherosclerotic lesions. The present chapter aims to detail the abovementioned issues. The present chapter also aims to promote a view that α-smooth muscle actin+ pericyte-like cells represent the key players in the development of atherosclerotic lesions.

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Keywords: Smooth muscle cells, Pericyte-like cells, 3G5 antigen, 2A7 antigen, Arterial wall, Intima, Atherosclerosis, Atherogenesis

1. Introduction

The cell composition of the human arterial intima has been intensely studied but is still not well understood [1]. The majority of cell population in normal and atherosclerotic intima is represented by cells expressing α-actins, which are thought to be smooth muscle cells [2]. According to the current paradigm, smooth muscle cells show striking plasticity responding to microenvironment signals and thus can be presented by different phenotypes [3-7]. Immune-inflammatory cells also reside in the intima of healthy arterial walls [8]. Resident immune-inflammatory cells (macrophages, lymphocytes, and dendritic cells) represent only a minority of the subendothelial cell population, but their proportion increases during the development of atherosclerotic lesions, reaching up to 20% of the total cell content [9].

Some antigens, which are absent in medial smooth muscle cells, were detected in intimal smooth muscle cells [10-13]. In particular, using 3G5 antipericyte antibody, presence of stellate-shaped pericyte-like resident cells in normal and atherosclerotic human aortic intima has been found [10, 11]. In all analyzed aortic tissue specimens, 3G5+cells were found to account for more than 30% of the total intimal cell population of undiseased intima. In the atherosclerotic lesions, the number of 3G5+ cells becomes notably lower than that in undiseased intima [11]. The use of 2A7 antibody that identifies "activated" pericytes revealed the presence of 2A7+ cells in atherosclerotic plaques, while no 2A7+ cells were detected in normal intima [12]. The strongest correlation was established between the number of pericyte-like cells and the content of intimal lipids [12]. The correlation coefficients between the number of pericyte-like cells and collagen content and intimal thickness were greater than the correlation coefficients for smooth muscle cells. On the basis of these findings, pericyte-like cells but not smooth muscle cells or other cell types have been declared to be the key cellular element driving the formation of atherosclerotic lesions [14].

The present chapter aims to detail the abovementioned issues. The present chapter also aims to promote a view that α-smooth muscle actin+ pericyte-like cells represent the key players in the development of atherosclerotic lesions.

2. Structural organization of human aortic intima

The wall of large arteries consists of three layers, namely, the tunica intima, the tunica media, and the tunica adventitia. Furthermore, the tunica intima of the adult human aorta consists itself of two layers separated by distinct boundaries [15-20]. The muscular-elastic layer, adjacent to the media (also called the Jores' layer), is separated from the media by the internal

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elastic lamina. The innermost intimal layer, adjoining the arterial lumen, is separated from the muscular-elastic layer by the internal limiting membrane. This innermost intimal layer is also called as elastic-hyperlastic [17], connective-tissue [19, 21-24], juxtaluminal [20], or proteoglycan-rich layer [21]; it is located between the internal limiting membrane and the endothelial lining (Figure 1).

It is well known that the thickness of the intima is greater in atherosclerotic lesions than in grossly normal areas, with intimal thickness reaching the maximum in atherosclerotic plaques. In fatty streak, the thickness of the muscular-elastic layer is the same as in uninvolved intima, while in atherosclerotic plaque, it is only 11% greater than in the normal intima [25]. In contrast to the muscular-elastic layer, the thickness of the proteoglycan-rich layer increases considerably in atherosclerotic plaque, forming an intimal protrusion into the lumen, which reduces blood flow through the aorta. On average, in fatty streak, the thickness of the proteoglycan-rich layer is almost two times and in the plaque almost four times as high as that in uninvolved intima [25]. Sometimes, the thickness of the proteoglycan-rich layer in the plaque can be 10- to 20-fold as high as that in a normal vessel [25].

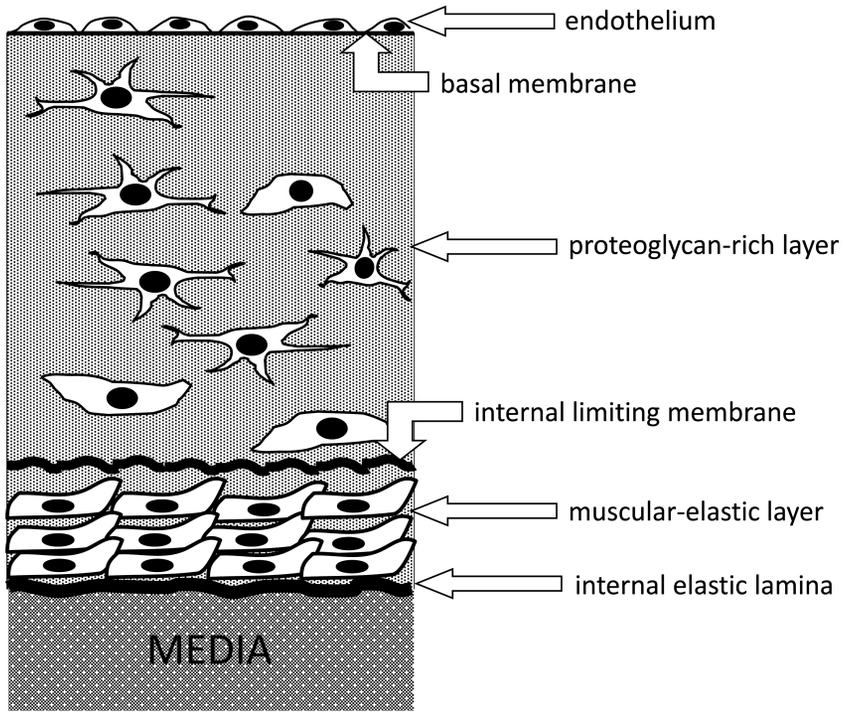
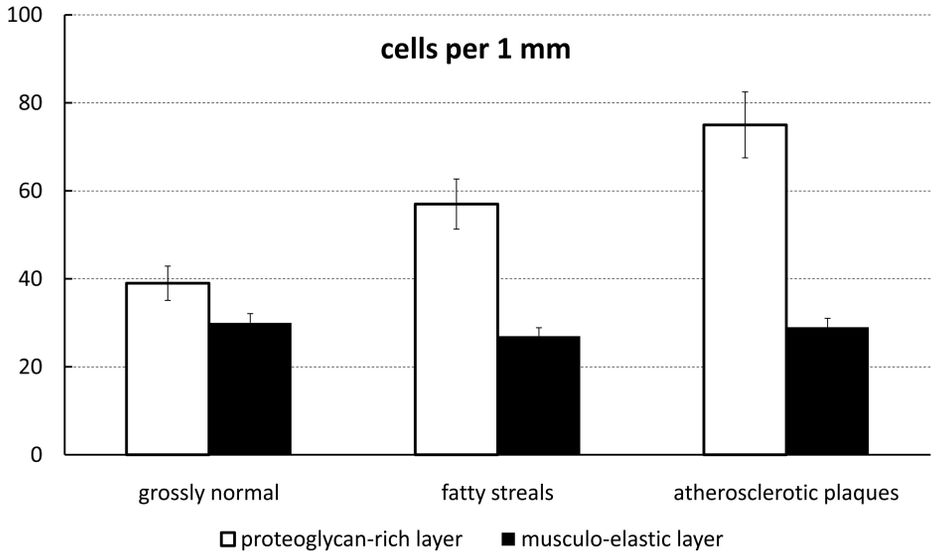


Figure 1. The intima is separated from the media by the internal elastic lamina. The innermost proteoglycan-rich sublayer of the human intima is separated from the muscular-elastic layer, adjacent to the media, by the internal limiting membrane. Intimal layers differ from each other in cell arrangement and by intercellular matrix organization. The muscular-elastic sublayer consists of several rows of longitudinally arranged elongated cells separated by elastic fibers, also longitudinally oriented. The connective tissue fibers of the proteoglycan-rich layer have no definite orientation to the vessel axis, and its cell population is morphologically heterogeneous, as was described as far as in the nineteenth century [24]. Histological and histochemical observations showed that in the normal intima, the muscular-elastic layer contains much more elastic fibers than the proteoglycan-rich layer, while the proteoglycan-rich layer contains more collagen and reticulin fibers [20, 23]. The layers differ from each other by the composition of glycosaminoglycans [20].

3. Cellular composition of normal and atherosclerotic intima of the human aorta

Cell numbers in the intimal layers of grossly normal areas and atherosclerotic lesions have been determined after alcohol-alkaline dissociation of tissue [26]. In the proteoglycan-rich layer of a fatty streak and an atherosclerotic plaque, the number of cells was found to be 1.5- and 2-fold higher than that in undiseased intima, while in the muscular-elastic layer of atherosclerotic lesions, the number of cells was found to remain practically unchanged in comparison with the normal intima [25].

Similar results were obtained during analysis of aortic cross sections [25]. Cell number in the muscular-elastic layer of uninvolved and atherosclerotic intima is similar, while the number of cells in the proteoglycan-rich layer of atherosclerotic lesions is twofold higher than in normal intima (Figure 2). Thus, atherosclerotic manifestations in the vascular wall coincide with an increase in the cell number of the proteoglycan-rich layer.



Cell number in the intimal layers was determined in suspension after alkali-alcohol dissociation on fixed tissue [25].
PG - proteoglycan-rich layer of the intima, ME - musculoelastic layer of the intima.
* - significant difference from normal, p<O.05

Figure 2. Cell number in different layers of the human aortic intima.

What are the cells populating different layers of the intima and what are the differences between these cells?

Cellular composition of the intima of human arteries has been studied for more than one century [27-30]. In classic works in this field, a special attention has been paid to the fact that the cell population inhabiting vascular walls is heterogeneous and consists of two subpopulations: resident vascular cells and round cells that are morphologically similar to peripheral blood monocytes and lymphocytes (inflammatory cells). In those studies, it was noted that

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resident subendothelial cells differ from typical smooth muscle cells of the media [28, 29]. These cells were referred to as intimal fibroblasts [27], mesenchymal reserve cells [28], pericytes or vascular cambium [30], etc. However, the application of electron microscopy studies led to the concept that *intimacytes* of major arteries are modified smooth muscle cells [31, 32]. Figure 3 shows typical ultrastructural features of cells that represent the main population among the subendothelial cells. The structural features of these cells include the presence of individual myofilaments and bundles of myofilaments in cell cytoplasm, the presence of "dense bodies" seen in the cytoplasm along the cell-surrounding membrane, and the presence of basal membrane surrounding the cell membrane extracellularly, a combination suggesting smooth muscle nature of these cells [31-33].

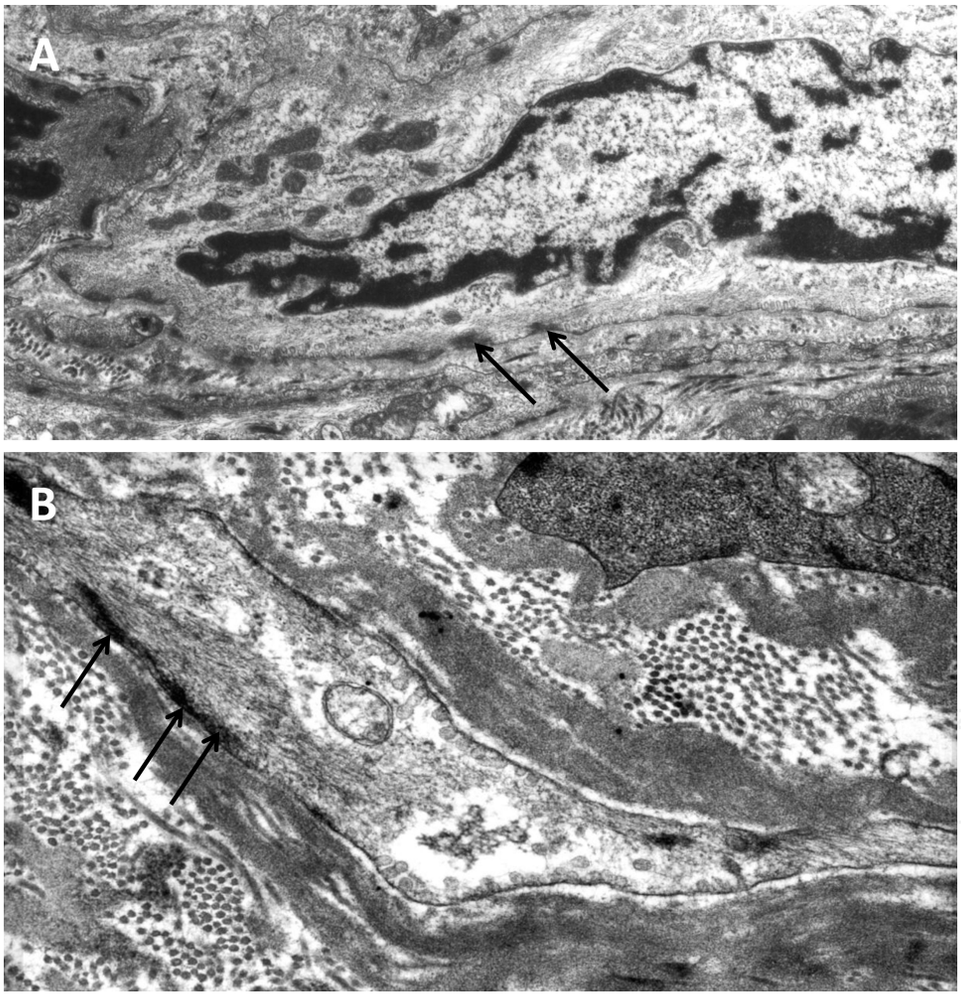


Figure 3. Electron micrographs showing ultrastructural appearance of *intimacytes* most frequently seen in the subendothelial space of undiseased aorta (**A**, **B**) (up to 90% of the total cells of the tunica intima of the undiseased aorta). Image (**A**): cell body; image (**B**): cell process. In (**A**, **B**), note the presence of myofilaments and the basal membrane; arrows show "dense bodies," a reliable ultrastructural criterion for the identification of smooth muscle cells. The presence of myofilaments and the basal membrane further supports the identification of the cells as smooth muscle cells.

4. Immunocytochemical typing of subendothelial cells

The use of antibodies specific to various types of mesenchymal cells has provided additional information regarding the cellular composition of normal and atherosclerotic intima. In addition to cells that expressed smooth muscle α-actin [34-37], immune-inflammatory cells such as macrophages [38-42], lymphocytes [40, 42-47], mast cells [48], and dendritic cells [49] were identified in the aortic intima.

Using a set of cell type-specific cytochemical markers, cells of undiseased and atherosclerotic intima have been characterized (Table 1). The majority of cell population in normal and atherosclerotic intima was found to be represented by cells expressing smooth muscle α-actin. About two-thirds of cells in the muscular-elastic layer expressed smooth muscle α-actin, while in the proteoglycan-rich layer, the proportion of these cells was lower (Table 1). Immune-inflammatory cells (lymphocytes and macrophages) were confined preferentially to the juxtaluminal part of the proteoglycan-rich layer. Their proportion was found to increase in atherosclerotic lesions, reaching 20% of the total cell content.

Examined area	Positively stained cells, %						
	Smooth muscle α-actin	CD45	CD68	3G5	2A7	Inflammatory cells (CD45+CD14)	Resident cells
Grossly normal [0][a]	47.6±2.3 (4)[b]	2.2±0.4 (3)	3.9±0.4 (5)	31.3±7.0 (4)	0.0±0.0 (3)	5.5±1.2 (8)	97.3±0.6 (8)
Initial lesions [I]	47.2±3.1 (3)	6.2±1.2 (4)	6.1±1.4 (4)	6.3±1.0* (3)	1.2±0.3 (3)	9.6±1.4* (6)	90.9±1.2 (6)
Fatty streaks [II]	42.2±3.1 (4)	5.0±0.9* (3)	13.2±0.8* (5)	11.7±2.0* (8)	3.0±0.7 (3)	13.4±1.5* (10)	89.4±1.1 (10)
Fibrolipid plaques [Va]	47.0±10. 9 (5)	6.2±1.8* (9)	13.1 ±2.3* (4)	5.0±0.7* (5)	27.0±3.1 (3)	18.7±2.0* (8)	84.7±1.4 (8)
Fibrotic plaques [Vc]	ND	ND	ND	ND	ND	6.4±1.9 (6)	94.9±1.6 (6)

[a]: Lesion type according to AHA Council on Atherosclerosis [50] is indicated in the square brackets.

[b]: The number of cases examined is indicated in the round brackets.

*: Significant difference from the percent of positively stained cells in grossly normal areas, $p<0.05$.

ND: Not determined.

Table 1. Immunocytochemical identification of cells in human aortic intima

The presence of pericyte-like resident cells in normal and atherosclerotic human aortic intima have been demonstrated [10, 11], using 3G5 antipericyte antibody [51]. The antigen for 3G5

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antibody represents O-sialoganglioside of the plasma membrane of brain capillary pericytes [51], which are known as a very stable, slowly renewed cell population [52]. It has been stated that this antigen is typical for quiescent pericytes [51]. Previously, 3G5-positive pericyte-like cells were identified in bovine aorta, in human undiseased intima, and in complicated atherosclerotic plaques with ectopic osteogenesis [10]. These cells account for more than 30% of the total intimal cell population (Table 1). On cross sections, cells bearing 3G5 antigen were found to present only in the uppermost subendothelial layer of the intima. On *en face* preparations, 3G5-positive cells with long processes form a network immediately under the endothelium (Figure 4) [1, 11-14]. The subendothelial localization of pericytes suggests that in the aorta and large arteries, 3G5-positive cells perform the functions similar to those of capillary pericytes [1, 11].

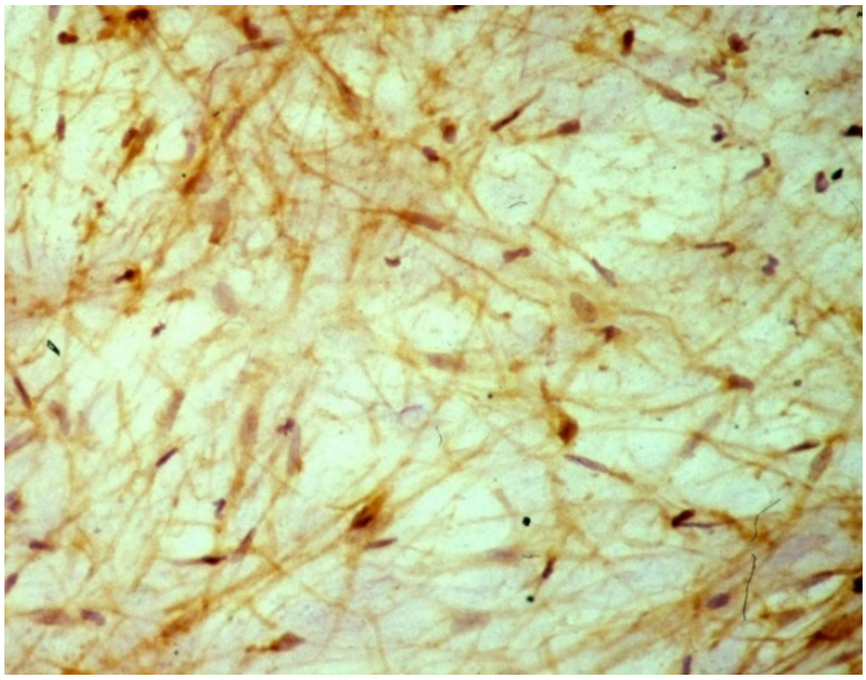


Figure 4. Positive cells by means of their long processes form a network immediately under the luminal endothelium in undiseased intima. *En face* specimen of the human aorta; immunohistochemistry.

It has been suggested that pericytes contribute to various pathophysiological processes associated with microcirculation dysfunctions, including diabetes, inflammation, wound healing, hypertension, tumor growth [52-54], and also regulate endothelial functions, including endothelial cell proliferation and ion and molecule exchange [53-57]. At the present time, the major predisposition of pericytes is assumed as precursors for other cells of mesenchymal origin, including smooth muscle cells [54], osteoblasts [56-59], chondrocytes [54], and adipocytes [60]. This is consistent with the earlier concepts that consider subendothelial cells as pluripotent cambial cells [30] and mesenchymal reserve cells [28]. The network formed immediately under the luminal endothelium by pericyte-like cells has been suggested to play a role in immune function of the arterial wall, especially taking into account that pericyte-like cells express HLA-DR antigen [1].

A comparison of the number of pericyte-like cells in atherosclerotic lesions with the number of pericyte-like cells in undiseased intima revealed that in atherosclerotic lesions, the number of the cells possessing 3G5 antigen is much lower than that in undiseased intima (Figure 5).

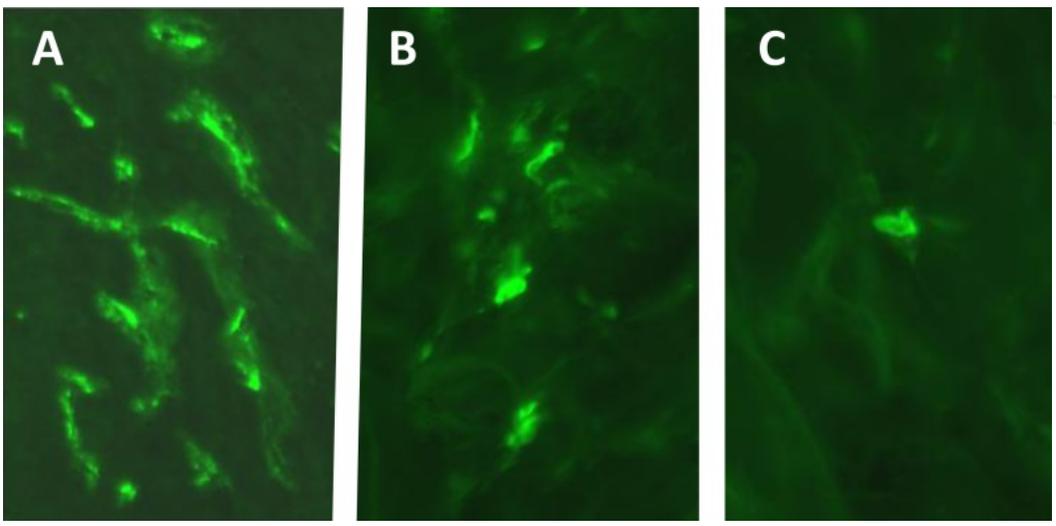


Figure 5. Visualization of 3G5+ cells in normal (undiseased) intima (**A**), in fatty streak (**B**), and in atherosclerotic plaques (**C**) by means of immunofluorescent analysis.

This could suggest that the number of pericyte-like cells in atherosclerotic lesions decreases. However, the results obtained in experiments with the use of antipericyte antibody 2A7, which recognizes another pericyte antigen, argue with such a suggestion. 2A7+ cells were found to appear in atherosclerotic plaques, while 2A7+ cells are absent in normal intima (Table 1). Anti-2A7 represents an antibody against melanomaassociated high-molecular-weight antigen (HMW-MAA), which is a chondroitin sulfate proteoglycan (also termed as melanoma proteoglycan), which is also present on pericytes in the areas of active angiogenesis (in granulation tissues in healing wounds, synoviitis, etc.) [61]. 2A7 is expressed by “activated” pericytes capable of proliferating [61].

The above-described findings led to a question: are 3G5 and 2A7 pericytic antigens expressed by the same cell or by different cell types? In order to answer this question, a culture of human brain pericytes was used for experiments. It has been found that in human brain pericytes, about 40% of cells express 3G5 antigen and 80% of cells express 2A7 antigen. Simultaneous staining for both antigens revealed 80% of positively stained cells indicating that all cells having 3G5 antigen expressed 2A7 antigen; however, there was a population of pericytes that expressed only 2A7 antigen. As was mentioned above, 2A7 and 3G5 antigens were described on the cells in different functional states: 3G5 antigen is typical for "quiescent" but 2A7 for "activated" pericytes, respectively. Activated 2A7-positive pericytes are capable of intense replication. It can be hypothesized that most of the cultured pericytes are "activated" (as a result of serum stimulation); therefore, only a part of these preserves 3G5 antigen, which is typical for quiescent pericytes.

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It is well known that lipid accumulation is one of the most pronounced manifestations of atherosclerosis. Lipid accumulation may influence the expression of pericyte antigens in the subendothelial cells. In fact, a 1.5- to 2-fold increase in the intracellular lipid content induced by modified low-density lipoproteins (modLDL) leads to a decrease in the proportion of 3G5-posilive cells (Figure 6). The total cell number remains unchanged, which indicates that a decrease in the proportion of 3G5+ cells does not result from selective death of these cells but is due to disappearance of 3G5 antigen caused by intracellular lipid accumulation. This occurs in atherosclerotic lesions, where the number of 3G5-positive cells is lower than in normal intima. It can be suggested that the functional state of the pericyte-like cells in the proteoglycan-rich layer of the intima is changed upon formation of the atherosclerotic lesions, which is accompanied by alterations in antigenic expression.

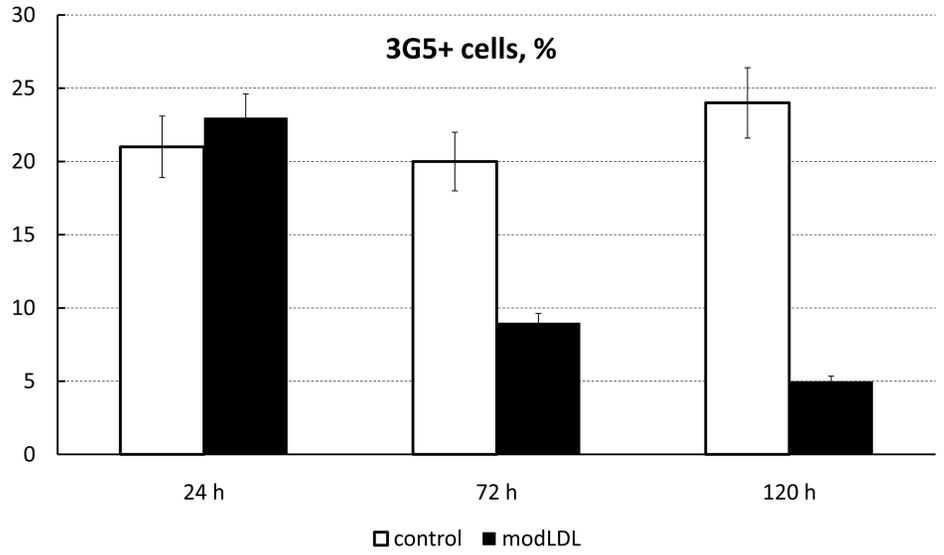


Figure 6. The effect of intracellular lipid accumulation on the expression of 3G5 pericyte-associated antigen in cultured cells from undiseased human aortic intima. On day 4, cells were washed with sterile PBS and incubated for 24, 72, hand 120 h in Medium 199 with 10% LDS (control), Medium 199 with 10% LDS + 100 μg/ml native LDL (not shown), or Medium 199 with 10% LDS +100 μg/ml modified (desialylated) LDL (modLDL). After incubation, cells were washed with PBS and stained with antibody against 3G5 pericyteassociated antigen. Total cell number and number of positively stained cells were calculated in each cell culture in triplicates. The data are representative of the three experiments performed. The data obtained with native LDL were similar to results without LDL and were not shown.

5. Inflammatory and resident cells

Immunocytochemical analysis of the cellular composition of the intimal layers showed that the cell population of the muscular-elastic layer is homogeneous, consisting predominantly of smooth muscle cells. These cells are similar to the medial smooth muscle cells, the majority of which react with antimuscle α-actin antibodies. The proteoglycan-rich layer of the intima is populated by resident and inflammatory cells. In the atherosclerotic lesions, the proportion of resident cells expressing smooth muscle α-actin is similar to that in uninvolved intima, while

the proportion of inflammatory cells increases, but these cells do not become predominating. Besides the smooth muscle cell antigen, resident intimal cells express pericyte antigens as well as the macrophage-associated antigen CD68.

What are the specific features of resident intimal cells? Morphological heterogeneity of intimal population consisting of processed cells of various shapes was described by Langhans [27], Schonfelder [62], Schlekunov [30, 63], Khavkin [64], Geer and Haust [19], and others. Elongated bipolar cells typical of the media predominate in the population of the muscular-elastic layer. At the same time, the cells of proteoglycan layer markedly vary in shape: they are elongated and stellate, with a variety of intermediate shapes. The presence of processed star-like cells [27] is the major characteristic of the proteoglycan-rich layer.

The morphological forms of resident subendothelial cells (elongated and stellate) have been described in primary cultures of enzyme-isolated cells from normal and atherosclerotic human aorta [65]. Elongated cells have a long body without processes or with small side processes. They are packed in compact cell layers, express α-actin, and have a well-developed contractile apparatus [65]. Stellate (pericyte-like) cells have a round body with three or more processes. They are unevenly distributed in the loose connective-tissue matrix of the intima. In addition to α-actin, some juxtaluminally located cells express 3G5 and 2A7 pericytic antigens and CD68 macrophage-associated antigen [13], a scavenger receptor [39]. ln atherosclerotic lesions, the cytoplasm of these cells is filled with lipids (foam cells) [66]. Electron microscopic studies revealed considerable numbers of synthetic organelles in these cells [14]. In the atherosclerotic lesions, the number of stellate cells increases sixfold while the total number or cells and the number of elongated cells increase only twofold. Thus, both quantitative and qualitative changes occur in cellular composition of the intima underlying atherosclerotic lesions.

6. Stellate cells and atherosclerosis-related manifestations

Is there any relationship between changes in cellular composition and the well-known manifestations of atherosclerosis such as intimal thickening and accumulation of lipids and collagen? The strongest correlation was established between the number of stellate cells, on the one hand, and the content of cholesteryl esters and total lipids, on the other [14]. The correlation coefficients between the number of stellate cells and collagen content and intimal thickness were somewhat lower; nevertheless, they remained greater than the correlation coefficients for elongated cells and the total cell content [14].

Stellate cells are located in the close proximity to endothelial lining; they are the first barrier for the compounds entering the vascular wall from the blood. This may account for the great extent of the involvement of the stellate cells in atherosclerotic manifestations in the vascular wall [1, 14].

The direct correlation between the number of stellate cells and atherosclerotic manifestations raised a number of questions. Why do cells become stellate? Are stellate cells specific to cell type or does this shape reflect the functional state of a cell? In the human body, there are other

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tissues containing stellate cells, for example, neurons and other nervous cells, dendritic cells of lymphatic follicles, Ito cells in the liver, mesangial cells in renal glomeruli, etc. [1]. These cells serve predominantly communicative functions via a system of their processes [1]. A similar function can be ascribed to stellate cells of subendothelial intima, which form an integrating communication network by their long branching processes [1].

In primary cultures, intimal cells are generally flattened and polygonal. In these cell cultures, arborization, i.e., formation of stellate cells, using cAMP elevators, was induced [12]. Almost all cells derived from the proteoglycan-rich layer become stellate, while the content of stellate cells is not higher than 50% in cultures from the muscular-elastic layer and about 10% in cultures from the media [12].

Arborization is accompanied by redistribution of connexin 43 (Cx43), a major protein of specific cell-to-cell contacts (gap junctions). This protein is localized on cell surface in specific structures, so-called Cx43 plaques. On nonarborized cells, small Cx43 plaques are unevenly distributed over the cell surface. In addition to small Cx43 plaques, on arborized (stellate) cells, large Cx43 plaques are localized predominantly on the ends of the cellular processes in the areas of the cell-to-cell contacts [12].

Thus, an increase in the intracellular cAMP content leads to arborization of subendothelial intima cells. Changes in the cell shape are accompanied by redistribution of connexin 43 plaques and, probably, by increase of the rate of the intercellular communication via gap junctions. This suggests that the stellate shape is important for the formation of cell-to-cell contacts in the intima. Subendothelial intimal cells differ from medial smooth muscle cells, which are poorly or not arborized, by the ability to form these contacts. Therefore, it is reasonable to suggest that subendothelial intimal cells are a specific cell type distinct from typical smooth muscle cells.

7. Cellular network in the intima

Based on the observations of *en face* preparations of the intima, Schonfelder [62] has suggested that stellate intimal cells are joined into a common network. He supposed that empty spaces between the interlaced cellular processes are the channels reaching deep into the intima.

The concept of a common cellular network in the intima of the human aorta was confirmed later [67]. Using scanning electron microscopy, it was shown that all cells in the proteoglycan-rich layer of uninvolved intima are interconnected in the horizontal plane and form a common network [67]. The cells are also joined to each other in the vertical plane. Thus, a three-dimensional cellular network is formed. All cell processes contact with the processes or bodies of other cells. Consequently, the proteoglycan-rich layer cannot be regarded as a sum of separated cells but as a unified cellular system. Empty spaces between the processes filled with the connective tissue matrix were also observed [67], as described by Schonfelder [62].

Cells of the muscular-elastic layer do not form a network characteristic of stellate cells in the proteoglycan-rich layer. Scanning electron microscopy showed that most of the cells have a

bipolar elongated shape characteristic of medial smooth muscle cells. In the muscular-elastic layer, densely packed cells form strata oriented at a small angle to each other [67].

A continuous vertical gradient in the ratio between the number of stellate subendothelial cells and typical elongated smooth muscle cells has been discovered [67]. The number of elongated cells decreases from the muscular-elastic layer to the endothelium, while the number of network-forming stellate cells increases.

In atherosclerotic lesions, the cellular system of the intima undergoes considerable changes. In fatty streaks, many stellate cells are generally laden with lipids [14, 25, 66]. This may account for an increase in their dimensions and formation of surface bleb-like protrusions by lipid droplets and vesicles. Vesicles in the extracellular space were also found; this suggests that these vesicles are propagated by gemmation (budding) from the surface of the cells and cell processes. Vesicle gemmation from the ends of the cell processes can obviously lead to the degradation of intercellular contacts and dissociation of the common network. In atherosclerotic plaques, changes in the cell system are more pronounced. In the superficial layers of the connective tissue cap, stellate cells are always settled as separate cells or small groups. Sometimes, these cells contain lipid inclusions. In deep layers of the intima, the number of processed cells increases, but they do not form a network. Interestingly, a three-dimensional cellular network typical of uninvolved intima was found in the intima next to the plaque shoulders displaying no visible atherosclerosis-related changes.

8. Intercellular communication

Thus, it was demonstrated that the cellular network is disintegrated in the proteoglycan-rich layer of atherosclerotic lesions [67]. Eventually, this disintegration results in complete separation of cells in an atherosclerotic plaque. It is an established fact that the regulation of cells forming highly differentiated tissue systems occurs via specialized cell-to-cell contacts, gap junctions [68, 69]. It was demonstrated that these contacts play an important role in the regulation of tissue homeostasis, providing the transport of cell metabolites, second messengers, and other biologically active molecules from cell to cell without entering the extracellular space. The presence of these specialized contacts is a specific feature of the differentiated cell systems with a high degree of intercellular integration [68-73]. Stellate shape of intimal cells and their interaction via the cellular processes may substantially contribute to the vascular wall regulation. It can be hypothesized that functional disturbances in gap junctions are one of the causes of atherosclerosis-related disintegration of cellular networks formed by the proteoglycan-rich layer intimacytes [73].

The degree of intercellular communication via gap junctions can be assessed by expression of proteins forming these contacts. Connexin 43 (Cx43) is the major protein of these contacts. This protein is localized on cell surfaces in so-called connexin plaques [73]. A primary culture of aortic cells was used to elucidate the causes of reduced intercellular communication in atherosclerotic lesions. In addition to the identification of Cx43, another approach can be employed in a cell culture, namely, the transfer of fluorescent dye from the injected cell to neighboring cells. Fluorescent dye is specifically distributed only via gap junctions, and the

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rate of the communication is evaluated by the number of contacting fluorescent cells. The rate of intercellular communication in cell cultures with various densities has been estimated [73]. It was revealed that both approaches correlated very closely with each other. It was found that the intensity of intercellular communication in cultures derived from grossly normal areas is 1.5-fold higher than that in cultures obtained from atherosclerotic lesions [73].

The presence of lipid-laden cells resembling so-called foam cells was a specific feature of cultures derived from atherosclerotic lesions [73]. Assuming that there is a relationship between the content of intracellular lipids and the rate of the intercellular communication, fluorescent dye was injected into cells with and without visual lipid inclusions. The rate of gap junctional communication of cells without lipids was similar to that of cells cultured from grossly normal areas [73]. The rate of communication of foam cells was twofold lower than that of lipid-free cells. The number of Cx43 plaques per lipid-laden cell was lower than per cell without lipids [73]. It can be suggested that lipid accumulation is a possible cause of reduced intercellular communication in the intimal cell system.

On the basis of the above data, it can be concluded that stellate cells are the principal cell type of the proteoglycan-rich layer. Stellate cells of subendothelial intima differ from typical medial smooth muscle cells. In contrast to elongated smooth muscle cells, they have several long processes capable of forming a network. Stellate cells have poor contractile organelles but possess a well-developed rough endoplasmic reticulum. These cells express a number of markers (pericyte antigens 3G5 and 2A7, macrophage antigen CD86, scavenger receptor) that are not expressed by typical smooth muscle cells. These markers may determine functional peculiarities of stellate cells. The number of stellate cells increases in arteriosclerotic lesions and strongly correlates with the major manifestations of atherosclerosis. In uninvolved intima, the cells of the proteoglycan-rich layer contact with each other by their processes forming a three-dimensional network. In atherosclerotic lesions, the rate of intercellular communication decreases and the contacts between cells are impaired, presumably as a result of lipid accumulation.

9. Atherosclerotic manifestations on the cellular level

The main manifestations of atherosclerosis are associated with alterations on the cellular level, namely, lipid accumulation leads to the formation of foam cells. Local increase in the cell number is a result of cell proliferation or migration. Accumulation of the connective tissue matrix is a consequence of synthesizing activity of the cells.

10. Lipidosis (intracellular lipid accumulation)

In grossly normal areas of the intima, lipids are accumulated predominantly in the extracellular space. Initial atherosclerotic lesions are characterized by the presence of cells with lipid inclusions [29]. The proportion of cells with lipid inclusions in uninvolved intima and in atherosclerotic lesions has been determined [66]. Fatty streaks have the highest content of lipid-

laden cells (up to 25%); these cells are located in the upper part of the proteoglycan-rich layer, comprising approximately two-thirds of it. By contrast, in atherosclerotic plaques, most of the cells with lipid inclusions are located in one-third of the proteoglycan-rich layer adjacent to the internal limiting membrane. In the muscular-elastic layer, the proportion of lipid-laden cells is the highest in atherosclerotic plaques, remaining not higher than 5%. Cells with lipid inclusions were found among all cell types of the aortic intima; however, their proportion among stellate cells reaches 30%, which is considerably higher than that among other cell types.

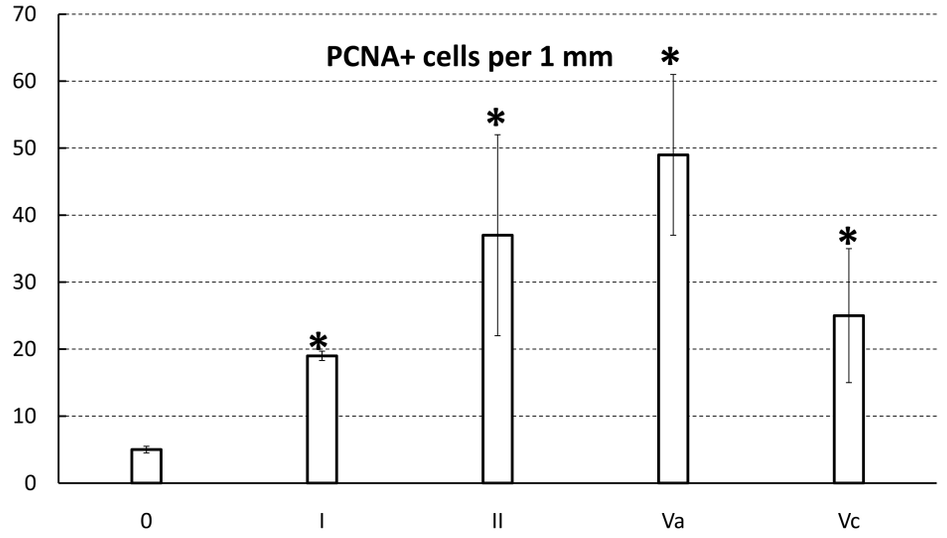
11. Proliferation (hypercellularity)

Cell number estimated on cross sections and in the suspensions obtained by alcohol-alkaline dissociation is twofold higher in atherosclerotic lesions compared to uninvolved intima [25]. The maximum cell number was revealed in lipid-rich lesions. In pronounced lipidrich lesions (fatty streaks and fibrolipid plaques), where the cell number is maximal, the number of both resident and inflammatory cells is increased. As the bulk of the intimal cell population (84-93%) is made of resident cells, the changes in the number of resident cells determine the increase in the cell content in atherosclerotic lesions. It should be noted that the cell number of fibrotic plaque is significantly lower than that of lipidrich atherosclerotic lesions.

For the identification of proliferating cells antibody to cyclin (proliferating cell nuclear antigen - PCNA), a protein expressed in the S-phase of cell cycle was used [74]. The number of proliferating cells in the lipidrich atherosclerotic lesions (fatty streak and fibrolipid plaques) is 10- to 20-fold higher than in uninvolved intima. The number of proliferating cells in fibrous plaques was found to be lower than in fatty lesions, being significantly higher than in uninvolved intima (Figure 7).

The proliferative index (the ratio between proliferating cells and the total cell number) for resident cells was considerably higher in all atherosclerotic lesions than in uninvolved intima [9, 74]. The maximum proliferative index (eight times as high as that in uninvolved intima) was recorded for fibrous plaques. Proliferative index of inflammatory cells is higher than that for resident cells; however, it is similar in uninvolved intima and atherosclerotic lesions and is comparable to that of peripheral blood leukocytes [9, 74]. From these findings, it can be suggested that the changes in cellularity of arterial intima is the result of resident cell proliferation and, probably, the migration of inflammatory cells. A “splash” of proliferative activity of resident cells takes place in lipid-rich lesions (fatty streaks and fibrolipid plaques). Proliferation of inflammatory cells was observed in the vascular wall too; however, proliferative index of inflammatory cells does not change in atherosclerotic lesions. Presumably, proliferative activity of inflammatory cells is a background process in atherosclerotic lesions and reflects the changes in the number of inflammatory cells as a result of their migration into subendothelial intima from blood stream. In contrast to resident cells, proliferative activity of inflammatory cells is not stimulated in atherosclerosis.

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0 – grossly normal (4), I – initial lesions (5), II – fatty streaks (4), Va - fibrolipid plaques (5), Vc - fibrotic plaques (6). The number of autopsy cases is indicated in the brackets.
*- significant difference from 0, $p<0.05$.

Figure 7. PCNA-positive cells in human aortic intima. On cross sections of human aortic intima, immunocytochemical identification of PCNA-positive cells was performed. Total cell number and number of cells with positively stained nuclei were calculated on each tissue section and the data from the same atherosclerotic lesions combined. Reproduced from Orekhov AN, Andreeva ER, Mikhailova IA, Gordon D. Cell proliferation in normal and atherosclerotic human aorta: proliferative splash in lipid-rich lesions. Atherosclerosis 1998;139(1):41-8 [74], with permission from Elsevier.

12. Fibrosis (synthesis and accumulation of extracellular matrix)

Accumulation of the extracellular matrix and formation of the connective tissue cap are the most clinically significant atherosclerotic manifestations. The total collagen content increases preferentially in the proteoglycan-rich layer but not in the muscular-elastic layer [25]. In uninvolved intima, cells producing collagen type I, the main interstitial collagen accumulated in atherosclerotic plaques, were not found [53]. In the proteoglycan-rich layer of atherosclerotic lesions, collagen-producing cells account from 6% (initial lesions) to 18% (fatty streaks) of total cell population [53]. These findings agree with immunohistochemical data on the localization of various collagen types in atherosclerotic lesions [25, 75, 76]. It was demonstrated that collagen is accumulated predominantly in the juxtaluminal intima.

The formation of the initial atherosclerotic lesions coincides with the emergence of cells producing type collagen I, the major interstitial collagen, which is accumulated in the cap of an atherosclerotic plaque. Formation of fatty streaks is accompanied by a considerable increase in the proportion of collagen-producing cells. Fibrollpid plaques have the maximum content of collagen-synthesizing cells, while in fibrous plaques the proportion of these cells is significantly lower, being comparable to that in initial lesion. Thus, in the sequence initial lesions - fatty streak - fibrolipid plaque fibrous plaque, a “splash” of synthetic activity of cells with the maximum in lipid-rich lesions (fatty streaks and fibrolipid plaques) has been detected [53].

Hypersecretion of extracellular matrix may be a cause or a consequence of disintegration of the intimal cellular system in atherosclerotic lesions. In initial lesions, where the cellular network is preserved, collagen-producing cells are not numerous and are integrated into a network [53]. The network is destroyed in fatty streaks and atherosclerotic plaques. The proportion of collagen-producing cells in these lesions is higher than in initial lesions, and the cells are located at the sites of the network disintegration [53]. This suggests that synthetic activity of cells increases considerably after they have lost contact with neighboring cells.

13. Mechanisms of cellular lipidosis

Bearing in mind that the "splash" of proliferative activity and collagen production occur in lipid-rich lesions, it can be suggested that there is a relationship between the main atherosclerotic manifestations: lipid accumulation (lipidosis), proliferation, and secretion of the extracellular matrix (fibrosis). Since the proliferative activity and the collagen-synthetic activity are the highest in the zones of lipidosis, it was important to find out how the accumulation of lipids in cells (cellular lipidosis) is related to other manifestations of atherosclerosis, namely, proliferation and fibrosis. To investigate this relationship, lipid accumulation was induced in cultured cells with maximum adequacy to cellular lipidosis occurring in the vascular wall [77, 78].

Disturbances in cell metabolism of lipids may be one of the causes of intracellular lipid accumulation. In the cells cultured from atherosclerotic lesions, the rate of the synthesis of main classes of lipids is higher than that in cells cultured from uninvolved human aortic intima [79]. The rate of lipid synthesis is directly correlated with the intracellular lipid content: the higher intracellular excess of fat, the higher the intensity of lipid metabolism in atherosclerotic cells [79]. However, these findings provide no answer to the following question: what is the cause of lipid metabolism dysfunction in the vascular wall that led to excessive lipid accumulation in vascular cells?

Low-density lipoprotein (LDL), the major lipid carrier in the blood, is the main candidate for the source of lipids that overload cells of atherosclerotic lesions. Considerable efforts have been concentrated on induction of lipid accumulation in cultured cells by adding LDL to the culture medium. However, most attempts failed: even high concentrations of LDL in culture medium induced no lipid accumulation [80]. This is due to reverse regulation of cholesterol metabolism in the cell, when the number of specific LDL receptors on the cell surface decreases in response to increased intracellular cholesterol content, which prevents LDL internalization via the receptor pathway [81].

It was demonstrated in several laboratories that intracellular lipid accumulation can be induced by LDL-containing insoluble complexes, such as LDL associated with glycosaminoglycans, proteoglycans, fibronectin, collagen, elastin, and other components of the arterial wall connective tissue matrix [77, 80, 82-92]. This pathway for intracellular lipid accumulation is quite possible, since all conditions are present in the vascular wall.

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In addition, LDL association (formation of complexes including several LDL particles) is a necessary and sufficient condition for the accumulation of intracellular lipids [86, 87]. Presumably, LDL associates, like LDL-containing insoluble associates, which are essentially large particles, are internalized into the cell not via the receptor pathway but by nonspecific phagocytosis. This may result in unregulated lipid deposition and lipid overload. Native LDL generally does not form associates, while chemically modified lipoprotein readily associates, forming rather large particles. It has been shown that all known chemical modifications of the lipoprotein, including naturally occurring multiple-modified LDL, stimulate LDL association [86-88].

Atherogenic modified LDL circulating in the blood, but not native LDL, induces lipid accumulation in cultured cells [88]. Modifications occur in protein, carbohydrate, and lipid moieties of an LDL particle; as a result, its size, charge, density, immunogenicity, and many other properties change [89]. As mentioned above, lipid accumulation caused by multiple-modified LDL is observed only when modified LDL forms associates. Similar to native LDL, unassociated modified LDL does not induce intracellular lipid accumulation [77]. A direct and strong correlation was established between the ability to induce lipid accumulation and the size of LDL associate [88].

Multiple-modified LDL has been isolated from the blood of patients with assessed atherosclerosis and from healthy subjects. However, the content of modified LDL in the blood of patients with atherosclerosis of any localization is considerably higher than in healthy subjects [90]. The positive correlation was revealed between the blood level of modified LDL and atherogenic potential of blood serum, i.e., its ability to induce intracellular lipid accumulation [90].

14. Relationship between lipidosis and other atherosclerotic manifestations

Blood serum and LDL isolated from blood of atherosclerotic patients capable to induce intracellular lipid accumulation can also lead to other atherogenic manifestations in cultured cells of subendothelial human intima [91]. Preincubation of cells with atherogenic serum or LDL, causing intracellular lipid accumulation, also stimulates cell proliferation and synthesis of collagen, glycosaminoglycans, and total protein [91, 93]. Thus, blood serum and LDL of atherosclerotic patients have a broad spectrum of atherogenic properties, causing all main manifestations of atherosclerosis: enhancement of cell proliferation, fibrosis, and lipidosis. Direct contact of modified LDL with cells is not necessary for stimulation of proliferation and production of the connective tissue matrix components. Increased proliferative activity and extracellular matrix production are preserved for several days after removal of atherogenic LDL from the culture medium [91]. This finding suggests that not the interaction between LDL and cells but rather the consequences of this interaction, i.e., lipid accumulation, which coincides with increased cell proliferation and extracellular matrix production, are the crucial aspect in the realization of atherogenic potential at the cellular level.

As mentioned above, lipid accumulation affects the integrity of cellular network in human aortic intima. In the lipid-rich lesions, where the content of lipid-laden and foam cells is high, rupture of the network have been observed. It should be noted that cells not contacting with their neighbors are often overladen with lipids [67].

A decrease in the rate of intracellular communication between cultured cells caused by modified LDL which induce intracellular lipid accumulation may account for the decreasing of cell contacts and their disruption. In fact, incubation of subendothelial intimal cells with modified LDL results in accumulation of intracellular lipids with a parallel reduction of the degree of intercellular communication [73]. It is reasonable to suggest that the reduction of cell-to-cell contacts is a consequence of lipid accumulation.

15. Atherogenic manifestations and cyclic nucleotides

Simultaneous and unilateral atherogenic modifications leading to accumulation of intracellular lipids, enhanced proliferation, and hypersecretion of extracellular matrix point to a relationship between these processes. This relationship may occur at the level of cellular regulation, since all these processes are regulated by cyclic nucleotides, universal cellular regulators. It is well known that cyclic 3',5'-adenosine monophosphate (cAMP) inhibits cell proliferation, lipid synthesis and interactions between LDL and cell receptors, and synthesis and secretion of proteins (e.g., collagen and glycosaminoglycans) but stimulates hydrolisis of lipids. On the other hand, cyclic 3',5'guanosine monophosphate (cGMP) has opposite effects on these processes [94, 95].

It was found that cAMP content in atherosclerotic cells (cells populating atherosclerotic lesions) is two- to eight-fold lower than in normal intimal cells, while cGMP content in atherosclerotic cells is more than twofold higher than in normal cells [94, 96]. It was demonstrated that changes in the content of cyclic nucleotides are associated with altered activity of enzymes involved in their synthesis and degradation (cyclases and phosphodiesterases, respectively) [97]. For example, the activity of adenylate cyclase, an enzyme synthesizing cAMP, in fatty streak is lower than in uninvolved intima, being the lowest in atherosclerotic plaque. At the same time, the content of phosphodiesterase, an enzyme hydrolyzing cAMP, remains unchanged, which accounts for cAMP drop in atherosclerotic cells [97]. On the other hand, the activity of guanylate cyclase, an enzyme that synthesizes cGMP, is higher in fatty streak than in uninvolved intima, while the activity of phosphodiesterase is unchanged [97]. In atherosclerotic plaques, the situation is different: the activity of guanylate cyclase is the same as in uninvolved intima, while the activity of cGMP phosphodiesterase is much lower [97]. These changes clearly explain increased cGMP content in atherosclerotic lesions.

On the basis of numerous data, it can be stated that a decrease in the intracellular content of cAMP and an increase in that of cGMP in atherosclerotic lesions should lead to the stimulation of the main atherosclerotic manifestations at the cellular level, i.e., cell proliferation, lipid accumulation, and extracellular matrix production [95].

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A question arises: what are the causes of the atherosclerosis-related changes in the intracellular contents of cyclic nucleotides? Upon incubation of human aorta intimal cells with modified LDL, a substantial lipid accumulation and parallel cAMP decrease and an increase of cGMP increase were observed, i.e., the exact changes in cyclic nucleotides occurring in atherosclerotic lesions (Table 2). It can be suggested that changes in the cyclic nucleotide system are caused by intracellular lipid accumulation.

	cholesterol	**cAMP**	**cGMP**
natLDL	105±14	90±10	96±10
modLDL	259±31	52±7*	254±26*

The data are presented as percent of control (cells without LDL).

*: Significant difference from the control, $p<0.05$.

Table 2. The effect of modLDL-induced intracellular cholesterol accumulation on the cAMP and cGMP content in human aortic intimal cells

Bearing in mind the data on cyclic nucleotides, a more precise scheme for the development of atherosclerotic manifestations at the level of subendothelial cells can be proposed. In the intima, modified LDL forms associates or LDL-containing complexes which activate phagocytosis. This reduces cell-to-cell contacts. Since modified LDL is internalized not by the regulated receptor pathway, lipids are accumulated in intimal cells, which decreases intracellular content of cAMP and increases that of cGMP. This stimulates lipid accumulation, cell proliferation, and extracellular matrix secretion. Presumably, these events occur at the early stages of atherogenesis. Assuming that further development of an atherosclerotic lesion proceeds in the same direction, intracellular lipid content, cell proliferation, and extracellular matrix production should constantly increase. This argues with the observations, cited above, that the intensity of atherosclerotic manifestations decreases in pronounced fibrotic atherosclerotic lesions in comparison with more early lipid-rich lesions and prompts further search for causes of the bell-shaped change in cellular lipidosis as well as in proliferative and synthetic activity observed in atherosclerosis.

16. Concluding remarks

The above-presented data allows us to suggest sequence events responsible for the initiation and the progression of atherosclerotic process. Modified LDL or/and their associates enter subendothelial intima from the blood. LDL associates or LDL-containing complexes stimulate phagocytosis, which reduces intercellular communication. LDL enters the cells via a pathway other than regulated receptor lipoprotein uptake and induces intracellular lipid accumulation. This lowers cAMP and elevates cGMP, stimulating lipid accumulation, cell proliferation, as well as synthesis and secretion of the extracellular matrix. Such a situation is typical of initial

atherosclerotic changes in the intima. Further accumulation of lipids internalized by phagocytosis leads to isolation of cells, disruption of cell-to-cell contacts, and disintegration of the cellular network characteristic of uninvolved intima. Foam cells laden with lipids appear. Further lipid accumulation in intimal cells and destruction of cell-to-cell contacts stimulate cell proliferation and extracellular matrix production. This is typical of pronounced lipid-rich lesions (fatty streak and fibrolipid plaque). Secreted collagen and other components of the extracellular matrix surround the matrix-producing cells and isolate them from other neighboring cells; this eventually leads to disintegration of the cellular network. The intensity of the major atherosclerosis-related processes (intracellular lipid accumulation, cell proliferation, and extracellular matrix production) decreases considerably. This occurs in a fibrous plaque.

Proceeding from this concept, intracellular lipid accumulation induced by modified LDL is the crucial event of atherogenesis. Lipid accumulation stimulates the major atherosclerotic manifestations; at the same time, associates of modified LDL destroy the cellular network in the intima by stimulating phagocytosis. This leads to the formation of pronounced lipid-rich lesions, namely, fatty streaks or fibrolipid plaques, where the intensity of atherogenic manifestations at the cellular level is maximal.

The intensity of cell functions activated in atherosclerosis is similar in uninvolved intima and atherosclerotic plaques. However, there is a principal difference in the state of cellular systems in these zones. Although functionally the cellular system in a fibrous plaque is similar to that of uninvolved intima, structurally it is changed to the extent indicating an irreversible transformation. Therefore, transformation of fibrous plaque to uninvolved intima is impossible. Clinically, it is unlikely that fibrous plaque is dangerous, since it is a stable, low-activity formation.

The situation is completely different in lipid-rich lesions, specifically in a fibrolipid plaque (atheroma). This is an unstable lesion with maximally or near-maximally active cellular processes. Clinically, these lesions are most dangerous, since they rapidly develop, and their growth leads to so-called rupture of the plaque which is accompanied by critical local changes in homoeostasis resulting in thromboembolic events, which is the cause of vascular catastrophes such as myocardial infarction, stroke, sudden death, etc. However, fibrolipid plaques and especially initial lesions are probably reversible, since changes in their cellular system presumably are not irreversible. Based on the suggestion that lipid accumulation is the key event in the initiation and development of atherosclerotic lesions, one may assume that prevention of lipid accumulation and removal of excessive fat from cells are most effective approaches to the prevention and reversion of atherosclerotic lesions preceding the fibrous plaque.

The above-provided data is indicative that α-smooth muscle actin+ pericyte-like cells represent the key players in the development of atherosclerotic lesions. Obviously, further studies are needed to understand the mechanisms of interaction between α-smooth muscle actin+ pericyte-like cells with other cell types during atherogenesis and evaluate how functioning of α-smooth muscle actin+ pericyte-like cells affects immune-inflammatory processes in atherogenesis. The involvement of pericyte-like cells in the regulation of immune-inflammatory

processes in atherosclerosis is supported by a finding of widespread expression of HLA-DR antigen by the vast majority of cells residing in the arterial subendothelial space [98].

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