

Cellular Mechanisms of Human Atherosclerosis: Cellular Composition of Normal and Atherosclerotic Intima: Focus on Pericytes

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INFLAMMATORY AND RESIDENT CELLS

Immunocytochemical analysis has demonstrated that cells of the muscular-elastic layer of intima represent a homogeneous population made mostly of smooth muscular cells. These cells are similar to the smooth muscular cells of the media that are almost uniformly positive for smooth muscular α -actin. Proteoglycan-rich layer of intima is populated both by resident cells and cells of hematogenous origin (inflammatory cells). In atherosclerotic lesions, percentage of the resident cells positive for smooth muscular α -actin remains stable, whereas percentage of inflammatory cells increases, however, never becoming predominant. Resident cells of the proteoglycan-rich layer are actively involved in the pathogenesis of atherosclerosis. These cells differ from typical smooth muscle cells, as they express markers of pericytes and a macrophage-associated antigen CD68.

What are the characteristics of the resident intimal cells? Morphological heterogeneity of this population has been described in the early works of Langhans, Schönfelder, Shchelkunov, Khavkin, Geer & Haust [1-6] and others. Alcohol-alkaline dissociation method was used to reveal cells of various shapes [7,8].

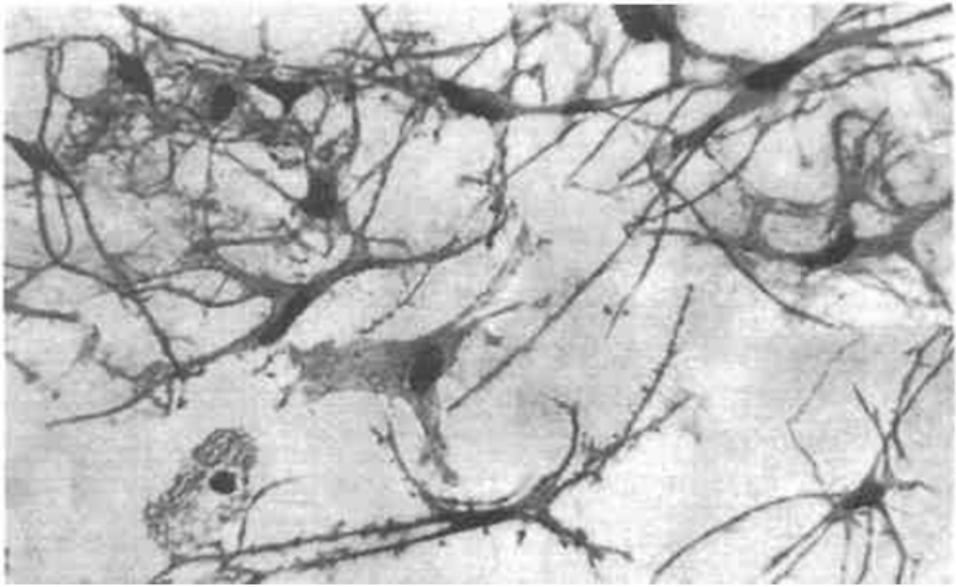


Figure 1: Heterogeneity of cells from the proteoglycan-rich intimal layer Cell suspension after alcohol-alkaline dissociation of fixed tissue. Toluidin blue staining. Magnification x 350.

Morphological heterogeneity of intimal cells, which has been previously described by many authors [6,1,9,2,5] can be observed in the cell suspension after dissociation of the extracellular matrix (Figure 1). Similar to the smooth muscle cells populating the media, cells of the muscular-elastic layer usually have elongated bipolar shape. In contrast, the proteoglycan-rich layer is populated by cells of various shape, from elongated to stellate. The presence of stellate cells with processes [1] is the main characteristic of the cell population of the proteoglycan-rich layer.

Polar morphological types of the subendothelial cell population can be characterized as follows. Bipolar cells have elongated bodies, usually without processes or with small lateral protrusions. They form dense cellular layers, express the smooth muscular marker α -actin and have a prominent contractile apparatus [8]. Stellate (pericyte-like) cells have rounded body and three or more processes. They are distributed without visible orientation in the connective tissue matrix of the subendothelial intima. Apart from α -actin, they express antigens typical for pericytes 3G5 and 2A7, macrophage antigen CD68 [10,11], which is a scavenger receptor. In atherosclerotic plaques, these cells have the highest lipid load (foam cells) [12]. Electron microscopy reveals large numbers of organelles in the cytoplasm of these cells [8]. In atherosclerotic lesions, total number of cells increases by 2 folds, whereas number of stellate cells increases by more than 6 folds [7]. As a result, atherosclerotic lesion development is associated not only with the increase, but also with qualitative change of the intimal cell population.

Data on the number of hematogeneous cells in atherosclerotic lesions are contradictory. Animal studies have demonstrated that macrophages are predominant in the intima in

atherosclerotic lesions [13,14]. Macrophages play a special role in atherogenesis because they can actively accumulate lipids becoming foam cells [15-17]. The discovery of numerous CD68-positive cells on vertical sections of human arterial wall with atherosclerotic lesions led to the concept that macrophages are the primary cell type responsible for the formation and progression of atherosclerotic plaques [18,19]. Kaartinen and co-authors [20] demonstrated high macrophage content in fatty streaks and fibrolipid plaques (23% and 42% respectively) in human aorta, which led them to the conclusion that inflammatory cells play the key role in atherosclerotic lesion development. Other authors have also demonstrated the relative increase of macrophage count in atherosclerotic lesions. However, macrophage content was not higher than 17% of total cell population, hence, changes of macrophage count was less prominent than reported by Kaartinen and co-authors [16]. It has to be noted that, although the involvement of macrophages in the pathogenesis of atherosclerosis is not disputed, they are not the predominant cell type in human aortic intima. Variability of the currently available data on the relative quantity of macrophages in atherosclerotic lesions could be explained by the fact that the results were obtained for different arteries (coronary artery, carotid artery, aorta). It is possible that difference in cellular content of atherosclerotic lesions reflect the existing difference in mechanisms of lesion development in different parts of the vascular system. Moreover, the observed difference in macrophage content could be explained by the use of certain antibodies for cell typing. Antibodies to CD14 lipopolysaccharide receptor recognize peripheral blood monocytes and monocyte-derived macrophages. Very few CD14-positive cells can be found in grossly normal intima, whereas in atherosclerotic lesions their number increases. Antibodies MAC3 that recognize tissue macrophages and activated monocytes allow detecting cells both in normal intima and atherosclerotic lesions. The maximal number of such cells was found in fatty lesions (fatty streaks and fibrolipid plaques), and was significantly higher than the number of cells expressing CD14.

The question on the relative amounts of inflammatory and resident cells in grossly normal intima and atherosclerotic lesions remains to be fully answered. Some authors have demonstrated that the percentage of smooth muscular cells decreases in atherosclerotic lesions, and the increase of total cell population is dependent on inflammatory cells. For instance, Aqel with co-authors [16] demonstrated that the number of macrophages in lesion areas increases in comparison to control and approaches the corresponding value for the smooth muscular cells. Another study reported different numbers. In fatty streaks, the ratio between smooth muscular cells and macrophages was estimated to be 3:1, which was in accordance with results obtained by Katsuda and co-authors [21] while studying cellular composition of fatty streaks in human aorta: 23:9.

Identification of lymphocytes, another type of inflammatory cells in the arterial wall, became a subject of numerous studies [22-25]. However, the current knowledge on the number of these cells in human aortic intima remains to be limited. Kaartinen and co-authors [20] studied the cellular composition of human intima reporting the following numbers of the lymphocyte content: 2% in grossly normal intima, 8% in fatty streaks, 7% in fibrolipid plaques.

Changes of resident and hematogenous cell count in the aortic intima were assessed using a mixture of antibodies CD45+CD14, specific for lymphocytes and macrophages, which allowed evaluating the total number of inflammatory cells. In atherosclerotic lesions, both resident and hematogenous cells were more numerous. Percentage of hematogenous cells increased to a larger extent (by 8 folds in comparison to grossly normal intima) than that of resident cells (by 3 folds). However, even such dramatic increase did not make the inflammatory cells predominant in atherosclerotic lesions, where their content did not exceed 20% of total cell population.

The number of resident and inflammatory cells in the subendothelial intima was assessed using the mixture of antibodies CD45+CD14 for staining vertical sections of human aorta. It was demonstrated that along the supposed course of atherosclerotic lesion progression: grossly normal-initial lesions-fatty streaks-fibrolipid plaques-fibrous plaques, the number of cells followed a bell-shaped curve, with maximal cellularity detected in fatty lesions. Similar picture was obtained for resident and inflammatory cells (Figure 2). Both in grossly normal intima and in atherosclerotic plaques resident cells represented the majority of cell population.

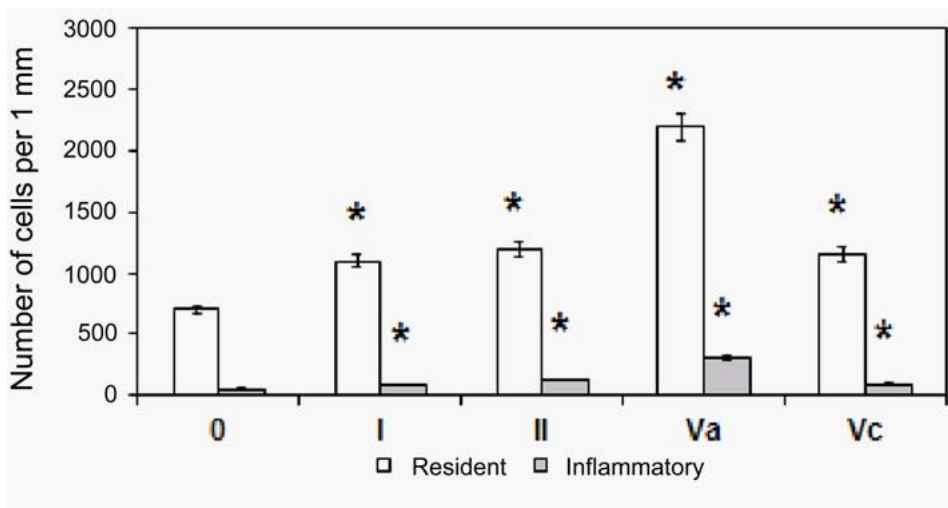


Figure 2: Number of resident and inflammatory cells in human aortic intima. Numbers of resident and inflammatory cells in human aortic intima were assessed by staining of vertical sections with antibodies specific for inflammatory cells (CD45+CD14), per 1 square mm of the luminal surface of the vessel. Resident cells were detected as all cells negative for antibody staining.

0 - grossly normal (4), I - initial lesions (5), II - fatty streaks (4), Va - fibrolipid plaques (5), Vc - fibrotic plaques (6). Number of analysed cases is presented in brackets.

* - Statistically significant different from grossly normal intima, $p < 0.005$

Estimation of the number of smooth muscular cells in atherosclerotic lesions and grossly normal intima is one of the key questions required for understanding the pathogenesis of

atherosclerosis. Immunocytochemical typing demonstrated that, while the total number of cells increased in atherosclerotic lesions, the percentage of smooth muscular cells remained unaltered (Table 4). In fatty streaks, a certain decrease of the number of smooth muscular cells was detected in comparison to uninvolved intima. Such decrease can be explained first by the fact that intracellular lipid accumulation is accompanied by loss of contractile filaments that are used for detection of this cell type, second, by possible re-distribution of actin filaments to the cell periphery, which hinders correct identification of cells by their nuclei.

The involvement of different intimal layers in atherogenesis is not equal [26]. Muscular-elastic layer adjacent to the media is not significantly altered, and main pathological processes linked to atherogenesis (lipid accumulation, collagen deposition, increase of cellularity) take place in the proteoglycan-rich layer. Qualitative and quantitative changes of the cellular content also occur mainly in the proteoglycan-rich layer. This layer hosts all the inflammatory cells, resident cells expressing pericyte markers, unusual for typical smooth muscular cells, smooth muscular cells expressing the macrophage antigen CD68. Most probably, such changes play an important role in the initiation and progression of atherosclerotic lesions.

STELLATE CELLS

One of the important questions for understanding of atherogenesis is revealing the possible link between changes of cellular composition and typical features of atherosclerotic lesion, such as intimal thickening, lipid accumulation and collagen deposition. Calculated correlation coefficients for these events are listed in Table 1. The highest correlation coefficients were obtained for stellate cells. The closest link was revealed between the number of stellate cells and total lipid and cholesterol esters contents. Correlation coefficients between the stellate cell count and collagen deposition and intimal thickening were lower, but still exceeded the corresponding values obtained for elongated cells and the total number of cells [8].

Stellate cells are located next to the lumen and represent the second barrier of the arterial wall after the endothelial lining. Such location is likely to explain the involvement of these cells in the early pathological changes in the arterial wall.

The most striking examples of stellate cells include neural cells and dendritic cells of lymphatic follicles, mesangial cells of renal glomeruli and others. Stellate shape allows maintaining intercellular communications in the tissue mediated by contact-forming processes. It can be speculated that stellate cells of the subendothelial intima have similar functions, forming a communicative network with their long processes.

Table 1: Correlation between the number of cells and atherosclerotic features (lipid accumulation, collagen deposition and intimal thickening).

	Stellate cells	Elongated cells	Total cell count
Collagen	0.80	0.54*	0.63*
Total lipids	0.95	0.66*	0.81*
Cholesterol	0.86	0.53*	0.68*
Cholesterol esters	0.96	0.72*	0.76*
Triglycerides	0.94	0.64*	0.80*
Phospholipids	0.90	0.57*	0.72*
Intimal thickening	0.73	0.50*	0.58*

* - statistically significant correlation for stellate cells, $p < 0.05$.

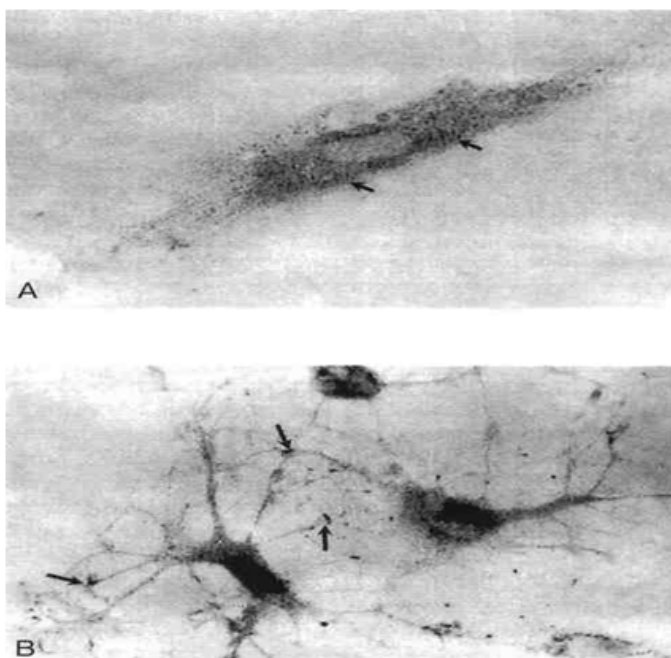


Figure 3: Connexin 43 (Cx43) in human aortic intimal cells in primary culture. ABC-
peroxydase, hematoxylin, Magnification x 200.

At day 7 of primary cell culture cells derived from grossly normal intima were treated with methylbutyl xanthine (MIX), 10^{-4} M. After 3 h of incubation, cells were washed, fixed in 4% formaldehyde and stained with antibodies specific for Cx43.

a – cells before treatment with MIX, Cx43 localized in small dots on the cell surface,

b – arborized cells after 3 h in incubation with MIX, Cx43 in the form of large patches located mostly at the ends of processes of arborized cells.

Arborization, hence, formation of stellate cells, could be induced in primary culture, where

cells usually have flat polygonal shape, by increasing the intracellular level of cAMP (Figure 3). In cultures from the proteoglycan-rich layer, almost 100% of cells acquired the stellate shape, while in cultures from muscular-elastic layer the percentage of arborized cells did not exceed 50%, and in cultures from the media - 10% [27].

Arborization of cells was associated with redistribution of connexin 43 (Cx43), the main protein of gap junctions, highly specialized intercellular contacts. This protein is located at the cell surface in structures that are called connexin plaques. On non-arborized cells, Cx43 plaques are small and evenly distributed. On arborized cells, apart from small plaques, larger Cx43-positive structures are present at the tips of cellular processes where intercellular contacts are formed (Figure 3).

Therefore, increase of the intracellular cAMP leads to arborization of the subendothelial intimal cells associated with redistribution of connexin plaques and, probably, enhancement of intercellular communication via gap junctions. It can be speculated that stellate shape of these cells is essential for intercellular contact formation in the arterial intima. The ability of intimal cells to arborize is not shared by the typical smooth muscular cells of the media that have reduced or virtually absent capacity for arborization.

In addition to the observations described earlier, the ability of intimal cells to arborize supports the suggestion that subendothelial intimal cells represent a distinct cell type, different from typical smooth muscular cells.

QUANTITATIVE CHANGES OF INTIMAL CELL POPULATION

Total cell count was assessed in grossly normal areas of intima and in atherosclerotic plaques on vertical sections of human aorta. In atherosclerotic lesions, number of intimal cells was higher than in control, and intimal thickness was significantly increased. To evaluate the changes of intimal cellularity, 1 mm-wide parts of the sections containing the whole depth of the intima were analyzed. In comparison with grossly normal areas, total cell count in initial lesions was 1.5 folds higher, reaching statistical significance (Table 2). Significant increase of cellularity was revealed also in fatty streaks and fibrolipid plaques (fibroatheromas), which contained 3 times more cells than uninvolved intima. At the same time, cell count in fibrous plaques was lower than in fibroatheromas and comparable to that in uninvolved intima.

Therefore, change of intimal cellularity follows a bell-shaped curve along the line: grossly normal intima-initial lesion-fatty streak-fibrolipid plaque-fibrotic plaque, with maximal cellularity reached in fatty lesions.

Cells of the proteoglycan-rich layer represent the most dynamic population in the arterial wall. Age-related intimal thickening is associated with an increase of cellularity in this layer. The highest density of proteoglycan-rich layer cells is observed at the age of 30-55 years, after that the number of cells decreases.

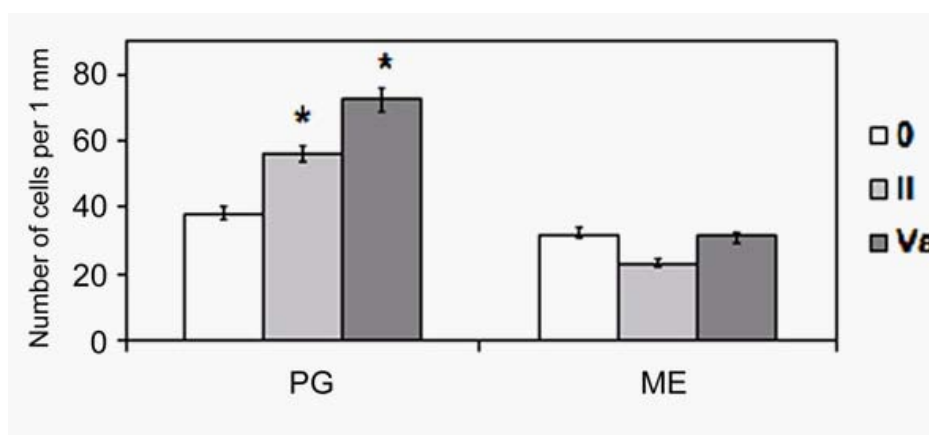
Table 2: Number of intimal cells in human aorta.

Lesion type	Total cellularity, cells per 1 mm	Number of resident cells per 1 mm	Number of henatogenous cells per 1 mm
0	780±60	660±60	40±6
I	1120±40*	1040±40*	80±6*
II	1320±60*	1180±40*	120±20*
Va	2560±220*	2200±180*	360±60*
Vc	1160±100*	1060±80*	80±80*

0 – grossly normal intima; I – initial lesion; II – fatty streak; Va – fibrolipid plaque; Vc – fibrotic plaques.

* - Statistically significant difference from grossly normal intima, $p < 0.05$.

The increase of proteoglycan-rich layer cellularity also occurs in other diseases. The most dramatic changes are associated with general septic and toxic processes, terminal pneumonia, diffused septic peritonitis and meningitis. Increased cellularity was also detected in glomerulonephritis and persistent hypertension. In contrast, carcinomas, myelosis and diabetes mellitus are associated with decreased cellular count [2].

**Figure 4:** Number of intimal cells in human aorta.

Number of cells in intimal layers was assessed on vertical sections of aortic wall stained with hematoxylin-eosin, counted per 1 mm of luminal surface of the vessel

0 - grossly normal intima (5), II - fatty streak (7), Va - fibrolipid plaque (6). Number of analyzed cases is presented in brackets.

PG – Proteoglycan-rich layer of intima, ME – muscular-elastic layer of intima.

* - Statistically significant difference from grossly normal intima, $p < 0.05$.

The cell count in grossly normal intima and atherosclerotic plaques was assessed using the

method of alcohol-alkaline dissociation of fixed tissue [9]. In the proteoglycan-rich layer, number of cells in fatty streaks and in lipofibrous plaques was increased by 1.5 and 2 folds respectively. At the same time, cellularity of the muscular-elastic layer and the media was not significantly changed [28]. Similar results were obtained on vertical sections of human aorta. Number of cells in the muscular-elastic layer was similar in uninvolved intima and atherosclerotic lesions, whereas the number of cells in the proteoglycan-rich layer was increased by 2 folds. Therefore, atherosclerotic lesion development is associated with an increase of cellularity of the proteoglycan-rich layer.

Along with the increase of total cell count in the proteoglycan-rich layer, atherosclerotic lesion development is associated with changes in the proportion of the main morphological cell types [29]. The number of stellate cells in the proteoglycan-rich layer dramatically increases in comparison with other cell types. The amount of elongated smooth muscular cells in atherosclerotic plaques increases only by 1.5 folds in comparison with grossly normal intima, whereas the number of stellate cells increases by 13 folds [26].

Study of the cellular composition of human arterial intima has a long history [1,26,30,31]. Resident subendothelial cells that are different from typical smooth muscular cells have been called intimal fibroblasts [1], mesenchymal reserve cells [30], pericytes or vascular cambial cells [3]. However, after a series of electron microscopy studies, an opinion was established that intimacytes of large arteries are modified smooth muscular cells [32,33].

Therefore, the total cell count increases in atherosclerotic lesion areas in comparison to uninvolved intima. According to a common hypothesis, hypercellularity is a key feature of atherogenesis [13,14]. However, the increase of cell count is less dramatic than has been considered before.

CELLULAR NETWORK

About 50 years ago, Schönfelder [2], while studying horizontal sections of intima, suggested that intial stellate cells can be connected into a network. He described empty channels between the interconnected processes that could penetrate deep into the intimal layer.

The concept of cellular network in the intimal layer was supported by the results of recent studies. Scanning electron microscopy revealed intercellular contacts in the proteoglycan-rich layer of normal aortic intima. All cells of the layer contacted with each other forming a horizontal network [34]. Cells located at different levels were also connected with each other, forming links between the networks of different levels and hence a three-dimensional network structure. All cellular processes formed contacts with processes or bodies of other cells. Therefore, proteoglycan-rich layer of the intima can be regarded as a system of interconnected cells. Free spaces between the cellular processes are filled with connective-tissue matrix.

It was demonstrated that cells of the muscular-elastic layer do not form a network typical for stellate cells of the proteoglycan-rich layer. Scanning electron microscopy studies have shown that most of the cells in this layer have elongated bipolar shape typical for smooth muscular cells

of the media. Cells in the muscular-elastic layer are densely packed forming layers positioned at small angles to each other.

There is a continuous vertical gradient of the ratio between stellate subendothelial cells with processes and elongated smooth muscular cells. The number of elongated cells decreases in the direction from the muscular-elastic layer to the endothelium, and the percentage of stellate network-forming cells correspondingly increases.

Cellular system of the intima is significantly altered in atherosclerosis. In fatty streaks, stellate cells are usually loaded with lipids [12,8,35]. Apparently, lipid accumulation is linked to the observed enlargement of cells, blebbing of the cell surface and formation of lipid droplets and vesicles. Extracellular vesicles, probably formed by budding of the cellular processes, can also be observed. It is possible that active vesicle formation at the tips of processes causes dissociation of intercellular contacts.

In atherosclerotic plaques, changes of the cellular system are even more pronounced. In the surface layer of the connective tissue cap of atherosclerotic plaque, stellate cells are always observed as isolated cells or present in small groups. Lipid inclusions can be seen in some cells. The number of stellate cells increases in deeper layers of the intima, but normal cellular network is nevertheless absent. Interestingly, normal three-dimensional cellular network can be found near the shoulder areas of atherosclerotic plaques, where no visible features of atherosclerotic lesion are present. These cells do not form contacts with fragments of cellular network present in the atherosclerotic plaque.

INTERCELLULAR COMMUNICATION

Atherosclerotic lesion formation is therefore associated with disconnection of the cellular network leading to complete isolation of intimal cells in atherosclerotic plaques. It is well known that regulation of interconnected cells in tissues can be mediated via a special form of intercellular contacts, gap junctions [36]. Gap junctions play an important role in regulation of tissue homeostasis, allowing transport of cellular metabolites, second messengers and other biologically active molecules between cells. Presence of gap junctions is a typical feature of differentiated cellular systems with high levels of intercellular integration [37-41]. As mentioned before, stellate shape and contact-forming of the intimal cells may be an important factor regulating the arterial wall function. Gap junction dysfunction can be one of the possible mechanisms of cellular network dissociation observed in atherosclerosis.

The degree of intercellular communication mediated by gap junctions can be estimated by the expression levels of contact-forming proteins. Connexin 34 (**Cx43**) is the principal protein of gap junctions, which is located on the cell surface in so-called connexin plaques. The number of connexin plaques is decreased by 3 folds in fatty streaks in comparison with uninvolved intima.

Importantly, in areas of atherosclerotic lesions, cells with lipid inclusions have less **Cx43** plaques those cells without visible lipid accumulation (Figure 5).

At the same time, in grossly normal intima, the number of **Cx43** plaques per cell is similar regardless of the cell location depth (Figure 6). In atherosclerotic lesions, the number of **Cx43** plaques per cell decreases in the surface areas of the proteoglycan-rich layer, while in the deeper areas, at the border with the muscular-elastic layer, this number is higher and similar to that of the uninvolved intima (Figure 6). These results are in accordance with previously reported observations that in atherosclerotic lesions, cellular network is disturbed in the surface layers, but remains intact in the deeper layers of the arterial intima.

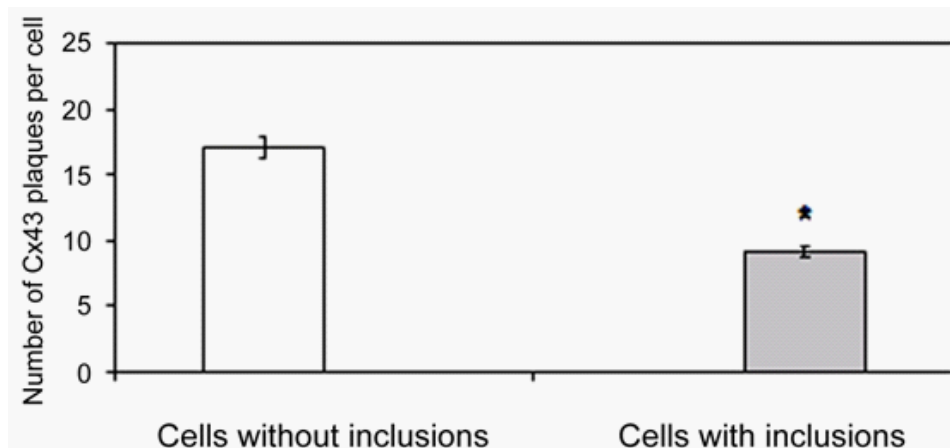


Figure 5: Number of Connexin 43 (**Cx43**) plaques on cells with and without lipid inclusions in human aortic intima.

Cx43 was visualized by immunocytochemistry on fixed sections. Sections were post-stained with filipin to reveal cholesterol esters in intracellular lipid inclusions. **Cx43** plaques were counted on cells with and without lipid inclusions.

* - statistically significant difference from cells without lipid inclusions, $p < 0.05$.

Study of the possible mechanism of disturbed intercellular communication in atherosclerotic lesions was performed on primary culture of aortic cells. Apart from staining for **Cx43**, level of intercellular communication can be estimated by tracing the transfer of fluorescent dye microinjected in one of the contacting cells into adjacent cells. Using fluorescent dye that can be specifically transferred through gap junctions allows evaluation of intercellular communication efficiency.

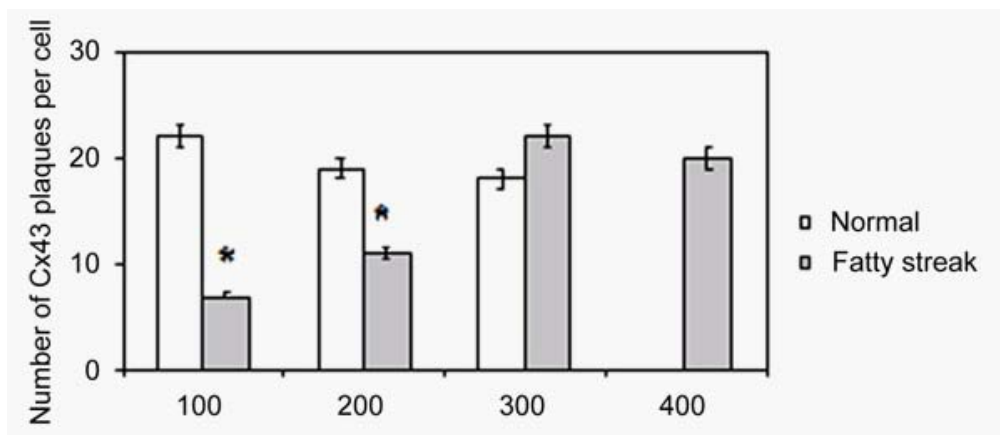


Figure 6: Number of Connexin 43 (Cx43) plaques on cells from normal intima and atherosclerotic lesions from human aorta.

Cx43 was revealed by immunocytochemistry on fixed sections of grossly normal areas and atherosclerotic lesions of human aortic intima. Number of **Cx43** plaques per cell was calculated for each sample. Number of **Cx43** plaques per cell was calculated for cells located at different distances from the vascular lumen (indicated in μm).

* - statistically significant difference from the normal intima, $p < 0.05$.

The level of intercellular communication was evaluated using both of the described methods in cultures with different density. The obtained results were consistent. Comparison of cultures derived from grossly normal areas and atherosclerotic lesions demonstrated that intercellular communication rate was 1.5 folds higher in cultures from uninvolved intima [35].

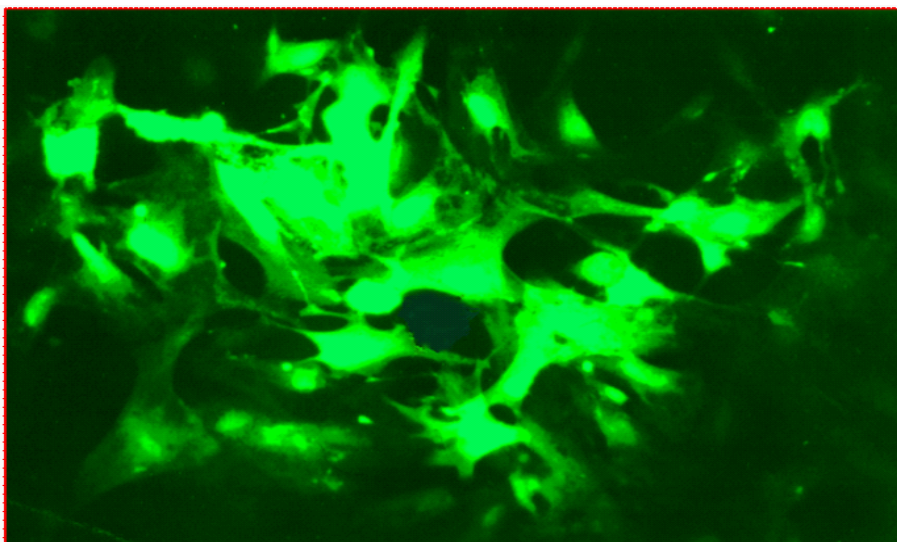


Figure 7: Distribution of fluorescence in primary culture of subendothelial cells from human aortic intima after injection of calcein into one of the cells.

Presence of cells with lipid accumulation (foam cells) is a typical feature of cultures obtained from atherosclerotic lesions. To test the hypothesis that lipid load is linked to the disturbance of intercellular communication, fluorescent dye was injected into cells without visible lipid accumulation and into cells filled with lipid inclusions (Figure 7). Level of intercellular communication of cells without inclusions was similar to that of cells cultured from grossly normal intima [35]. Strikingly, intercellular communication of cells with lipid inclusions was reduced by 2 folds (Figure 8). The number of **Cx43** plaques was also reduced in cells with lipid accumulation in comparison to cells containing no visible lipid inclusions (Figure 8). It can be concluded that intracellular lipid accumulation is one of the possible reasons of the decreased intercellular communication in atherosclerotic lesions.

Based on the discussed observations, it can be concluded that stellate cells represent the principal cell type in the proteoglycan-rich layer of the arterial intima. These cells are different from the typical smooth muscular cells of the media by their shape, presence of several long processes and the ability for network formation. They contain few if any contractile organelles, but have a well-developed endoplasmic reticulum. Stellate cells express a number of antigens that distinguish them from the smooth muscular cells and may reflect their functional difference: pericyte-specific antigens 3G5 and 2A7 and macrophage-specific scavenger receptor **CD68**. The number of stellate cells increases in atherosclerotic lesions, which correlates with main pathological features of atherogenesis.

In the proteoglycan-rich layer of normal intima, interconnected cells form a three-dimensional network. In atherosclerotic lesions, intercellular communication rate decreases, most probably, as a result of intracellular lipid accumulation.

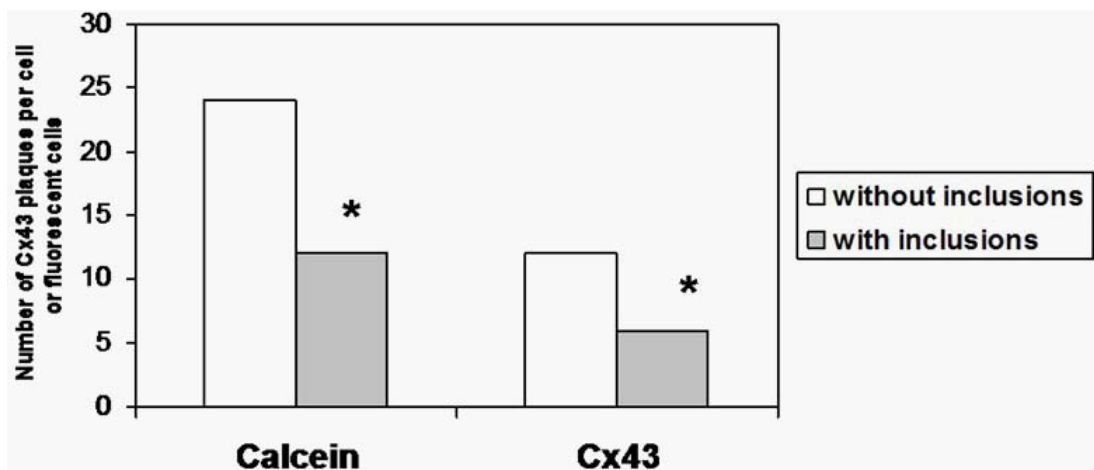


Figure 8: Intercellular communication in cells with and without lipid inclusions.

Intercellular communication was assessed in primary culture of human aortic intimal cells by microinjection of fluorescent dye calcein (left) as described before [35] and by counting the number of Cx43 plaques (right).

Fluorescent dye was injected into cells with lipid inclusion and without visible lipid inclusions, and the number of fluorescent cells contacting with the injected cell was counted.

Anti-Cx43 staining was performed and Cx43-positive plaques were counted on cells with lipid inclusion and without visible lipid inclusions revealed by filipin staining.

* - statistically significant difference from cells without lipid inclusions, $p < 0.05$.

AKNOWLEDGEMENTS

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References

- Langhans TH. Beitrage zur normalen und pathologischen Anatomie der Arterien. Virchows Arch [A]. 1866; 36: 187-226.
- Schönfelder M. Ortolgie und Pathologie der Langhans-Zellen der Aortenintima des Menschen. Pathol Microbiol. 1969; 33: 129-145.
- Shchelkunov SI. Intima of small arteries and veins. Arch Biol Sci. 1935; 35: 609-637.
- Shchelkunov SI. Main principles of cell differentiation. Meditsina, Moscow, 1977.
- Khavkin TN. On the development of atherosclerotic lesions in human aorta. Arch Pat. 1950; 12: 23-33.
- Geer JC, Haust MD. Smooth muscle cells in atherosclerosis. Karger, Basel, 1972.
- Orehov AN, Andreeva ER, Krushinsky AV, Smirnov VN. Primary cultures of enzyme-isolated cells from normal and atherosclerotic human aorta. Med Biol. 1984; 62: 255-259.
- Orehov AN, Andreeva ER, Krushinsky AV, Novikov ID, Tertov VV, Nestaiko GV, et al. Intimal cells and atherosclerosis. Relationship between the number of intimal cells and major manifestations of atherosclerosis in the human aorta. Am J Pathol. 1986; 125: 402-415.
- Krushinsky AV, Orehov AN, Smirnov VN. Stellate cells in the intima of human aorta. Application of alkaline dissociation method in the analysis of the vessel wall cellular content. Acta Anat. 1983; 117: 266-269.

10. Andreeva ER, Pugach IM, Gordon D, Orekhov AN. Continuous subendothelial network formed by pericyte-like cells in human vascular bed. *Tissue Cell*. 1998; 30: 127-135.
11. Andreeva ER, Pugach IM, Orekhov AN. Subendothelial smooth muscle cells of human aorta express macrophage antigen in situ and in vitro. *Atherosclerosis*. 1997; 135: 19-27.
12. Andreeva ER, Orekhov AN, Smirnov VN. Quantitative estimation of lipid-laden cells in atherosclerotic lesions of the human aorta. *Acta Anat*. 1991; 141: 316-323.
13. Stadius ML, Rowan R, Fleischhauer JF, Kernoff R, Billingham M, Gown AM. Time course and cellular characteristics of the iliac artery response to acute balloon injury. An angiographic, morphometric, and immunocytochemical analysis in the cholesterol-fed New Zealand white rabbit. *Arterioscler Thromb*. 1992; 12: 1267-1273.
14. Tsukada T, Rosenfeld M, Ross R, Gown AM. Immunocytochemical analysis of cellular components in atherosclerotic lesions. Use of monoclonal antibodies with the Watanabe and fat-fed rabbit. *Arteriosclerosis*. 1986; 6: 601-613.
15. Stary HC. Changes in components and structure of atherosclerotic lesions developing from childhood to middle age in coronary arteries. *Arteriosclerosis*. 1994; 89: 17-32.
16. Aqel NM, Ball RY, Waldmann H, Mitchinson MJ. Identification of macrophages and smooth muscle cells in human atherosclerosis using monoclonal antibodies. *J Pathol*. 1985; 146: 197-204.
17. Roessner A, Herrera A, Höning HJ, Vollmer E, Zwadlo G, Schürmann R, et al. Identification of macrophages and smooth muscle cells with monoclonal antibodies in the human atherosclerotic plaque. *Virchows Arch A Pathol Anat Histopathol*. 1987; 412: 169-174.
18. Gown AM, Tsukada T, Ross R. Human atherosclerosis. II. Immunocytochemical analysis of the cellular composition of human atherosclerotic lesions. *Am J Pathol*. 1986; 125: 191-207.
19. van der Wal AC, Das PK, Bentz van de Berg D, van der Loos CM, Becker AE. Atherosclerotic lesions in humans. In situ immunophenotypic analysis suggesting an immune mediated response. *Lab Invest*. 1989; 61: 166-170.
20. Kaartinen M, Penttilä A, Kovanen PT. Mast cells of two types differing in neutral protease composition in the human aortic intima. Demonstration of tryptase- and tryptase/chymase-containing mast cells in normal intimas, fatty streaks, and the shoulder region of atheromas. *Arterioscler Thromb*. 1994; 14: 966-972.
21. Katsuda S, Boyd HC, Fligner C, Ross R, Gown AM. Human atherosclerosis. III. Immunocytochemical analysis of the cell composition of lesions of young adults. *Am J Pathol*. 1992; 140: 907-914.
22. Alpers CE, Gordon D, Gown AM. Immunophenotype of vascular rejection in renal transplants. *Mod Pathol*. 1990; 3: 198-203.
23. Emeson EE, Robertson AL Jr. T lymphocytes in aortic and coronary intimas. Their potential role in atherogenesis. *Am J Pathol*. 1988; 130: 369-376.
24. Hansson GK, Jonasson L, Lojsthe B, Stemme S, Kocher O, Gabbiani G. Localization of T lymphocytes and macrophages in fibrous and complicated human atherosclerotic plaques. *Atherosclerosis*. 1988; 72: 135-141.
25. Hansson GK, Seifert PS, Olsson G, Bondjers G. Immunohistochemical detection of macrophages and T lymphocytes in atherosclerotic lesions of cholesterol-fed rabbits. *Arterioscler Thromb*. 1991; 11: 745-750.
26. Orekhov AN, Andreeva ER, Tertov VV, Krushinsky AV. Dissociated Cells from Different Layers of Adult Human Aortic Wall. *Acta Anat*. 1984; 119: 99-105.
27. Andreeva ER, Rekhter MD, Romanov YuA, Antonova GM, Antonov AS, Mironov AA, et al. Stellate cells of aortic intima: II. Arborization of intimal cells in culture. *Tissue Cell*. 1992; 24: 697-704.
28. Orekhov AN, Andreeva ER, Tertov VV. The distribution of cells and chemical components in the intima of human aorta. *Soc Med Rev A Cardiol*. 1987; 1: 75-100.
29. Orekhov AN, Karpova II, Tertov VV, Rudchenko SA, Andreeva ER, Krushinsky AV, et al. Cellular composition of atherosclerotic and uninvolved human aortic subendothelial intima. Light-microscopic study of dissociated aortic cells. *Am J Pathol*. 1984; 115: 17-24.
30. Maximow A. Binbegewebe und blutbildende Gewebe; in Anonymous *Handbuch der mikroskopischen Anatomie des Menschen*. Arch Exper Zellforsch, 1927.
31. Anichkov NN. Pathologic anatomy. Part II. Heart and vascular system. Second edition, Medgiz, Moscow-Leningrad. 1947.
32. Campbell JH, Campbell GR. Biology of the vessel wall and atherosclerosis. *Clin Exp Hypertens A*. 1989; 11: 901-913.
33. Haust MD, More R, Movat HZ. The Role of Smooth Muscle Cells in the Fibrogenesis of Atherosclerosis. *Am J Pathol*. 1960; 37: 377-389.

34. Rekhter MD, Andreeva ER, Mironov AA, Orekhov AN. Three-dimensional cytoarchitecture of normal and atherosclerotic intima of human aorta. *Am J Pathol.* 1991; 138: 569-580.
35. Andreeva ER, Serebryakov VN, Orekhov AN. Gap junctional communication in primary culture of cells derived from human aortic intima. *Tissue Cell.* 1995; 27: 591-597.
36. Trautmann A. Functions of gap junction channels in the open and closed states. *Biochem Soc Trans.* 1988; 16: 534-536.
37. Bény JL, Connat JL. An electron-microscopic study of smooth muscle cell dye coupling in the pig coronary arteries. Role of gap junctions. *Circ Res.* 1992; 70: 49-55.
38. Bény JL, Gribi F. Dye and electrical coupling of endothelial cells in situ. *Tissue Cell.* 1989; 21: 797-802.
39. Davies PF. Vascular cell interactions with special reference to the pathogenesis of atherosclerosis. *Lab Invest.* 1986; 55: 5-24.
40. Gabella G. Smooth muscle cell junctions and structural aspects of contraction. *Br Med Bull.* 1979; 35: 213-218.
41. Loewenstein WR. Junctional intercellular communication and the control of growth. *Biochim Biophys Acta.* 1979; 560: 1-65.