

# Cellular Mechanisms of Human Atherosclerosis: Key Events

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Main manifestations of atherosclerosis are linked to changes at the cellular level and include lipid accumulation leading foam cell formation, local increase of cellularity as a result of cell proliferation and migration and deposition of extracellular matrix.

## LIPIDOSIS

Macroscopically unaltered areas of intima are characterized by mainly extracellular lipid deposition. However, even the earliest stages of atherosclerotic lesions contain cells with prominent lipid inclusions [1-3]. Maximal numbers of cells with lipid inclusions are observed in fatty streaks (up to 25%), where such cells are located in the upper two thirds of the proteoglycan rich layer [4]. In contrast, in atherosclerotic plaques, most of the cells with lipid depositions are located deeper in the intima, in the proximity of the internal limiting membrane.

In muscular-elastic sublayer, percentage of lipid-loaded cells is the highest in atherosclerotic plaques, but even there it does not exceed 5%. Cells with lipid inclusions can belong to all the morphological cell types described in the aortic intima. However, the majority of them (up to 30%) are stellate cells (Figure 1).

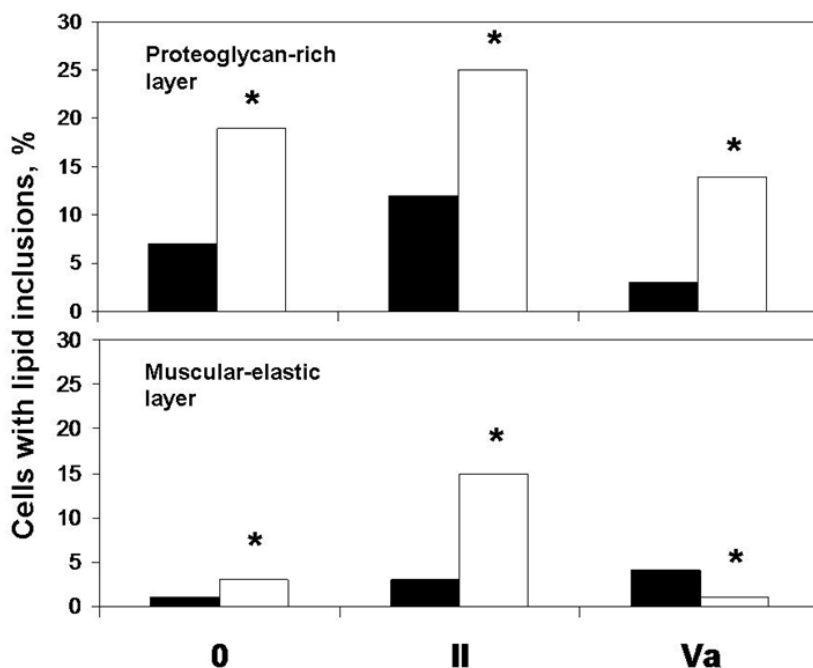


**Figure 1:** Percentage of cells of different types with lipid inclusions in human aortic intima.

Percentage of cells with lipid inclusions was assessed in samples after alcohol-alkaline dissociation of fixed tissue (proteoglycan-rich layer and muscular-elastic layer). Black = elongated cells, white = stellate cells.

\* - statistically significant difference from the percentage of elongated cells with lipid inclusions.

One of the possible mechanisms leading to intracellular lipid accumulation is alteration of cellular metabolism. Cells cultured from atherosclerotic lesions are characterized by increased synthesis of main classes of lipids than cells from uninvolved human aortic intima [5,6]. Lipid synthesis rate closely correlates with lipid content of cells: the higher the intracellular lipid accumulation, the more intensive is the lipid metabolism in atherosclerotic cells [5,6]. These observations, however, provide no insight into the possible causes of metabolic changes and lipid accumulation in the arterial wall.

Low Density Lipoprotein (**LDL**), the principal lipid carrier in blood, is regarded as the main source of lipid accumulation in atherosclerotic lesions. Numerous experiments aimed to demonstrate the direct role of LDL in the development of cellular lipidosis were based on incubation of cultured cells with LDL. These attempts were mostly unsuccessful, as LDL failed to cause significant lipid accumulation even at high concentrations [7,8]. This could be explained by the existence of cellular regulatory mechanism, which reduces the surface expression of LDL receptors in response to intracellular cholesterol accumulation therefore reducing the receptor-mediated LDL uptake [9].

Intracellular lipid accumulation can be induced by insoluble LDL-containing complexes [7,8,10-13]. Such complexes include LDL aggregates with glycosaminoglycans, proteoglycans, fibronectin, collagen, elastin and other components forming the connective tissue matrix of the arterial wall. Such pathway of lipid accumulation is plausible because it corresponds to conditions that exist in the arterial wall.

Moreover, association of several LDL particles was shown to be necessary and sufficient to induce intracellular lipid accumulation [14,15]. Apparently, relatively large LDL aggregates penetrate cells using similar mechanism as insoluble LDL-containing aggregates via receptor-independent pathway, such as phagocytosis. Such by-pass of the regulated endocytic pathway can lead to uncontrolled lipid accumulation. Native LDL is not prone to association, while chemically modified LDL readily associates forming relatively large particles.

It was demonstrated that all known types of chemical modification of LDL, including multiple modification of LDL, which takes place in human bloodstream, stimulate LDL association [16,17]. On the other hand, in absence of association, even modified LDL causes no lipid accumulation in cultured cells [16,17].

Therefore, a subfraction of atherogenic modified LDL circulates in human blood and causes lipid accumulation, while native LDL is not atherogenic and does not cause lipid accumulation in atherial wall cells [18]. LDL modification affects protein; lipid and carbohydrate moieties of the LDL particle are results in changes of the particle size, density, electrical charge, immunogenicity and other properties [19]. As mentioned above, lipid accumulation caused by multiply modified LDL occurs only if LDL particles form aggregates. Non-associated modified LDL, just as native LDL particles, does not cause intracellular lipid accumulation [16,17]. There exists a direct and strong correlation between the associate size and ability to induce intracellular lipid accumulation [16,17].

Subfraction of multiply modified LDL is present in blood of both atherosclerotic patients and healthy subjects. However, its relative content is significantly increased in the blood of patients with confirmed atherosclerosis [18]. There is a direct correlation between modified LDL content and atherogenic properties of blood serum, hence its ability to induce intracellular lipid accumulation [18].

## PROLIFERATION

Evaluation of intimal cell count on thin sections and in suspension after alcohol-alkaline dissociation [20,21] shows a 2-fold local increase of cellularity in atherosclerotic lesions [22,23]. The maximal cell count was detected in fibrolipid plaques. In fatty lesions (fatty streaks and fibrolipid plaques) numbers of both resident and inflammatory cells are significantly increased. Human arterial intima is formed mostly by resident cells (84-93%), and the increase of their number is the main reason of the increase of total cellularity of atherosclerotic lesions in comparison with uninvolved intima [22,23]. Noteworthy, cellularity of fibrotic plaques is lower than that of fatty lesions.

Proliferating cells can be detected using antibodies to Proliferating Cell Nuclear Antigen (PCNA), which is expressed during the S-phase of the cell cycle (Figure 2).

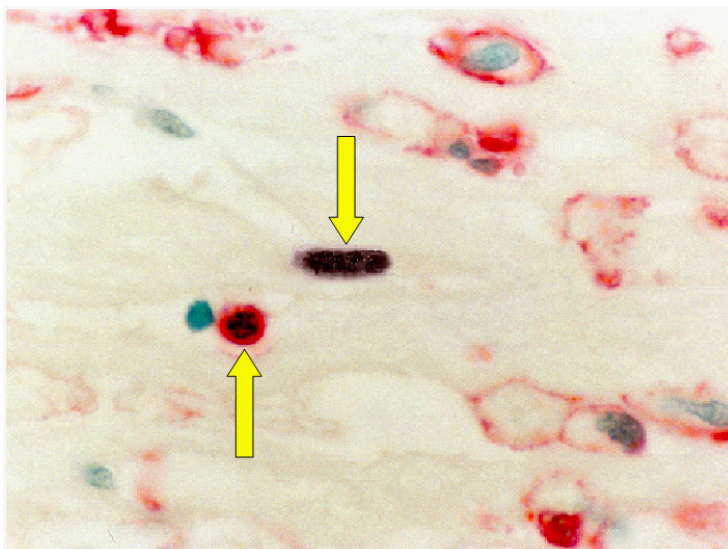


**Figure 2:** Immunohistochemistry reveals PCNA-positive cells in human aortic intima (indicated with arrows).

The highest number of proliferating (PCNA-positive) cells was detected in fatty lesions: fatty streaks and fibrolipid plaques that contained 10 to 20 folds more dividing cells than grossly normal regions [22,23]. Number of proliferating cells in fibrotic plaques was lower than in fatty lesions, but higher than in grossly normal regions (Figure 3).

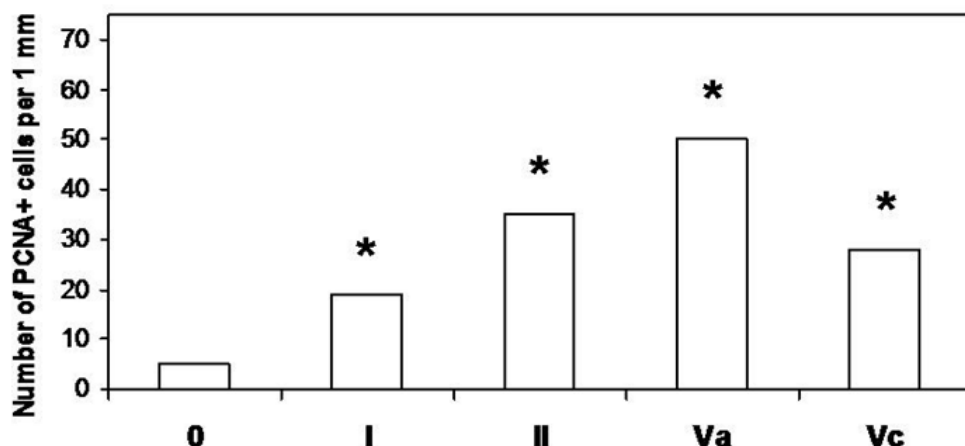


**Figure 3:** Number of PCNA-positive cells in human aortic intima in different types of atherosclerotic lesions. Proliferating cells were detected on vertical sections of grossly normal intima and of atherosclerotic lesions using antibodies to PCNA.

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

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

Proliferative index (ratio of proliferating cells to the total cell count) of resident cells in atherosclerotic lesions was significantly higher than in uninvolved intima [22,23]. The highest proliferative index was detected in fibrolipid plaques, where it was approximately 5 folds higher than in healthy tissue (Figure 4). Proliferative index of inflammatory cells was higher than that of resident cells, but its value in atherosclerotic lesions was unaltered in comparison to uninvolved intima and was similar to proliferative index of peripheral blood leukocytes.

Taken together, these observations indicate that increased cellularity of atherosclerotic lesions can be explained by increased proliferation of resident cells and, possibly, migration of the inflammatory cells from the bloodstream. Fatty lesions (fatty streaks and fibrolipid plaques) are characterized by a splash of proliferative activity of resident cells. Proliferation of inflammatory cells also takes place in the arterial wall, however, proliferative index of these cells remains unaltered in atherosclerotic lesions in comparison to uninvolved areas. Proliferative activity of the inflammatory cells is therefore a background process that remains constant in atherosclerosis.

## FIBROSIS

Accumulation of extracellular matrix and fibrous cap formation is the most clinically relevant manifestation of atherosclerosis. As mentioned earlier, collagen content increases exclusively in the proteoglycan-rich layer of the arterial intima. In grossly normal areas, intimal cells do not produce collagen type I, the principal interstitial collagen accumulated in atherosclerotic plaques. However, cells, producing this type of collagen account for 6% to 18% of total cell population in the initial lesions and fatty streaks respectively [24]. These results correspond to the immunocytochemical data of distribution of different types of collagen in atherosclerotic [25]. Juxtaluminal layer of intima is the main site of collagen accumulation.

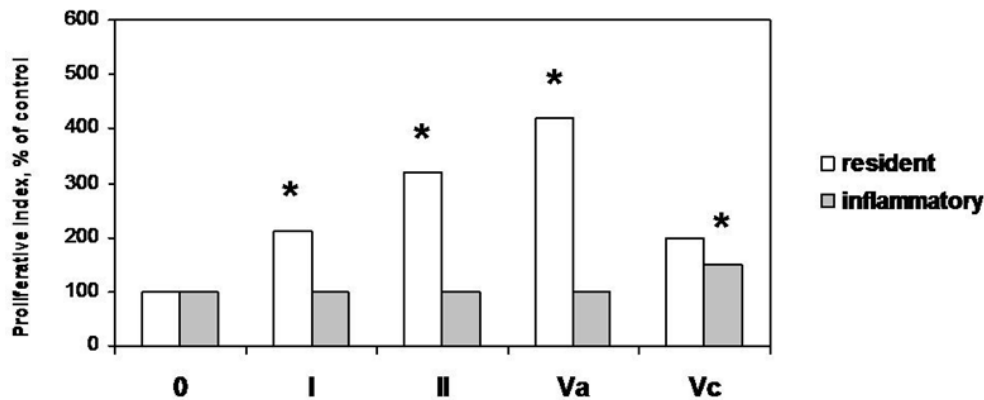


**Figure 4:** Proliferative index of resident and hematogenous cells of human aortic intima.

Proliferating cells were revealed in vertical sections of grossly normal areas and atherosclerotic lesions by staining with antibodies specific for PCNA. Cell typing was performed by double staining for relevant markers. Hematogenous cells were detected using a mixture of anti-CD45 and anti-CD14 antibodies. Resident cells were negative for these markers. Proliferative index was calculated for each cell population.

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

White –resident cells, grey – hematogenous cells.

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

Atherosclerotic lesions at early stages, such as initial lesions, already contain cells producing the main interstitial collagen of the fibrous cap – collagen type I [24]. In fatty streaks, the number of such cells significantly increases. Maximal numbers of collagen-synthesizing cells is found in fibrolipid plaques. In fibrotic plaques, this number is lower and comparable with that in the initial lesions. There is therefore a splash of collagen-producing activity in fatty lesions (fatty streaks and fibrolipid plaques) [24].

Hyper secretion of extracellular matrix may be a cause or a consequence of the disturbed integrity of intimal cellular system in atherosclerotic lesions. Few collagen producing cells are present in initial lesions, where all cells are connected into a network. In fatty streaks and fibrolipid plaques cellular network is disintegrated. Such areas contain more collagen-producing cells, many of them located at the sites of network disruption [24]. This observation indicates that loss of intercellular contacts may be linked to the increased synthetic activity of cells.

## **LIPIDOSIS IN RELATION TO OTHER MANIFESTATIONS OF ATHEROSCLEROSIS**

Proliferative and collagen-producing splash in fatty lesions underscores the possible link between the main manifestations of atherosclerosis: lipid accumulation (lipidosis) and proliferation and extracellular matrix formation (fibrosis). Such link may be important, because both proliferative activity and fibrosis reach their maximum in areas with highest lipid accumulation.

Blood serum and LDL obtained from atherosclerosis patients not only induce lipid accumulation in cultured intimal cells, but also demonstrate other properties relevant to atherosclerosis. Incubation of cells with serum or LDL stimulates proliferation and enhances collagen synthesis and production of glycosaminoglycans and total protein [26]. Therefore, blood serum and LDL from atherosclerotic patients have a broad spectrum of atherogenic properties inducing all the main manifestations of the disease at the cellular level: proliferative splash, lipidosis and fibrosis. Direct contact of cells with modified LDL is dispensable for the enhancement of proliferation and extracellular matrix synthesis. Increased proliferative and synthetic activity is retained for several days after removal of LDL from the cultural medium [8,26]. It can be therefore suggested that the principal atherogenic activity at the cellular level belongs not to the interaction of LDL with cells, but to the consequences of this interaction, such as lipid accumulation.

Intracellular lipid accumulation affects the integrity of cellular network in human aortic intima. In fatty lesions, where many cells contain lipid inclusions and many foam cells are present, cellular network is disintegrated. Cells containing lipid inclusions loose contacts with their neighbors [27]. The results presented above demonstrate that lipid accumulation induced by modified LDL affects intercellular communication of cultured cells [28]. It could be suggested that weakening of

intercellular connections occurs as a result of intracellular lipid accumulation, however, studies of phagocytosis in cultured cells contradict to this suggestion. Incubation of subendothelial intimal cells with latex beads of similar size as modified LDL aggregates led to a decrease of intercellular communication to a similar extent as incubation with modified LDL (Figure 5). Latex beads were internalized by cultured cells via phagocytosis, causing, however, no lipid accumulation (Figure 5). Therefore, intercellular connections are disturbed not because of modified LDL-induced lipid accumulation, but as a result of increased phagocytic activity, which can also be induced by LDL associates.

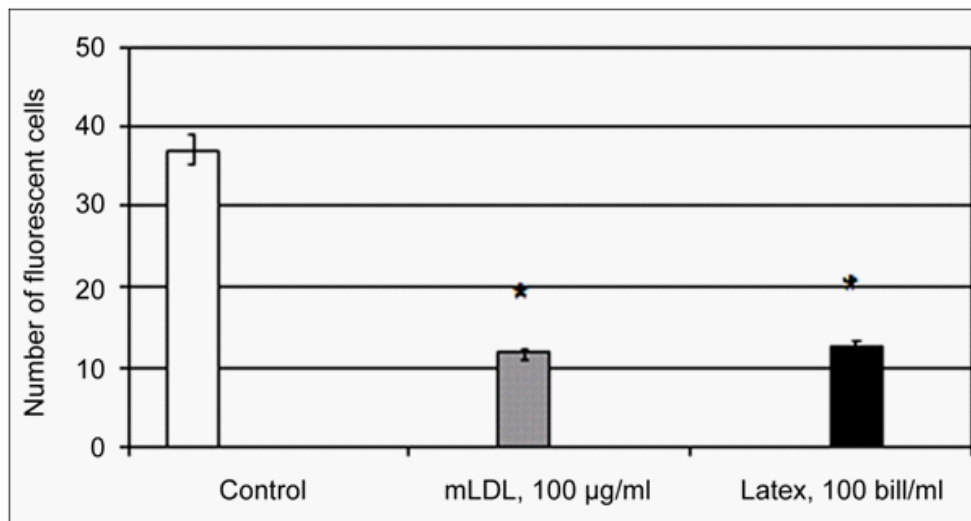


**Figure 5:** Influence of modified LDL and latex beads on intercellular communication in primary culture of human aortic intima cells.

Intercellular communication was evaluated by calcein tracing [28].

Fluorescent dye was injected into cells, and number of fluorescent cells contacting with injected cells was counted.

\* - statistically significant difference in comparison with cells without lipid inclusions,  $p < 0.05$ .

Importantly, latex beads-induced stimulation of phagocytic activity did not lead to stimulation of proliferation or protein synthesis. Therefore, these atherogenic manifestations can be attributed to lipid accumulation rather than to increased phagocytic activity.

Based on the provided data, the following model of cellular manifestations of atherosclerosis can be proposed. Modified LDL penetrates the sub endothelial intimal layer, where particles associate with each other or with the components of extracellular matrix. These associates enter sub endothelial intimal cells by means of phagocytosis in an uncontrolled manner, which leads to intracellular lipid accumulation followed by increased proliferation and extracellular matrix secretion. At the same time, stimulation of phagocytosis causes disruption of intercellular connections.

## ATHEROGENIC MANIFESTATIONS AND CYCLIC NUCLEOTIDES

Atherogenic manifestations that develop in parallel and lead to intracellular lipid accumulation, stimulation of proliferation and extracellular matrix secretion indicate the existence of a link between them. Such link is possible at the level of cellular regulation. Indeed, the abovementioned processes are regulated by the cyclic nucleotide system. Cyclic 3',5'-Adenosine Monophosphate (**cAMP**) inhibits cell proliferation, lipid and protein synthesis, protein secretion (including collagen and glycosaminoglycans) and interaction of LDL with cellular receptors and enhances lipid hydrolysis. Cyclic 3',5'-Guanosine Monophosphate (**cGMP**) has a reversed influence on these processes [29,30].

It has been demonstrated that cAMP concentration is 2 to 8 folds lower in atherosclerotic cells (cells populating atherosclerotic lesions) than in cells from grossly normal intima, and cGMP concentration is almost 2 folds higher [31,32]. These changes are linked to altered activity of the enzymes responsible for synthesis and degradation of cyclic mononucleotides, cyclases and phosphodiesterases respectively. For instance, the activity of adenylate cyclase is decreased in fatty streaks and even more decreased in fibrolipid plaques in comparison to uninvolved intima, whereas the activity of phosphodiesterase activity remains constant, leading to the decrease of cAMP concentration in atherosclerotic lesion cells. On the other hand, the activity of guanylate cyclase is increased in fatty streaks in comparison to normal intima, while the activity of phosphodiesterase degrading cGMP remains constant [33]. In atherosclerotic plaques, the activity of guanylate cyclase is unaltered, but the activity of cGMP phosphodiesterase is significantly lower [33]. Such changes explain the observed increase of cGMP in atherosclerotic lesions.

Based on the accumulating data, it can be concluded that the described changes of cAMP and cGMP levels in vascular wall cells lead to the activation of main manifestations of atherosclerosis at the cellular level: proliferation, lipid accumulation and extracellular matrix synthesis [32].

What causes the changes of cyclic mononucleotide concentrations in atherosclerotic lesions? It has been demonstrated that incubation of human aortic intimal cells with modified LDL resulted in a significant decrease of the cAMP and increase of cGMP levels in parallel with cholesterol accumulation. This reflects the situation described for atherosclerotic lesions of the arterial wall (Table 7). It can be concluded that intracellular lipid accumulation alters the regulation of cyclic nucleotide system.

Taking into account that the obtained data on altered cyclic nucleotide concentrations, the model of cellular mechanisms of atherogenesis can be adjusted as follows. Modified LDL associates that stimulate phagocytosis in the sub endothelial intimal cells disrupt intercellular communication. Because modified LDL enters cells bypassing the regulated endocytic pathway, it causes lipid accumulation followed by a decrease of cAMP and increase of cGMP levels. This leads to further lipid accumulation, stimulates proliferation and extracellular matrix production. These events are likely to take place at the initial stages of atherosclerosis. If the described

processes continued during the later stages of the lesion development, it could lead to constant increase of lipid accumulation, cellular proliferation and extracellular matrix deposition. These processes, however, are less intense in pronounced atherosclerotic lesions in comparison to early stages. Therefore, the proposed scheme does not explain the atherosclerotic lesion formation completely. The observed bell-shaped curve of lipid accumulation, proliferation and synthetic activity depending on the lesion stage re-quires a different explanation.

## INTERCELLULAR COMMUNICATION AND ATHEROGENESIS

Atherosclerosis develops gradually from light changes in the intima that can be revealed only microscopically or with specific histochemical techniques (initial lesions) to pronounced clinically significant lesions that can be seen by naked eye. According to modern classification [34], the following sequence of events in atherosclerosis development can be distinguished: grossly normal intima – initial lesions – fatty streaks – fibrolipid plaques (atheromas) – fibrotic plaques. There is currently no data that disproves the proposed sequence. Moreover, no principally different classification of the pathological events has been proposed so far. The described sequence of atherogenesis stages is presented on Figure 6. Typical features of atherosclerotic process, such as lipid accumulation, cell proliferation, collagen synthesis and intercellular communication can be put on such graph according to the intensity of the pathological change. Correspondingly, changes of lipid load, proliferative activity and collagen synthesis follow a bell-shaped curve. Proliferative activity and collagen synthesis increase from nearly zero level in grossly normal intima to maximal values in fatty lesions (fatty streaks and atheromas), but decrease again in fibrotic plaques. Number of cells with lipid inclusions steadily increases reaching maximum in fibrolipid plaques, then decreases abruptly, becoming even lower than in grossly normal intima.

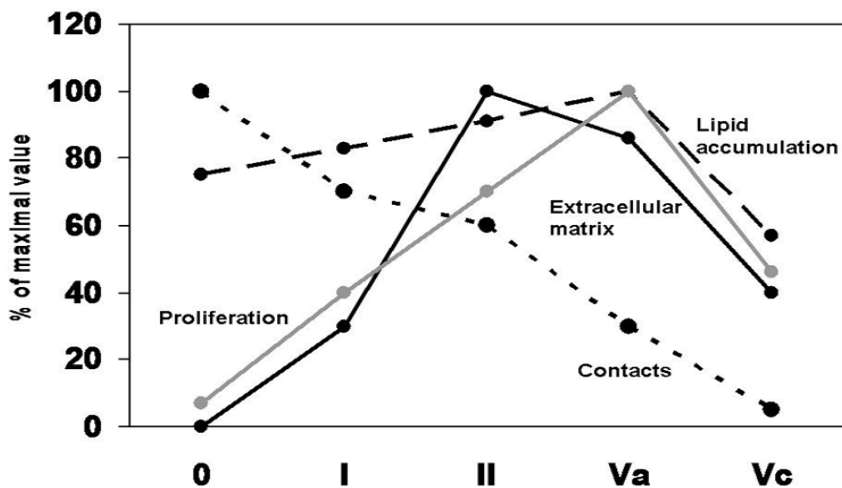


**Figure 6:** Atherogenic manifestations in human aortic intima.

Altered function of cells in atherosclerosis observed on vertical sections of human aortic intima and in primary culture of intimal cells, expressed as % of maximal value registered for each function

0 – normal intima, I – initial lesions, II – fatty streaks, Va – fibrolipid plaques, Vc – fibrotic plaques.

On such graph, intercellular communication rate steadily decreases almost to the zero level in fibrotic plaques, where complete disintegration of cellular network takes place. It can be speculated that functions of cells that play the key role in atherogenesis depend on intact intercellular communication, and this dependence also follows a bell-shape curve. This hypothesis was studied on cultured cells from healthy human intima with varying degree of intercellular contact communication. Cells isolated from human aortic intima were seeded at different densities (Figure 7). In very dispersed cultures, no contacts were formed between cells (intercellular communication was almost absent). In dense cultures, most of the cells formed contacts with neighbors, and the degree of intercellular communication was the highest. Intermediate cultures have also been studied (Figure 7).

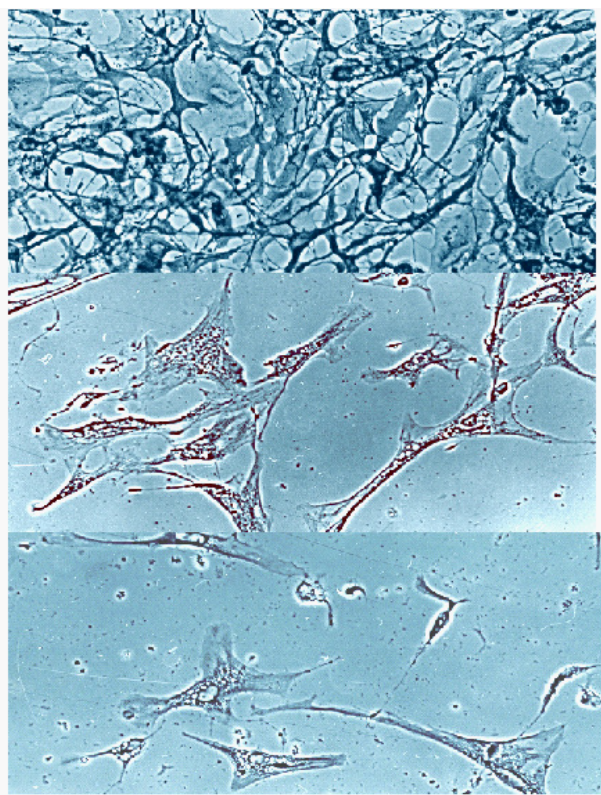


**Figure 7:** Culture of human aortic sub endothelial cells with different density.

High density – upper plate, intermediate density – middle plate, low density – lower plate.

Such cultures seeded with different density imitate the situation in the arterial wall, where cellular network formed by cellular processes, which is present in normal tissue, is gradually disintegrated and, finally, completely disrupted in atherosclerotic plaques. This results in isolation of intimal cells from each other (Figure 8).

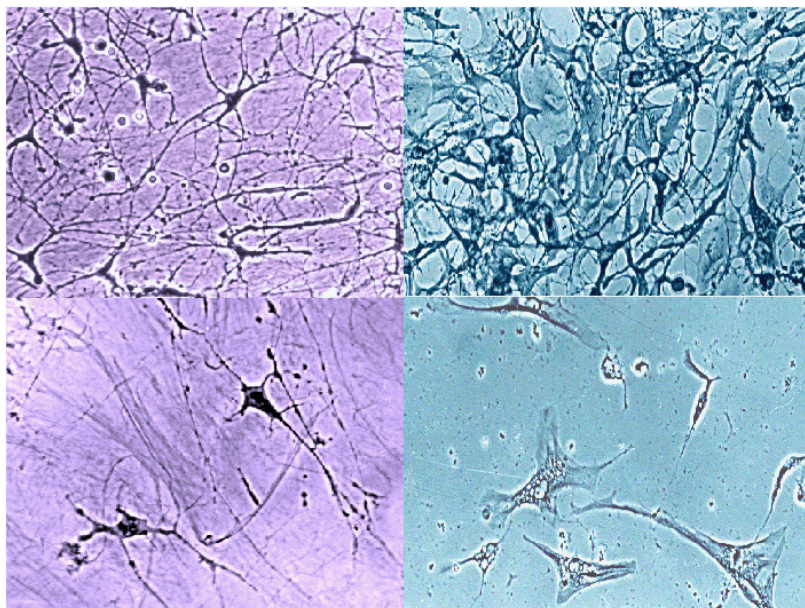


**Figure 8:** Different density of cells in the arterial wall (left) and in culture (right).

In very dispersed cultures, where almost no intercellular contacts are formed, proliferative activity and protein secretion were minimal (Figure 9). Intercellular communication rate and both of the measured functions increased with increasing culture density (Figure 9). Maximal proliferative activity and protein secretion were detected in cultures with well developed intercellular contacts. In cultures with higher density, proliferative activity and protein secretion decreased, and in the most dense cultures, these levels were comparable with the corresponding values obtained for very dispersed cultures (Figure 9).

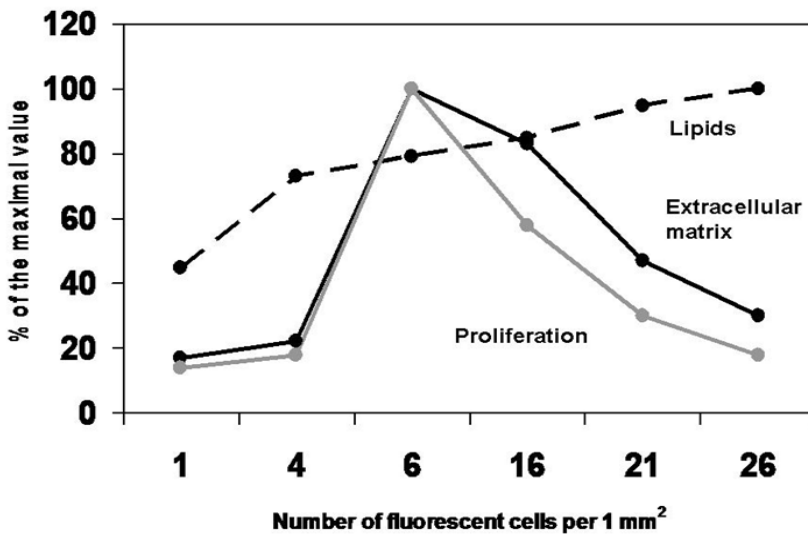


**Figure 9:** Dependence of atherogenic manifestations from intracellular communication rate in cultured cells.

Cells obtained from grossly normal human aortic intima were seeded with varying density. The obtained cultures had different intracellular communication rate assessed by tracing the injected fluorescent distribution to neighboring cells. At day 6 of culture, modified LDL-induced cholesterol accumulation, proliferative activity and protein synthesis were measured.

Cholesterol accumulation in cultured cells was not dependent on the intercellular communication rate in relatively dense cultures, but was significantly lower in cultures where virtually no intercellular contacts were present (Figure 9). Therefore, main functions of intimal cells related to atherogenesis depend on the rate on intercellular communication. Such dependency is not straight-forward and, for proliferative activity and protein synthesis follows a bell-shaped curve. Intracellular lipid accumulation does not depend on the intercellular communication rate, but is minimal in cultures with absent intercellular contacts.

The observed bell-shaped dependency of cellular functions on intercellular communication rate suggests that changes of these features in atherosclerosis are linked to dysfunction of cellular contacts, since the development of atherosclerotic lesion is associated with disintegration of the cellular network in the intima.

Changes of cellular functions in atherosclerosis are presented on Figure 10. Shown are changes in human aortic intima (right graph) and in cultured intimal cells with difference culture density (left graph). Intercellular communication rate is presented as decreasing from left to right to facilitate the comparison with the atherosclerotic lesion development, where minimal intercellular communication is observed in fibrotic plaques where no cellular network remains.

Changes of proliferative activity, extracellular matrix synthesis and lipid accumulation are so similar on the two graphs that the role of intercellular communication in changes of cellular functions associated with atherosclerosis appears evident. The fact that cellular lipidosis does not depend on the intercellular communication rate until the contacts are virtually absent confirms the absence of a direct link between these two events. On the other hand, this observation suggests that stimulation of phagocytosis associated with modified LDL accumulation in intimal cells is the main reason of intercellular contact disintegration, and not vice versa.

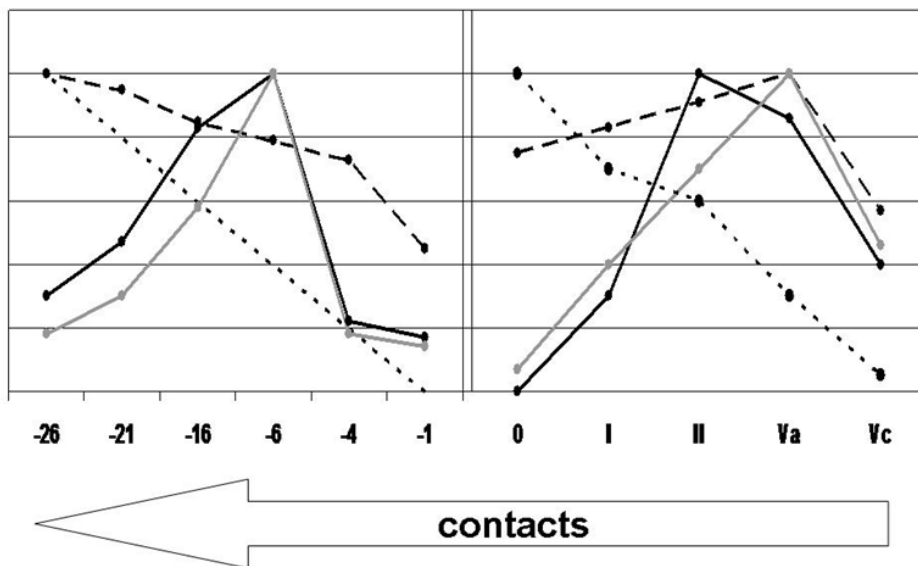


**Figure 10:** Intercellular communication and manifestations of atherosclerosis in human aortic intimal cells.

Line types correspond to the legend of Figures 6 and 9.

## CELLULAR MECHANISMS OF ATHEROGENESIS

In summary, the presented concepts attempt to explain the major changes observed in human aortic intima during atherogenesis. The possible scheme of the pathological events is presented below (Figure 11).

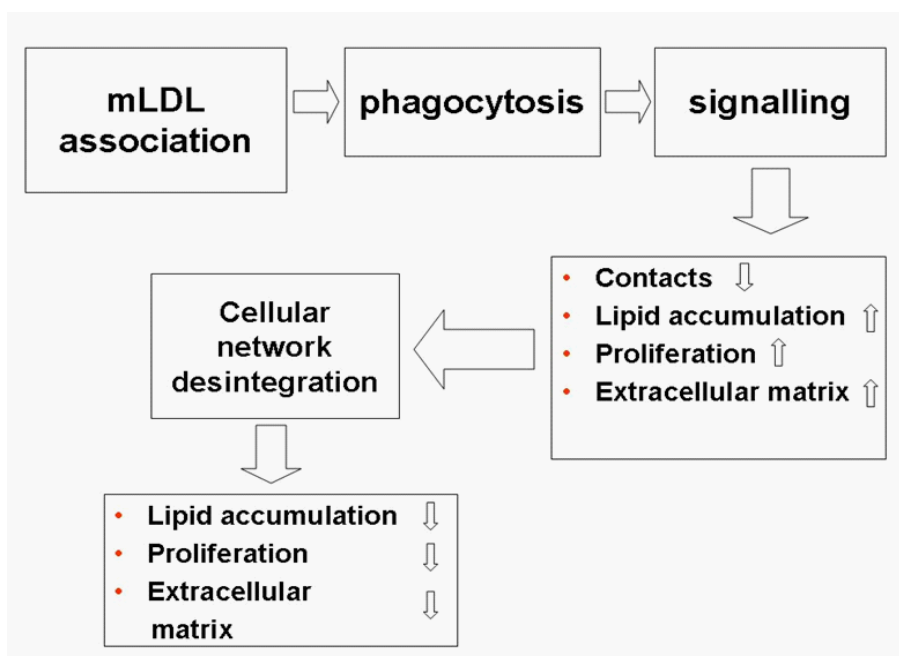


**Figure 11:** Key events in atherogenesis.

Modified LDL and/or their associations penetrate to the sub endothelial intima from the bloodstream. LDL associates or LDL-containing complexes activate phagocytosis leading to disintegration of intercellular contacts. LDL enters the cells bypassing the regulated receptor-mediated endocytosis and cause intracellular lipid accumulation. This causes the decrease of cAMP and increase of cGMP levels, which, in turn, leads to further lipid accumulation, enhanced proliferation and extracellular matrix synthesis such chain of events is likely to occur in early atherosclerotic lesions. Further phagocytosis-mediated lipid accumulation in the intimal cells causes their dissociation from each other until a complete loss of intracellular contacts and disintegration of the cellular network in the intima. Foam cells are formed with cytoplasm, completely filled with lipid inclusions. Following the lipid accumulation and dissociation of intercellular contacts, further stimulation of proliferative activity and extracellular matrix synthesis takes place. This is typical for pronounced fatty lesions (fatty streaks and fibrolipid plaques). Secreted collagen and other extracellular matrix components surround the producing cells further increasing their isolation from each other. The activity of main atherosclerotic processes, such as lipid accumulation, cell proliferation extracellular matrix production drops at this stage, which corresponds to fibrotic plaques.

The proposed scheme is presented on Figure 12. Intracellular lipid accumulation induced by modified LDL is regarded as the primary event in atherogenesis. It stimulates other pathological processes at the cellular level. At the same time, aggregations of modified LDL disrupt the cellular network in the intima by triggering phagocytosis. These processes lead to the development of pronounced fatty lesions, such as fatty streaks and fibrolipid plaques, where the maximal intensity of atherogenic manifestations is observed.

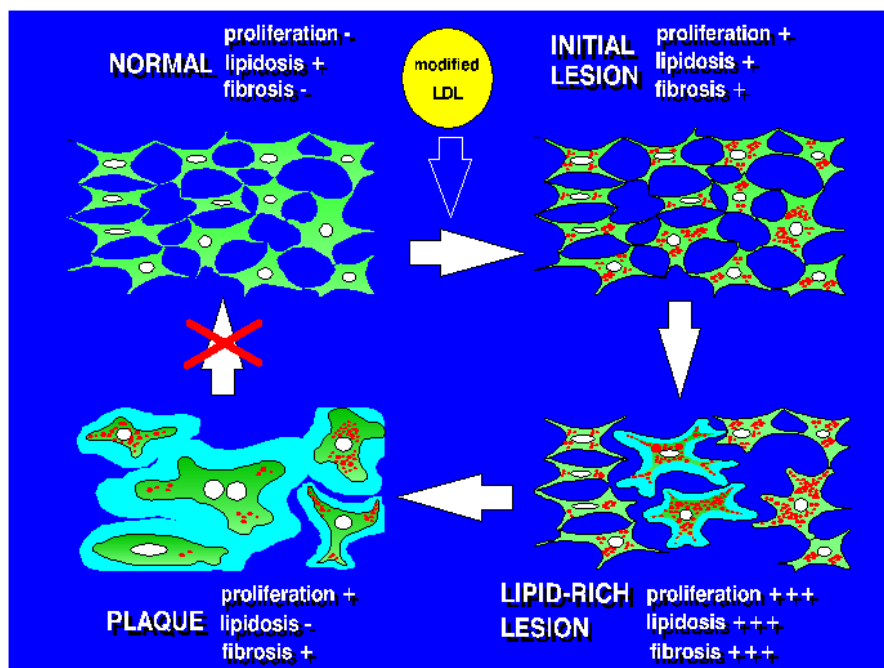


**Figure 12:** Cellular mechanisms of atherogenesis.

The intensity of cellular functions activated in atherosclerosis are similar in grossly normal intima and fibrotic plaque (the final stage of atherogenesis). However, the cellular system state is very different in these situations. In fibrotic plaques, cellular system is profoundly altered, and its restoration appears to be impossible. From the clinical point of view, fibrous plaques are rather benign, being stable and inert. A different picture is observed in fatty lesions, especially in fibrolipid plaques (atheromas). These are unstable structures, in which all the cellular processes are maximally active. Clinically, these structures, also called unstable (vulnerable) plaques, are most dangerous because of their rapid development. Their rapid growth may lead to plaque rupture associated with critical local changes of hemostasis causing thromboembolism and cardiovascular events with fatal consequences, such as myocardial infarction, stroke, sudden death and others. However, such fibrolipid plaques and, especially, initial lesions can apparently be reversed, since changes of the cellular system in such lesions are not terminal.

Given that intracellular lipid accumulation is the key event in the pathogenesis of atherosclerosis, it can be suggested that the most effective approaches to atherosclerosis prevention and treatment will target the lipid accumulation in the arterial wall and promote discharge of the lipid deposits from arterial intimal cells.

## AKNOWLEDGEMENT

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