

Cellular Mechanisms of Human Atherosclerosis: Cell Types of Intimal Population

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IMMUNOCYTOCHEMICAL TYPING OF INTIMAL CELLS

Modern approach to cell type identification is mostly based on detection of antigens specific for a given cell type. The development of monoclonal antibody technology revolutionized cell type identification (typing). Currently, immunocytochemical typing using monoclonal antibodies is the basic instrument for identification of tissue cell populations. As any other method, immunocytochemistry has its limitations. However, formal classification of cell types is currently based mostly on this technique.

There are numerous works describing cellular composition of human and animal arterial wall using immunocytochemical typing. The aims of these works included identification of cell types using specific antibodies (presence of smooth muscular cells, macrophages, lymphocytes and mast cells has been demonstrated in the intimal layer), localization of different cell populations, selection of antibodies suitable for revealing certain cell subpopulations (detection of T helpers and T suppressors) and studying the nature of different cell phenotypes (for instance, it was

demonstrated that foam cells can originate both from macrophages and smooth muscular cells). Therefore, this method allowed characterizing the cellular composition of healthy and atherosclerotic arterial intima.

Development of antibodies specific to markers of different types of mesenchymal cells allowed broadening our knowledge on cellular composition of the intimal layer of large arteries and its changes in atherosclerotic lesions.

Apart from smooth muscular cells that express muscular α -actin [1-4], intimal layer contains cells of hematogenous origin: macrophages [5-9] lymphocytes [10-12,7,8,13] mast cells [14] dendritic cells [15].

Detailed immunocytochemical analysis of cellular population of human aortic intima in grossly normal areas and in atherosclerotic lesion sites has been performed using a set of antibodies specific to different cell types (Table 1). This set of immunocytochemical markers allowed characterization of cells from normal and affected intima (Table 2).

Smooth muscle cells were identified with antibodies against muscular α -actin. On en face preparations, cells, positive for this marker, have rounded nuclei and cytoplasm with 2 or more processes (Figure 1).

In fatty lesion areas, muscular actin is revealed mostly in cellular processes. Quantitative analysis of cell populations of certain phenotype was performed on vertical sections of the arterial wall. On such sections, cells of the proteoglycan-rich layer containing α -actin have elongated shape and are located parallel to the luminal edge of the section. In the muscular-elastic layer, positively stained cytoplasm has a shape of a wide ring or half-ring around the nucleus (Figure 2a).

Both in grossly normal intima and in atherosclerotic lesions, around 50% of cells were positively stained for muscular α -actin (Table 2). Correspondingly, the other half of the cell population could not be identified using this smooth muscle marker.

Table 1: Antibodies and reagents for immuocytochemistry.

Antibodies and conjugates	Source	Working dilution	Conditions
2B11,PD7/26 (CD45) leykocyte antigen	DAKO	1:200	1h, 20°C
TÜK4 (CD14) monocyte-macrophage differentiated cell antigen	DAKO	1:20	1h, 20°C
3G5 antigen of brain capillary pericytes	ATCC, Rockville, MD, USA	1:50	1h, 20°C
2A7 antigen of pericytes from angiogenesis sites	Dr. M. Verbeek	Culture medium	1h, 20°C
asm-1 smooth muscle α -actin	Boehringer Mannheim GmbH, Mannheim, Germany	1:200	1h, 20°C
Biotinylated horse anti-mouse IgG	Vector Laboratories, Burlingame, CA, USA	1:200	30 min., 20°C
Streptavidin-peroxidase	Vector	1:100	30 min., 20°C
Streptavidin—alkaline phosphatase	Vector	1:1000	30 min., 20°C
FITC-streptavidin	Vector	1:200	30 min., 20°C

Table 2: Immunocytochemical characterization of cells from human aortic intima.

Antigen	% of positively stained cells			
	0	I	II	Va
Smooth muscle α -actin	47.6 $\pm$ 2.3 (4)	47.2 $\pm$ 3.1 (3)	42.2 $\pm$ 3.1 (4)	47.0 $\pm$ 10.9 (5)
CD45	2.2 $\pm$ 0.4 (3)	6.2 $\pm$ 1.2 (4)	5.0 $\pm$ 0.9* (3)	6.2 $\pm$ 1.8* (9)
CD68	3.9 $\pm$ 0.4 (5)	6.1 $\pm$ 1.4 (4)	13.2 $\pm$ 0.8* (5)	13.1 $\pm$ 2.3* (4)
3G5	31.3 $\pm$ 7.0 (4)	6.3 $\pm$ 1.0* (3)	11.7 $\pm$ 2.0* (8)	5.0 $\pm$ 0.7* (5)
2A7	0.0 $\pm$ 0.0 (3)	1.2 $\pm$ 0.3 (3)	3.0 $\pm$ 0.7 (3)	27.0 $\pm$ 3.1 (3)
Hematogenous cells CD45+ CD14	5.5 $\pm$ 1.2 (8)	9.6 $\pm$ 1.4* (6)	13.4 $\pm$ 1.5* (10)	18.7 $\pm$ 2.0* (8)
Resident cells	97.3 $\pm$ 0.6 (8)	90.9 $\pm$ 1.2 (6)	90.9 $\pm$ 1.2 (6)	84.7 $\pm$ 1.4 (8)
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Immunocytochemical study of various cell types was performed on vertical sections of grossly normal aorta and areas with atherosclerotic lesions. Nuclei were post-stained with methyl green. In each sample, the total number of cells and cells, positively stained with corresponding antibodies were analyzed. Numbers of analyzed samples are given in brackets.

0 - normal; I - initial lesions; II - fatty streak; Va - fibrolipid plaque.

* - statistically significant difference from uninvolved human aortic intima, $p < 0.05$.

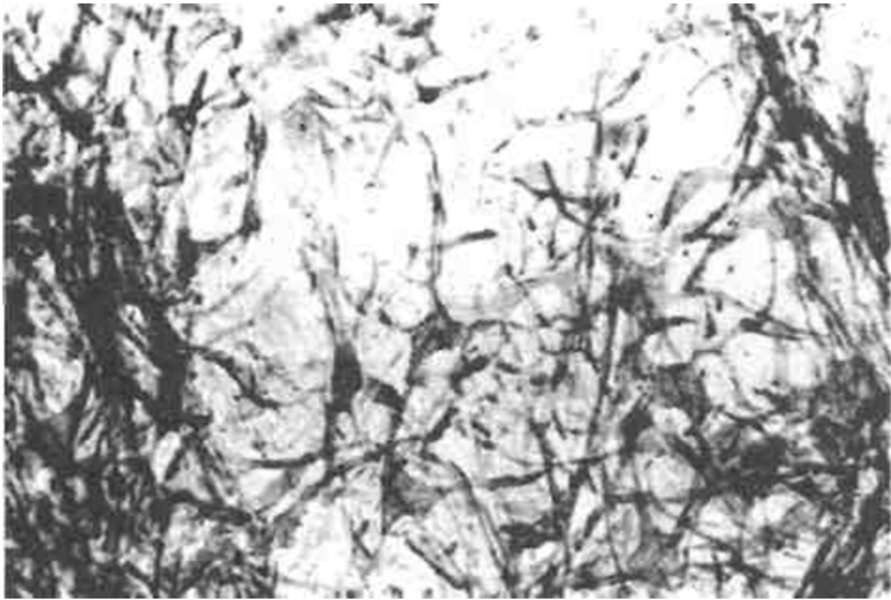


Figure 1: *En face* preparation of the proteoglycan-rich layer of human aortic intima. Immunocyto-chemical staining revealed smooth muscular α -actin. ABC-peroxidase. Magnification x 150.

It was found that about two thirds of cells in the muscular-elastic layer express muscular α -actin, whereas the proportion of such cells in the proteoglycan-rich layer is much smaller. Inflammatory cells (lymphocytes and macrophages) are localized only in the subendothelial part of intima in the proteoglycan-rich layer. Their content increases in atherosclerotic lesion sites, where it can reach about 20% of total cells.

Importantly, a considerable part of resident subendothelial cells does not express smooth muscular α -actin. It could be explained by possible phenotypical modulation of intimal cells in process of physiological adaptation and in course of pathological changes of the arterial wall. Presence of a well-developed contractile apparatus is not essential for intimal cells of aorta and other large arteries, because these blood vessels maintain the tonus via different mechanisms. In such circumstances, resident intimal cells can possibly exert other functions, such as trophic and metabolic support and regulation of the immune response in the vascular wall.

Following the authors of classical works [16-21], it can be hypothesized that these cells represent a population, which is functionally and, probably, ontologically different from typical smooth muscular cells of the media. For instance, Bobryshev et al. [15] described stellate cells in human aortic intima that express dendritic cell antigens S-100 and CD1a.

Antigens unusual for typical smooth muscular cells of the media have been revealed in resident intimal cells. In atherosclerotic lesions, some cells express the antigen 2A7, which is typical for pericytes from sites of active angiogenesis (Figure 2c). No such cells can be observed in uninvolved intima. The maximal numbers of 2A7-positive cells are detected in fibrolipid plaques

(Table 2). Antibodies 2A7 against High Molecular Weight Melanoma-Associated Antigen (**HMW-MAA**), which is a proteoglycan with chondroitin-sulfate moieties, positively stain not only tumor cells, but also areas where active angiogenesis is present [22]. Such areas include, for instance, granulation tissue formation during wound healing and synovitis. This antigen is expressed on “activated” pericytes that are capable of proliferation.

Taking into account the possible link of intimal cells to pericytes, antibodies to another pericyte antigen, 3G5, can be used for subendothelial cell identification. 3G5 antigen is an O-sialoganglioside of the cell membrane expressed on pericytes of brain capillaries, which is a very stable population with slow turnover. It was suggested that this antigen should be regarded as marker of resting pericytes [23]. Using anti-3G5 antibody, pericyte-like cells were revealed in uninvolved human and bovine intima, as well as in complicated atherosclerotic plaques with ectopic bone formation [24]. Anti-3G5 staining allowed revealing pericyte-like resident cells in human aortic intima both in grossly normal areas and in atherosclerotic lesions. In uninvolved intima, 3G5-positive pericyte-like cells account for more than one third of the total cell population (Table 2). On vertical sections, 3G5-positive cells are detected only in the uppermost, subendothelial intimal layer. On horizontal thin sections, it can be seen that 3G5-positive cells with processes form a cellular network below the endothelium [25,26]. Such location of the pericyte-like cells indicates that, in large arteries, such as aorta, they exert functions similar to those of capillary pericytes.

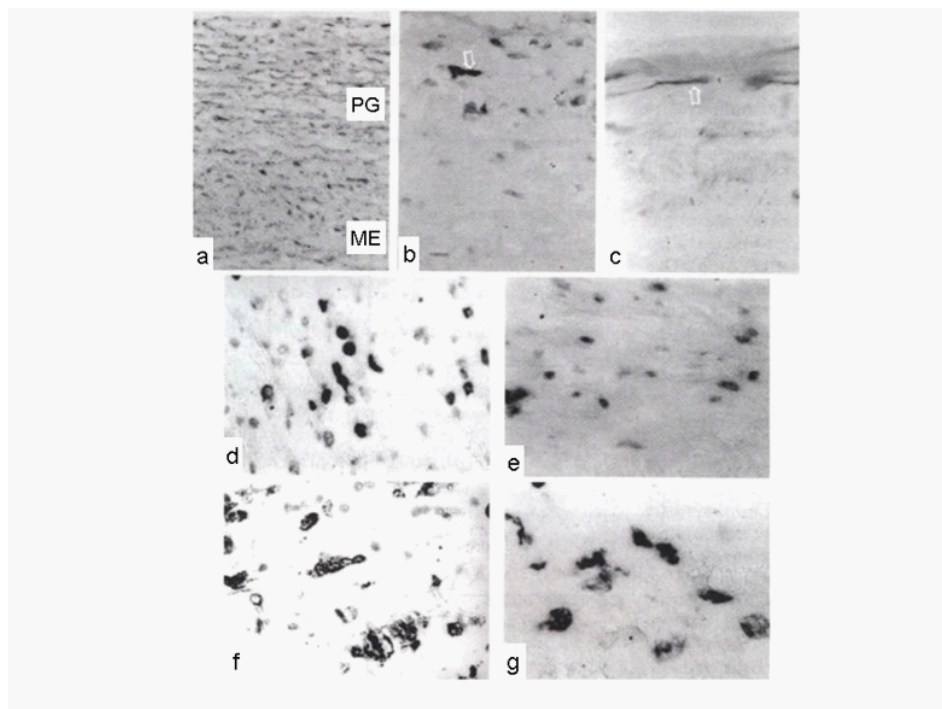


Figure 2: Immunocytochemical analysis of cell populations from human aortic intima. Vertical sections.

- a - Smooth muscular α -actin in the intimal cells. ABC-peroxydase, methyl green. Magnification x 300.
- PG - Proteoglycan-rich layer of the intima.
- ME - Muscular-elastic layer of the intima.
- b - Cells, positive for pericyte marker 3G5 (indicated with arrows). ABC-peroxydase, methyl green Magnification x 450.
- c - Cells, positive for pericyte marker 2A7 (indicated with arrows). ABC-peroxydase, methyl green. Magnification x 450.
- d - Lymphocytes in the proteoglycan-rich layer of intima. Identification was performed using anti-bodies against a common leukocyte antigen CD45. ABC-peroxydase, methyl green. Magnification x 450.
- e - Intimal macrophages positive for CD14 antigen. Alkaline phosphatase, methyl green. Magnification x 450.
- f - Cells, positive for CD68 antigen associated with macrophages. ABC-peroxydase, methyl green. Magnification x 450.
- g - Intimal macrophages positive for MAC3 antigen of tissue macrophages. Alkaline phosphatase, methyl green. Magnification x 450.

Cells positive for 3G5 are very rare in the muscular-elastic layer of intima. Most of them are detected in the subendothelial part of the proteoglycan-rich layer (Figure 2b). The percentage of 3G5-positive cells decreases in fatty lesions in comparison to uninvolved intima from 31% to 5% (in fibroateroma) (Table 2).

It has been suggested that pericytes can be actively involved in various processes that are associated with disturbed microcirculation: diabetes, inflammation, wound healing, hypertension and neoplasia (Sims DE, 1991), and can also coordinate endothelial functions, including endothelial cells proliferation and molecule and ion exchange [27-29]. One of the most important pericyte features is their ability to become precursors of other cell types of mesenchymal origin: smooth muscular cells [30], osteoblasts [31,32,27], chondrocytes [29], adipocytes [33]. This conforms to the earlier concept of the pluripotent nature of subendothelial cells representing the cambial [20], or mesenchymal reserve [17].

In atherosclerotic lesions, the number of cells positive for the pericyte marker 3G5 is significantly decreased in comparison to uninvolved intima. At the same time, the number of resident intimal cells is increased in the lesion sites. It could be concluded that pericyte-like cells are depleted from atherosclerotic lesions. However, study of the distribution of another pericyte antigen, 2A7, contradict to this conclusion. The number of 2A7-positive cells was increased in atherosclerotic plaques, whereas no 2A7 expression could be observed in uninvolved intima (Table 2). Such

expression pattern indicates that atherosclerotic lesion formation is associated with changes in the functional state of pericyte-like intimal cells accompanied by antigen expression alterations.

Another important question is whether or not the expression of different antigens is present in the same type of cells? To answer this question, cultured pericytes from brain capillaries - a homogeneous cell population, has been studied. Around 40% of these cells expressed 3G5 antigen, and 80% - 2A7 antigen. Simultaneous staining for 3G5 and 2A7 antigens also resulted in positive signal from about 80% of cells. Therefore, all cells positive for 3G5 express 2A7 antigen, however, there is a subpopulation of pericytes expressing only 2A7 antigen. As mentioned above, 3G5 and 2A7 pericyte antigens have been described for cells in different functional states. Antigen 3G5 is typical for “quiescent” pericytes, and 2A7 - for “activated” pericytes capable of intensive proliferation. It can be suggested that in cell culture conditions large part of pericytes becomes activated (as a result of exposure to serum factors and increased proliferative activity), and only some cells still retain the expression of 3G5 antigen.

Lipid accumulation is one of the key features of atherosclerosis. It was hypothesized that lipid accumulation can also influence the antigen expression by the subendothelial cells. Indeed, the 1.5 - 2-fold increase of the intracellular lipid content induced by Modified Low-Density Lipoproteins (**mLDL**) led to a significant decrease of the number of 3G5-positive cells (Figure 3).

The total number of cells remained constant, indicating that the decrease of 3G5-positive cells was not a result of selective cell death, but rather the consequence of the expression pattern alteration associated with intracellular lipid storage. This corresponds to the situation in atherosclerotic lesions, where 3G5-positive cells are less numerous than in normal intima. It can be therefore suggested that atherosclerotic lesion formation is linked to functional changes of pericyte-like cells of the proteoglycan-rich intimal layer that are reflected by alteration of protein expression pattern.

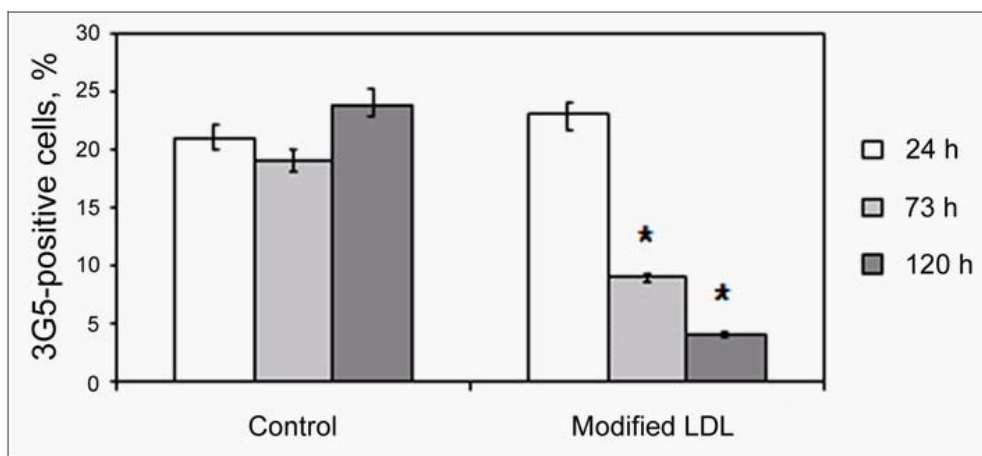


Figure 3: Influence of the intracellular lipid accumulation on the expression of 3G5 antigen in primary culture of cells from grossly normal human aortic intima.

At day 4 of culture, cells were washed and medium 199 was added, containing 10% FCS (fetal calf serum) (control); 10% FCS + 100 µg/ml apoB native LDL; 10% FCS + 100 µg/ml apoB modified LDL for 24, 72 and 120 h. After incubation, cells were washed and stained with antibodies to 3G5 pericyte antigen. Total number of cells and the number of positively stained cells were counted in 3 repetitions for each condition. Shown are representative results of one of the 3 similar experiments.

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

Another important feature of subendothelial intimacytes that can play a role in their involvement in the pathogenesis of atherosclerosis is the expression of macrophage antigen CD68. In primary culture of human aortic intimal cells, number of cells, double-positive for α -actin and CD68 increases after induction of intracellular lipid accumulation by mLDL [24]. It is therefore possible that presence of CD68 antigen is not a marker of monocytic origin, but rather of phagocytic activity of cells, which increases upon atherosclerotic lesion formation. Among the CD68-positive cells, branched cells, morphologically distinct from typical macrophages could be observed. Double staining with antibodies to α -actin and CD68 revealed actin filaments in the branched processes of these cells. The number of CD68-positive cells increases in the intima of atherosclerotic lesion sites in comparison to uninvolved intima (Table 2). At the same time, the number of double-positive cells increases in fatty lesions. Cells of haematogenous origin, or inflammatory cells, can be detected both in uninvolved intima and in atherosclerotic lesions. Antibodies to CD45 antigen allow revealing T and B lymphocytes. Cells positive for this marker have a typical shape: rounded nucleus surrounded of cytoplasm (Figure 5e). A small part of cells expressing CD45 antigen can be detected in fibrolipid plaques.

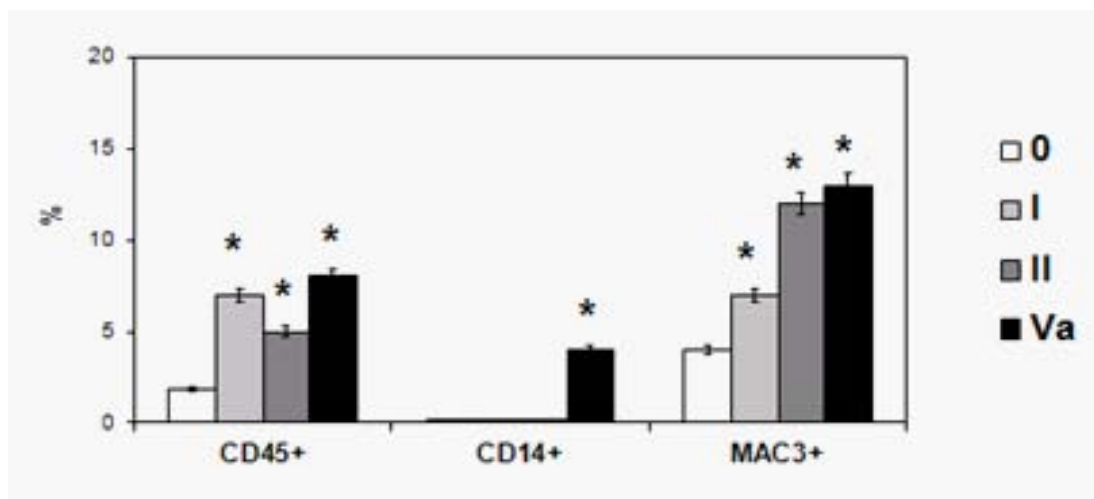


Figure 4: Inflammatory cells in the intima.

* - statistically significant difference from the control (grossly normal human aortic intima), $p < 0.05$.

Identification of another type of inflammatory cells, monocytes/macrophages, was performed using several antibodies. Most of the CD68-positive cells have the same morphology as CD14-positive cells (Figure 5f). Antibodies to CD14 revealed cells of monocytic origin with typical morphological features of macrophages: rounded or bean-shaped nucleus and ovoid cytoplasm with slightly irregular borders. The number of CD14-positive cells increases only slightly in atherosclerotic lesions as compared to normal intima, and remains low even in fibrolipid plaques (Figure 4).

As described above, CD68 cannot be regarded as specific marker of tissue macrophages in the blood vessel wall. Therefore, MAC3 antibodies, which do not stain smooth muscular cells, were used for this purpose. MAC3-positive cells have rounded or bean-shape nuclei and ovoid cytoplasm, sometimes with short processes. The number of MAC3-positive cells is increased in atherosclerotic lesions in comparison with grossly normal intima, with the maximal amount reached in fibrolipid plaques (Figure 4). However, even in atherosclerotic lesions, monocytes/macrophages and other inflammatory cells are not predominate cell types and do not exceed the number of pericytes and smooth muscular cells.

Using a set of antibodies for markers expressed by various cell types in the arterial wall allowed characterizing quantitative and qualitative changes that occur in resident and inflammatory cells in the same blood vessels. Smooth muscle cells, macrophages and lymphocytes are present in visibly uninvolved intima and in atherosclerotic lesions. Contrary to common opinion, no domination of monocytes/macrophages was observed among cells populating atherosclerotic lesions. A distinct cell type expressing pericyte antigens accounts for a large part of cellular population of the intima. These pericyte-like cells and smooth muscular cells are the basic cell types in the intima. In atherosclerotic lesions, these cells accumulate lipids and start proliferating. This is accompanied by changes in the expression of pericyte-specific antigens. Study of the cellular composition of the arterial intima has a long history [16,17,34,18]. Classical works demonstrate that the population of the arterial wall cells is heterogeneous and comprised of two large subpopulations: subendothelial cells with processes - resident cells, and rounded cells, morphologically similar to peripheral blood lymphocytes - migrated inflammatory cells. Subendothelial resident cells differ from typical smooth muscular cells of the media.

SUBENDOTHELIAL CELLS IN PRIMARY CULTURE

Cell culture is a convenient model for studying the mechanisms of atherogenesis. Primary culture, in which cells have not yet started proliferating and retain their main features, is especially interesting. It has been demonstrated that human subendothelial aortic cells start proliferating only at day 10-14 of culture [35]. This allows considering early primary cultures (5-7 days after seeding) as maximally relevant.

Immunocytochemical analysis with antibodies specific for various cell types was performed at day 7 of culture (Table 1). In all studied cultures, the majority of cells expressed muscular α -actin

(Figure 5a). There were also cells, positive for the pericyte-specific 3G5 antigen. These cells were large, well-spread on the substrate and had a polygonal shape (Figure 5b). The percentage of 3G5-positive cells reached 50% in cultures from grossly normal intima and was significantly reduced in atherosclerotic lesions (Table 3). Double staining with antibodies against 3G5 antigen and α -actin demonstrated that 3G5-positive cells also express the smooth muscular marker. In the studied cultures, cells expressing another pericyte antigen 2A7 were also identified. These cells were also large, resembling the 3G5-positive pericytes morphologically. The percentage of these cells varied between cultures obtained both from grossly normal intima and atherosclerotic lesions (Table 3).

Cells resembling peripheral blood monocytes, positive for CD14 (Figure 5d) and CD45 (Figure 5e) were also identified in the studied cultures. Staining of cultures from normal tissue and atherosclerotic lesions for the macrophage antigen CD68 delivered interesting results. CD68 antigen was localized in small cytoplasmic granules, which quantity varied from cell to cell. In cultures from grossly normal intima, most of the CD68-positive cells morphologically resembled peripheral blood macrophages. Cells, different from monocytes/macrophages were also observed in these cultures. They were larger, with a well-spread elongated body and processes. They resembled therefore smooth muscular intimal cells. The number of visible CD68-positive granules was moderate (Figure 5f). Cultures from fatty streaks and atherosclerotic plaques contained large rounded or oval cells with cytoplasm filled with CD68-positive granules, as well as smooth muscle-like cells containing large numbers of CD68-positive granules in the perinuclear area. Double staining demonstrated that these cells were also positive for smooth muscle α -actin.

Number of CD68-positive, α -actin-positive and double-positive cells was measured in cultures from grossly normal areas and atherosclerotic lesions (Table 4). In cultures from atherosclerotic lesions, number of cells expressing CD68 antigen was increased in comparison with cultures from grossly normal intima. Number of double-positive cells was increased as well. In contrast, percentage of cells expressing only α -actin proportionally decreased in cultures from atherosclerotic lesions.

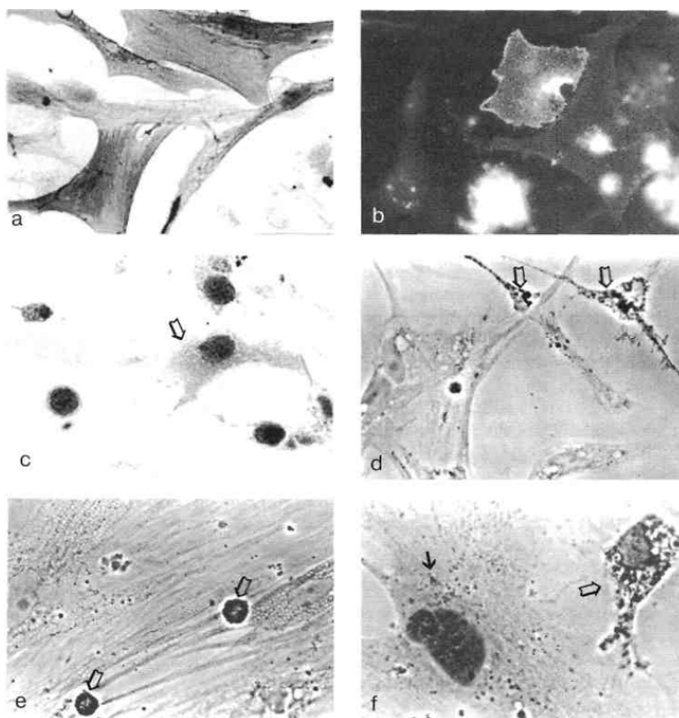


Figure 5: Immunocytochemical analysis of cells from primary culture of human aortic intima.

a - Smooth muscle α -actin. ABC-peroxydase. Magnification x 500.

b - Cells, positive for pericyte marker 3G5. FITC. Magnification x 500.

c - Cells, positive for pericyte marker 2A7 (indicated with arrows). ABC-peroxydase, hematoxylin. Magnification x 500.

d - Intimal macrophages expressing CD14 antigen (indicated with arrows). ABC-peroxydase. Phase contrast. Magnification x 500.

e - Lymphocytes in primary culture (indicated with arrows). Antibodies against the common lymphocyte antigen CD45, alkaline phosphatase. Phase contrast. Magnification x 500.

f - Cells expressing CD68 macrophage-associated antigen. ABC-peroxydase, hematoxylin. Magnification x 600.

→ - resident cell.

→- hematogenous cell.

Table 3: Immunocytochemical characterization of human aortic intima cells in primary culture.

Antigen	% of positive cells		
	0	II	Va
Smooth muscle α -actin	89.6 $\pm$ 6.7	77.5 $\pm$ 15.5	77.7 $\pm$ 11.8
CD45	3.6 $\pm$ 0.4	6.7 $\pm$ 1.1*	12.8 $\pm$ 2.8*
CD68	5.2 $\pm$ 1.3	14.5 $\pm$ 1.9*	13.9 $\pm$ 3.1*
3G5	45.8 $\pm$ 10.9	17.3 $\pm$ 3.2*	7.8 $\pm$ 1.9*
2A7	24.1 $\pm$ 9.9	13.3 $\pm$ 4.0*	9.2 $\pm$ 2.2*

0 - grossly normal intima; I - initial lesions; II - fatty streak; Va - fibrolipid plaque.

* - statistically significant difference from the control (grossly normal human aortic intima), $p < 0.05$.

Table 4: Expression of smooth muscle α -actin and CD68 in human aortic intimal cells in primary culture.

Lesion type	Smooth muscle α -actin (%)	CD68 antigen (%)	Non-identified cells (%)	α -actin + CD68 (%)
grossly normal intima (0)	75.1 $\pm$ 2.6	5.2 $\pm$ 1.3	5.7 $\pm$ 1.2	14.5 $\pm$ 4.1
fatty streak (II)	52.4 $\pm$ 8.5*	14.5 $\pm$ 1.9*	8.0 $\pm$ 1.1	25.1 $\pm$ 3.3*
fibrolipid plaque (Va)	63.1 $\pm$ 7.0*	13.9 $\pm$ 3.1*	8.4 $\pm$ 1.8	14.6 $\pm$ 4.8

* - statistically significant difference from the control (grossly normal human aortic intima), $p < 0.05$.

Therefore, primary cell culture allowed revealing the important feature of the subendothelial intimal cells, which can explain their involvement into the pathogenesis of atherosclerosis. It turned out that macrophage-specific antigen CD68 can be expressed not only by cells of hematogenous origin, but also by resident subendothelial cells. It was demonstrated that the percentage of cells, double positive for CD68 and α -actin is higher in cultures obtained from atherosclerotic lesions. This leads to the suggestion that CD68 is not the marker of cells of monocyte origin, but of phagocytic activity, which is increased in course of atherosclerotic lesion development.

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