Structural Organization of Reconstituted Human Arterial Smooth Muscle Tissue

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Abstract We used human arterial smooth muscle cells (SMCs) that had been reorganized three-dimensionally into aggregates, so-called spheroids, as a model system that might more closely correspond to arterial smooth muscle in vivo than do conventional monolayer cultures. After reaggregation the presence of serum in the culture medium strongly promoted the maintenance of spheroidal SMCs. With access to fresh serum, the spheroids developed into highly organized structures with an outer laminated shell of spindle-shaped SMCs and a more porous core of rounded or polygonal SMCs. After several weeks in culture, extracellular matrix components appeared and the tissue assumed features characteristic of maturing intimal repair tissue. Many cells had features of programmed cell death (apoptosis). This feature may be important because it may indicate that regression of arterial smooth muscle tissue may be a more strongly controlled process than hitherto realized. Without access to fresh serum, the spheroidal tissue showed degenerative features, much like those in atherosclerotic lesions, ie, the presence of foam cells, cellular debris, and some cell death. It is possible that this situation in vitro resembles that of atherosclerotic tissue in vivo, in which retention of plasma constituents is a conspicuous feature. In some respects, therefore, the small sample of human arterial tissue represented by the spheroid may represent an in vitro analogue of the arterial wall, which may undergo maturation or degenerative atherosclerosis-like changes depending on exogenous factors. The spheroidal SMC system may therefore also be a suitable model for in vitro studies of atherogenesis. (Arterioscler Thromb. 1994;14:644-651.)

Key Words • vascular smooth muscle • arteries • atherosclerosis • humans • cell survival • foam cells • cell differentiation • cell death • extracellular matrix • apoptosis

Our concepts of the biology of the arterial wall are largely based on results from in vitro studies that have mainly employed cell culture techniques, and almost all investigations of this kind have used monolayer cultures. With respect to the smooth muscle component in the vascular wall, this situation in vitro may be dissimilar to conditions in vivo, in which the smooth muscle is a highly organized, three-dimensional tissue. For this reason, results from two-dimensional cell culture studies may not be directly applicable to the in vivo situation. Excessive intimal thickening is a primary feature of atherosclerosis and of several nonatherosclerotic, arterial stenotic conditions as well, eg, restenosis after balloon dilatation, neointimal occlusion of coronary bypass and other grafts, and chronic vascular rejection after transplantation, and much interest has been paid to the characteristics and regulation of growth of smooth muscle cells (SMCs). However, virtually all in vitro results concerning SMC growth have been based on monolayer cultures. Several systems have been used to simulate three-dimensional in vivo growth. Of these systems, cellular agglomerates, so-called spheroids, have been used frequently in tumor research for studies of cell growth (for review, see Reference 5). That growth in spheroids is analogous to growth in vivo in some respects is suggested by the finding that growth of normal, nontransformed cells is generally inhibited when they are densely packed in spheroids as they are in vivo for nonactivated tissues. Despite the high cellularity, transformed cells, however, generally grow even in spheroids in similarity to tumor cells in vivo. Arterial smooth muscle tissue has a spontaneous tendency to form spheroids under certain conditions in vitro. A well-known and characteristic property of SMCs in culture is their tendency to form multilayered regions ("mounds," "hills," or "nodules"), which contain a subpopulation of SMCs with a low capacity for adhesion. Selection of this subpopulation strongly promotes multilayer formation in culture to a degree that pieces of multilayered tissue become detached and form free-floating spheroids. Spheroids are also formed from SMCs after reaggregation of enzyme-dispersed cells. The latter system with rat, rabbit, and chicken SMCs has mainly been used for studies of the electrophysiological and contractile properties of reaggregated cells. We have recently described some structural and biological changes that occur during the very early phase after reaggregation of human SMCs. The possibility that SMC spheroids might more closely imitate the situation in the arterial wall and thus be more applicable to the in situ situation during intimal thickening than the conventional monolayer system prompted us to investigate the conditions for sustained cultivation of human SMC spheroids and to study the structural organization during the development of spheroids under various conditions. Methods Arterial wall samples were obtained from the abdominal aorta from traffic victim kidney donors. The arterial wall samples were cut open and immersed in Ham's F10 nutrient...
FIG 1. Semilog plot showing effect of serum concentration on recovery of human arterial smooth muscle cells after 1 week as agglomerates (spheroids). The cells (20,000 per well) were placed in agarose-coated quadruplicate wells for each concentration of serum in nutrient medium MCDB 104 (GIBCO) with 2.6 mmol/L L-glutamate, 50 μg neomycin, 5 μg transferrin (Collaborative Research), 50 μg ascorbic acid, and 1 mg ovalbumin (grade V, Sigma Chemical Co) per milliliter, after which the cells spontaneously aggregated into spheroids. After 1 week the spheroids were disaggregated enzymatically and the cells counted. Arrow and short vertical bar on the y axis represent the value for serum-free medium. For further details see "Methods."

FIG 2. Transmission electron photomicrographs of smooth muscle cells agglomerated to spheroids and incubated for 3 days to 1 week under conditions indicated in Fig 1 legend and with serum- and fibronectin-free medium. A, Survey at low magnification with a typical small spheroid that formed under serum-free or low-serum conditions. Cells are very densely vacuolized, and the intercellular space is wide and contains abundant debris. S indicates surface. B, Higher-magnification view of markedly vacuolized cytoplasm with numerous regions of fused but not membranous vacuoles that are suggestive of cytoplasmic defects (magnification x2270 [A] and x9600 [B]).
medium was changed weekly except for experiments that involved serum starvation.

To release the cells from spheroids for cell counting, the spheroids from each well were centrifuged, the supernatant was discarded, the pellet was washed once with Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS and suspended in 0.9 mL of Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS; 100 \(\mu\)L of a solution of 15 to 20 mg collagenase per milliliter (type I, Worthington-Cooper, Millipore Corp) in Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS was added. The suspension was incubated at 37°C for 45 minutes. The suspension was centrifuged at 180g for 5 minutes. The sediment was resuspended in 1.0 mL of 0.25% trypsin solution (DIFCO), and incubation was continued for an additional 10 to 15 minutes at 37°C. The suspension was disaggregated further by gentle suction (three times) through a 2.0-mL siliconized measuring pipette, after which 2.0 mL of culture medium was added. The cells were centrifuged and washed once more with culture medium. The number of cells was determined by counting the total cell number in aliquots, the volume of which was chosen to contain 100 to 300 cells. Counting was done in open wells with a grid engraved on the bottom plate and with an inverted phase-contrast microscope that permitted selective counting of intact cells and exclusion of cell fragments and debris.

For transmission electron microscopy, the spheroids were centrifuged and the pellet was fixed in 3% glutaraldehyde solution, rinsed, postfixed in 1% Os\(_2\)O\(_4\) solution, rinsed, dehydrated in a graded series of ethyl, immersed in propylene oxide, embedded in epoxy resin (agar resin 100, Agar Aids), and sectioned in an ultramicrotome. The sections were stained with uranyl acetate and lead citrate and examined in a Philips MI 400 transmission electron microscope.

Results

Serum Dependence

The recovery of SMCs after aggregation to spheroids was directly related to serum concentration, as demon-

Fig 4. Facing page. Transmission electron photomicrographs of smooth muscle cells agglomerated to spheroids and incubated for 1 week with a high serum content in the medium. A, Survey at low magnification of spheroid incubated with 60% serum in the medium. Spheroids were larger than in cells cultured with a low serum content in the medium and were stratified, with a superficial shell-like zone of elongated, spindle-shaped, relatively densely arranged smooth muscle cells and a core of more rounded, less densely arranged smooth muscle cells. Some cells, especially in the core region, were smaller, with condensed chromatin (long arrows) and tabulated or fragmented nuclei. These cellular changes are indicative of apoptosis.17-19 There were also even smaller bodies with fragments of condensed chromatin (short arrows) characteristic of cells in a late phase of apoptosis (apoptic bodies17-19). B, Higher-magnification view of superficial zone of spheroid cultured at high serum concentration (60%). In the superficial zone smooth muscle cells were sparsely vacuolized, had abundant rough endoplasmic reticulum, and contained few lipid droplets. Extracellular space was rather narrow and debris scarce. C, Higher-magnification view of core of a spheroid (100% serum). In this region cells were more rounded and had variable numbers of digestive vacuoles, secondary lysosomes, and multivesicular bodies, and some cells contained lipid droplets. Membrane residues in digestive vacuoles indicate autodigestion. Endoplasmic reticulum seemed generally less developed in deeper regions of spheroids. Mitochondria were generally well preserved. Especially in this region some cells had condensed chromatin (long arrow) and lobulated or fragmented nuclei (double arrow) indicative of apoptosis (see also A). Extracellular space was wide and contained large amounts of cellular debris, rounded structures with the appearance of apoptotic bodies (short arrow) with condensed and fragmented chromatin, and sparse cytoplasmic elements (magnification \(\times 785\) [A] and \(\times 7400\) [B]).
strated in Fig 1. Without serum (arrow), a small fraction of the cells was preserved, but with increasing serum concentration as much as 50% of the cells was recovered 1 week after aggregation.

The structural changes in the spheroids also were related to serum concentration and were studied by transmission electron microscopy for serum concentrations of 0%, 1.5%, and 5% in the medium after 3 and 7 days and of 40%, 60%, 80%, and 100% pure serum as the medium after 7 days. Without serum the cytoplasm developed numerous vacuoles (Fig 2A), many of which were fused but partially separated by incomplete, irregular strands of residual cytoplasm (Fig 2B). The vacuoles were not membranous, a feature indicative of cytoplasmic defects. The cells contained varying numbers of lipid droplets, and some cells had a foam cell-like character. For spheroids in serum-free medium, the cells were rather loosely packed and the
extracellular space was generally wide and contained large amounts of cell debris.

With 1.5% and 5% serum, increasingly larger portions of the cytoplasm were preserved. With 1.5% serum (Fig 3A) the cytoplasm was almost as heavily vacuolated as with serum-free medium, but most vacuoles appeared to be regular membranous digestive or autophagic vacuoles or multivesicular bodies (Fig 3B) and were not merely “holes” in the cytoplasm as in serum-free medium (compare with Fig 2B). Lipid droplets were abundant in some cells.

**Tissue Development in Spheroids**

SMCs agglomerated into spheroids and incubated for 1 week with a high serum content in the medium yielded larger spheroids, which were stratified with a superficial shell-like zone of elongated, relatively densely arranged SMCs and a core of more rounded SMCs (Fig 4A). In the superficial zone the SMCs were sparsely vacuolated and had abundant rough endoplasmic reticulum and few lipid droplets (Fig 4B). The extracellular space was rather narrow, and the amount of debris was small.

In deeper regions of the spheroids the cells were more rounded and had numerous digestive vacuoles, secondary lysosomes, and multivesicular bodies, and some cells contained lipid droplets (Fig 4C). Membrane residues in some digestive vacuoles indicated that autophagy had occurred. The endoplasmic reticulum seemed generally less developed in deeper regions of the spheroids. The extracellular space was wide and contained accumulations of cellular debris.

Some cells especially in the core region were smaller, with lobulated or fragmented nuclei and condensed chromatin (Fig 4A and 4C). These cellular changes were clearly indicative of programmed cell death, ie, apoptosis. There were also even smaller bodies, with fragments of condensed and fragmented chromatin, characteristics of cells in a late phase of apoptosis, (ie, apoptotic bodies; Fig 4A and 4C).

After 3 weeks of incubation with medium containing 60% serum that had been changed weekly, the spheroids had a prominent outer shell-like region with flattened cells organized in a laminated fashion (Fig 5A). Rough endoplasmic reticulum and bundles of microfilaments were abundant. The cells of the inner core-like region were rounded or polygonal (Fig 5B) and some had secondary lysosomes (Fig 5B) and sparse lipid droplets, but foam cells were not encountered. Microfilaments or rough endoplasmic reticulum was less abundant in cells of the core region. Extracellular space increased with depth in the spheroid, and in the core region the cells were dispersed in a predominantly amorphous matrix and cell contacts were sparse (Fig 5A). Bundles of filamentous material appeared extracellularly in both the shell and core regions (Fig 5C) and some of it was cross striated, suggesting early formation of collagen fibers (Fig 5C).

To study the importance of fresh medium and serum, cultures of spheroids were incubated for 3 weeks in medium containing 60% serum without any change of medium. There was a tendency for organization of the spheroids, with a prominent outer shell-like and an inner core region, but it was much less pronounced (Fig 6A). Even the cells of the shell region were rounded or polygonal, and many cells in both regions had a foam cell character, with numerous lipid droplets (Fig 6A through 6C) and lysosomes. The extracellular space was very wide toward the deeper parts of the spheroids and contained cell debris and some microfilamentous material, but cross striation, indicative of collagen formation, was not encountered.

**Discussion**

Investigations of cells cultured in vitro have greatly contributed to our understanding of cell and tissue physiology. However, as generally employed, ie, as monolayer cultures, growth conditions of these cells may be remote from those in vivo. This is especially true for monolayer cultures, which grow in three-dimensional patterns in vivo. Several systems have been constructed to imitate three-dimensional in vivo growth, and of these cellular agglomerates, spheroids have been frequently used in tumor research for studies of cell growth.5,14 That growth in spheroids may be analogous to growth in vivo in some respects is suggested by the finding that growth of normal nontransformed cells is generally inhibited when they are densely packed in spheroids,3,14 as they are in vivo for nonactivated tissues. Despite a high cellularity, however, transformed cells generally grow even in spheroids in similarity to tumor cells in vivo.

In an earlier report we have demonstrated that after their aggregation into spheroids in medium containing 5% serum, most human arterial SMCs degenerate to SMC foam cells.11 In contrast, such changes are much less apparent with human lung fibroblasts. Similar changes occur in monolayer cultures of SMCs in conjunction with focal development of multilayered growth.6,7 Human arterial SMCs, therefore, seem especially sensitive to conditions with high cellular density. This is interesting not only in terms of cell biology, it might be an important indication of SMC behavior in situations with potentially high cellular density in the arterial wall, such as during repair, and possibly contribute to the formation of atherosclerotic lesions, which are characterized by foam cells and tissue necrosis.

SMC spheroids require a high serum concentration in the culture medium for maintenance in vitro, in contrast to what is required for monolayer SMC cultures. Very high serum levels may be toxic to monolayer SMC cultures, but no such effects were observed for spheroids cultured with 100% serum, which indicates that the two systems are also markedly different in this respect.

With time, the arterial smooth muscle spheroid develops into a highly organized structure with an outer, dense, and laminated shell-like region with spindle-shaped cells and a more porous, inner core region with rounded or polygonal cells and extracellular matrix. This temporal series of changes in the spheroid has many features in common with the in vivo changes that occur in the artery during repair and progressive nonatherosclerotic intimal thickening, which has been conceived as adaptive reinforcement of the arterial wall.15,16

The electron microscopic investigations on the development of the smooth muscle in the spheroids disclosed changes in many cells that are distinctive features of programmed cell death, ie, apoptosis (for a review of apoptosis see References 17 and 18). In contrast, we did
not find changes indicative of nonspecific cell necrosis in the organized, maturing spheroids. To our knowledge apoptosis in SMCs has not been reported earlier, and its presence in this cell type may be important. It may indicate that regression of human arterial smooth muscle may be a much more organized and strongly con-
trolled process than hitherto realized. It is known that apoptosis is important in many situations involving rapid modeling of tissues and organs,\(^7\) for instance, during normal embryonic and fetal development, during regression of hyperplastic tissue, and during normal involution and pathological atrophy of adult tissues.\(^9\) That the hyperplastic arterial intima after experimental arterial injury rapidly regresses after repair\(^5\) and that regression under certain circumstances may occur in both experimental and human atherosclerotic lesions are well documented (for a review see Reference 20). The possibility that the initiation and further regression of hyperplastic arterial intimal tissue is under programmed control may give new clues for understanding the nature of persistent or increasing intimal thickening in atherosclerosis and nonatherosclerotic conditions, such as progressive intimal thickening with age, restenosis after balloon dilatation, and chronic vascular rejection.

When the spheroids were deprived of fresh serum and medium, the tissue degenerated like that in athero-
sclerotic lesions. It is possible that this situation resembles that in atherosclerotic tissue, in which retention of plasma constituents is a conspicuous feature. The small sample of human arterial tissue represented by the spheroid may therefore in some respects represent an in vitro analogue to the arterial wall, which may undergo maturation or degenerative atherosclerosis-like changes depending on exogenous factors. The spheroidal SMC system may, therefore, also be a suitable model for in vitro studies of atherogenesis.

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References

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