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A scanning electron microscopic study of phenotypic plasticity and surface structural changes of aortal smooth muscle cells in primary culture

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Phenotypic modulation of smooth muscle cells (SMCs) from a contractile to a synthetic state characterised by active proliferation appears to be an early event in the pathogenesis of atherosclerosis. A similar transition occurs when SMCs are established in culture. In this study the phenotypic plasticity and surface structural changes of aortal smooth muscle cells during the transition from the contractile to the synthetic state and during maturation have been structurally assessed by scanning electron microscope (SEM). The experiments were performed on SMCs obtained from aorta of neonatal rats after enzymatic digestion and then cultured on glass cover slips. SEM observations revealed a three-dimensional appearance characteristic for different stages of SMCs. Intensively proliferating cells from monolayer region were large, polygonal in shape with lamellipodia and well spread. Long, uniform in diameter, finger-like microvilli were densely arranged on the surface of these cells. In the thickened region of culture, the cells were rather small, generally spindle-shaped, not well spread, with low density of short, bubble-like microvilli on the surface. Numerous plasma membrane structural alterations in apoptotic cells were observed by SEM: loss of cellular adhesion, smoothing, shrinkage and outpouching of membrane segments have been recognised as markers associated with the cell injury and death. It was concluded that scanning microscopy observations would allow a more complete understanding of SMCs and their changes in culture and atherosclerotic disease.

key words: apoptosis, microvilli, phenotypic modulation, SEM

INTRODUCTION

Smooth muscle cells display multifunctional capacity for contraction, migration, proliferation, synthesis and secretion of extracellular matrix (ECM) components both in vivo and in vitro [1, 6, 12]. In the early stages of atherosclerosis and also in tissue culture the SMCs modify from a differentiated contractile phenotype to an immature, synthetic phenotype [6, 12, 13]. It is obviously known that the SMCs express different phenotypic features in primary culture and thus the population of these cells is heterogeneous in their morphology and growth. Cells isolated from newborn animals have a greater proliferative capacity than adults and maintain, at

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least in part, the potential to express differentiated features in culture [1]. Thus, cultured newborn rat aortic SMCs furnish an in vitro model for the study of several aspects of SMCs differentiation.

Ultrastructurally the synthetic phenotype is characterised by an increase in membranous organelles (such as a prominent Golgi apparatus, extensive endoplasmic reticulum and mitochondria) and a decrease in intracytoplasmic microfilaments. The cells in contractile phenotype have less abundant membranous organelles, but a large part of their cytoplasm is densely filled with thin myofilaments, with a tendency to arrange in arrays [13–15]. The contractile filaments and cytoskeleton elements in SMCs interact with each other, with the plasma membrane, other membranous organelles, and with subcellular structures or molecules to generate and control cell: movement, shape, adhesion or division [1, 2, 13]. According to phenotypic diversification cytoskeleton proteins rearrange and therefore the ultrastructure of the cell surface differs significantly due to the physiological condition of the cell. The microvilli on the SMCs surfaces may reflect a high metabolic activity of proliferating cells and may play an important role in the process of atherogenesis.

The aim of the present study was to examine morphological changes and growth characteristics of aortal SMCs in primary culture using scanning electron microscopy technique. We also used scanning microscope for analysis of apoptosis, a physiological form of cell death present in many disease conditions when the balance of mitosis versus apoptosis is altered.

MATERIAL AND METHODS

The investigation was carried out according to the requirements of the Ethics Committee for Animal Care of Poland.

Cell culture

Smooth muscle cells were obtained from the media of neonatal Wistar rats' aorta. Rats were purchased from the same animal breeding company. SMCs isolation, purification and culturing were conducted according to the method described before [14, 15]. The cells were examined from 5 to 9 days after inoculation when they grew logarithmically in primary culture.

Immunocytochemical staining

The analysis of cytoskeleton of *in vitro* cultured SMCs was performed employing the direct immunofluorescence method. The cells attached to the cover slips were washed with PBS, fixed by immersion in absolute methanol for 5 min at -20°C, and air-dried. Prior to incubation with antibodies, the specimens were washed with PBS and blocked for 2 min. in PBS containing 1% foetal bovine serum. The culture was incubated for 30 min at room temperature with the anti-α-tubulin monoclonal antibody conjugated with FITC (Sigma, F2168) diluted 1:200 in PBS. After gentle washing in PBS the cells were mounted in glycerol and examined with a Nikon Eclipse 800 microscope equipped for epifluorescence using the appropriate filter set.

Scanning electron microscopy

Cells on cover slips were washed twice with PBS, fixed in 2% glutaraldehyde in 0.1 M Na-cacodylate — sucrose buffer (pH 7.2, 37°C) for 5 min. Next, the fixative mixture was replaced with a fresh one for 1 h at room temperature, gently agitated and once more changed for a further 24 h at 4°C. Fixed cells were rinsed three times in the same buffer and post-fixed with 1% OsO_4 in 0.1 M Na-cacodylate — sucrose buffer, at room temperature for 1 h. Dehydration was carried out in increasing concentrations of ethanol, up to 100%. The cover slips were carefully displaced from the culture dish with a preparation needle and dried by the critical point method using liquid CO_2. Finally, specimens were coated with gold in a sputter-evaporator (Balzers), and stored under vacuum until they were examined and imaged in a Philips XL 30 scanning electron microscope operated at an accelerating voltage of 10 kV.

RESULTS

The SMCs were attached to the glass cover slips and spread out within 48 h after seeding in culture. After that intensive proliferation and "hills and valleys" formation was observed. Focally multilayered regions appeared as mounds surrounded by monolayer. The changes were noted in the cytoskeleton structure of SMCs involved in the different expression of α-tubulin as well as the different arrangement of microtubules (description in Fig. 1, 2).

Proliferating cells (synthetic phenotype) from monolayer region were large, polygonal in shape and well spread (Fig. 3, 4). Lamellipodia appeared as a flap-like, branched structures with mitochondrial protrusions and radial fibrils of actin cytoskeleton (Fig. 5). In some cells (e.g. dividing) numerous, long and thin filopodia, with many intercellular junctions were observed (Fig. 6, 7).

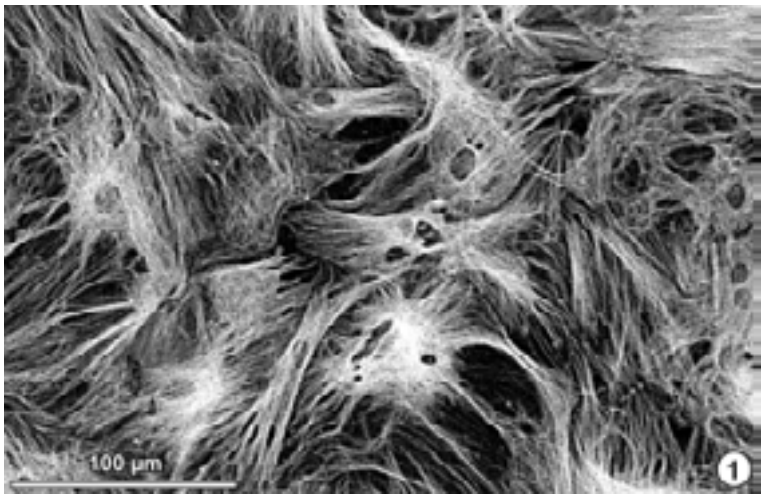

Figure 1. SMCs in primary culture. Synthetic phenotype — highly expressed three-dimensional network of α-tubulin in the cells. Note the radial distribution of microtubules in the flat, stellate cells. Direct immunofluorescence.

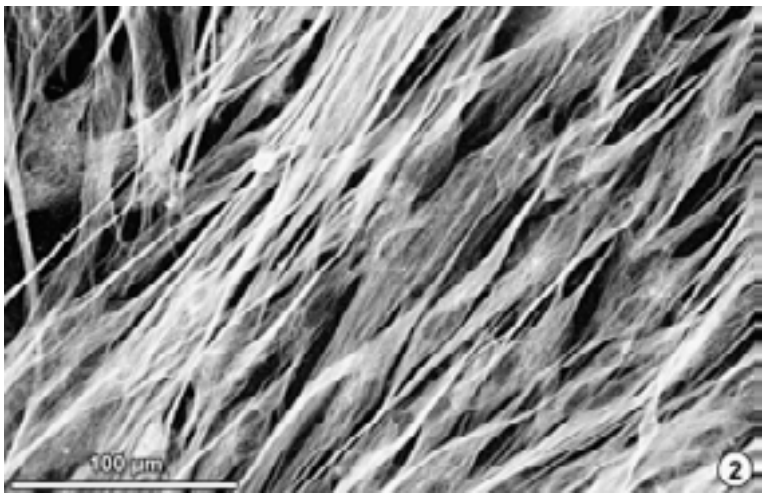

Figure 2. SMCs in primary culture. Contractile phenotype — low expression of a-tubulin in the elongated, spindle-shaped cells. The microtubules are longitudinally arranged. Direct immunofluorescence.

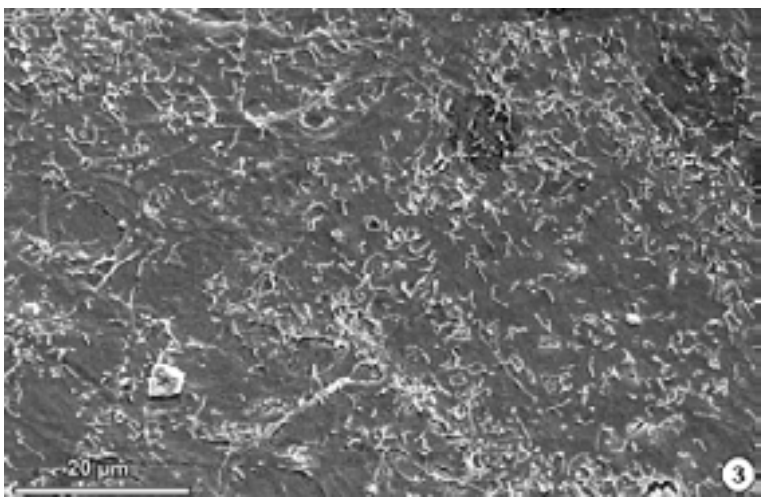

Figure 3. SMCs attached to glass cover slips maintain proliferative phenotype. The well-preserved, finger-like microvilli are densely arranged on the cell surface. SEM.

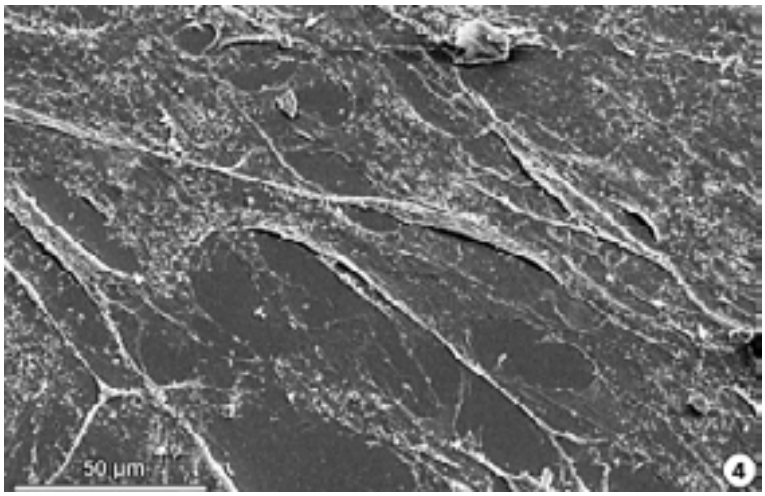

Figure 4. Large, polygonal in shape and well spread SMCs. Note the junctions between the cells. SEM.

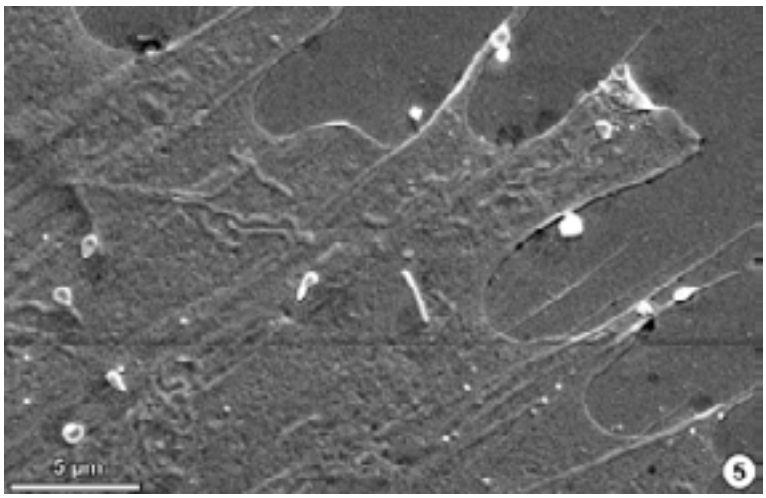

Figure 5. Flap-like lamellipodia with mitochondrial protrusions and elements of cytoskeleton are visible. SEM.

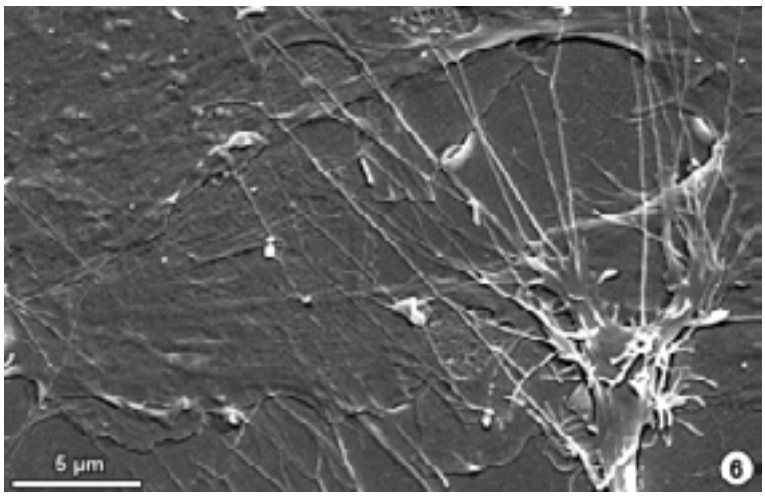

Figure 6. Good preservation of numerous, long and thin filopodia, with many intercellular junctions. SEM.

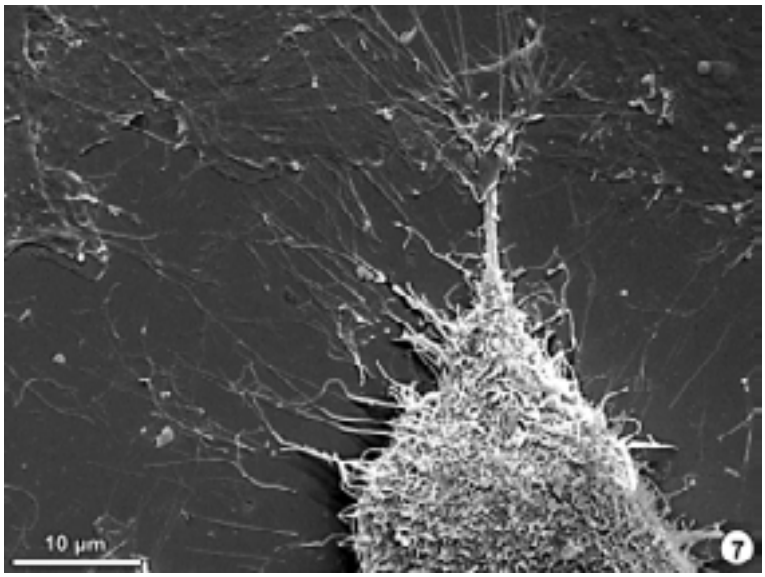

Figure 7. SMC undergoing mitosis. The long, thin, and densely arranged microvilli cover the well-preserved cellular surface. SEM.

The contractile phenotype cells were rather small, generally spindle-shaped and not well spread. The elongated cells were circumferentially oriented, ran parallel to one another and formed a continuous polylayer (Fig. 8, 9).

Microvilli were recognised as common surface structures of the cultured cells. The microvilli were categorised as long, short or blunted and their distribution varied significantly. Long, uniform in diameter, finger-like microvilli, were densely arranged on the surface of cells revealing synthetic phenotype (Fig. 3, 4). Lower density of bubble-like microvilli on the surface of cells revealing contractile phenotype was observed (Fig. 9).

When the SMCs spontaneously undergone to apoptosis they showed numerous plasma membrane structural modifications. The initial changes, such as a loss of pericellular adhesion to substrate, their further rounding and smoothing caused by losing of microvilli on the cell surface, were monitored by SEM (Fig. 10). At higher magnifications irregular, disorganised, fragmented microvilli-like formations of various size were observed. Blebbing, shrinking and outpouching of membrane segments were the next specific phenomena at the cell surface, closely associated with SMCs apoptosis (Fig. 11). Finally, the apoptotic bodies detached from the cells surface were visible.

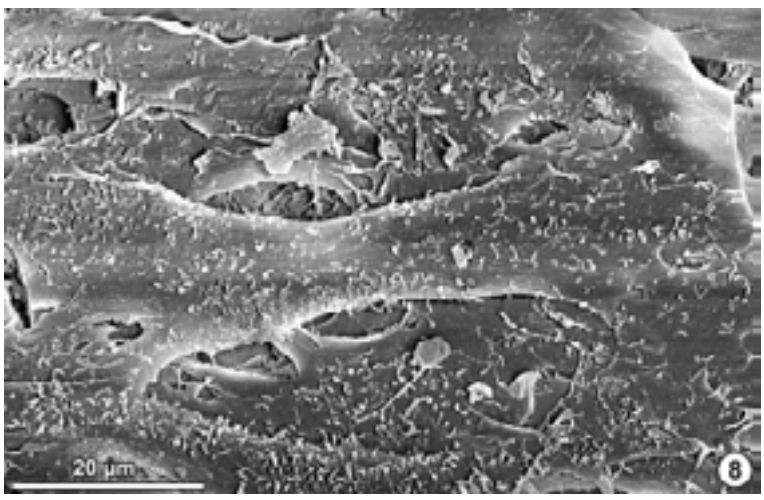

Figure 8. SMCs forming a multilayer. SEM.

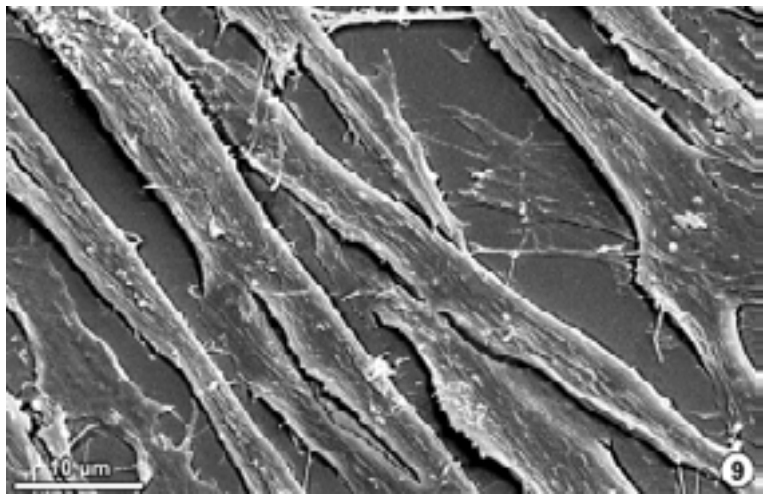

Figure 9. Spindle-shaped SMCs are not well spread and run parallel to each other. Short, sparsely distributed microvilli on the cell surface are visible. SEM.

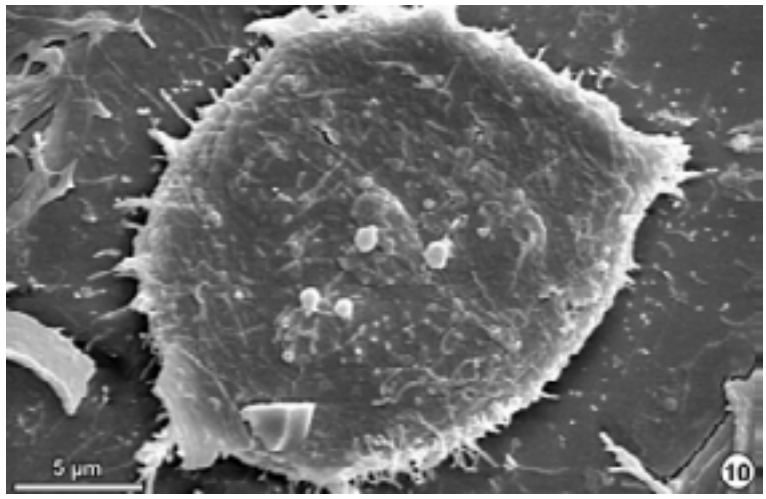

Figure 10. SMC at early stage of apoptosis. Typical features associated to apoptosis such as a reduced cellular adhesion, rounding and smoothing are visible. SEM.

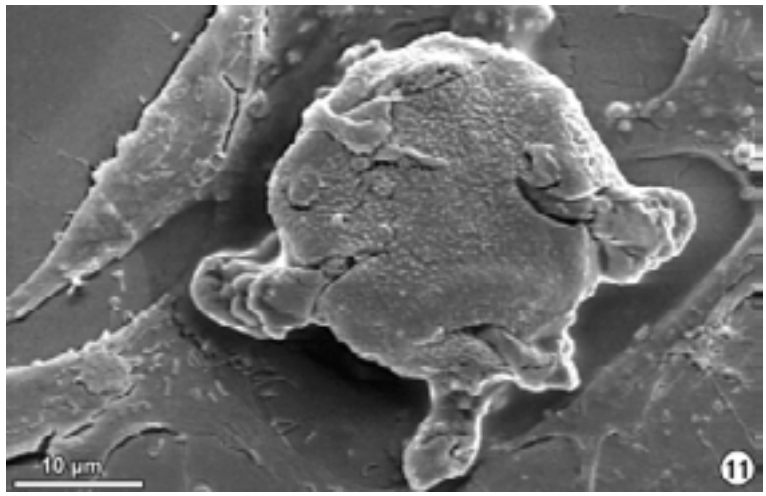

Figure 11. SMC at pronounced stage of apoptosis. Large blebs are present on the cell surface. SEM.

DISCUSSION

It is essentially well accepted that vascular SMCs exhibit two distinct phenotypes and a change from the contractile to the synthetic phenotype plays a key role in the development of atherosclerotic disease [6, 12, 13]. To describe the process of modulation and differentiation of SMCs in primary culture we previously used transmission electron microscopy [14, 15]. Many morphological modifications become progressively visible in the internal structure of the cells, which suggests their surface ultrastructure may also differ to some extent, due to the metabolic state of each cell. In the present study our efforts were focused on the researching cell membrane and its plasticity during a variety of cellular activities: adhesion, motility, multilayer formation, cell division and cell death.

Standard techniques for electron microscopy and optimal methods for SEM, which allow a single cell and cell cultures to be successfully exposed, are different [4, 8, 10, 11]. We modified the procedure for preparing animal cells grown on cover slips [10], as was described under Material and Methods. From the methodological point of view, it was beneficial to use a stepwise fixation of specimens processed for SEM at appropriate temperature and adequate time. A selection of dehydrates and drying techniques to obtain good quality of the cell surface with negligible degree of cell shrinkage was also made. After use of the modified method neither evident distortion nor collapse of the most delicate structures, such as a microvilli, were observed.

Scanning electron microscopic observations confirmed numerous ultrastructural features of SMCs connected with transition from the contractile (G_0 phase) to the synthetic phenotype (G_1 phase) in the culture [12]. As the cell cycle progresses, an increase in cell volume is accompanied by an increase in cell plasticity and mobility. The migration activity in culture involves the extension of lamellipodia, attachment to the extracellular matrix and the generation of force that pulls the cell forward. According to Fukui et al. [5] this process takes place in late G_1 phase. On the other hand, the SMCs before mitosis round up, and become less adhesive.

Proliferation of SMCs and their programmed cell death both play a role in the early stages of atherosclerosis [6, 7, 9]. As far as we know, SEM analysis of apoptosis is mainly referred to the study of plasma membrane modifications on the cell surface [3]. To our best knowledge, the presented results have given the first evidence that the apoptotic SMCs in culture exhibit alterations of their surface connected with rounding, smoothing, shrinking and blebbing.

We conclude that the scanning electron microscopic observations provide complementary, easily accessible information concerning several aspects of SMCs modulation and differentiation. It would allow a more complete understanding of SMCs and their changes in culture and atherosclerotic disease.

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REFERENCES

1. Bochaton-Piallat ML, Gabbiani F, Ropraz P, Gabbiani G (1992) Cultured aortic smooth muscle cells from newborn and adult rats show distinct cytoskeletal features. Differentiation, 49: 175–185.
2. Chitaley K, Webb RC (2001) Microtubule depolymerization facilitates contraction of vascular smooth muscle via increased activation of RhoA/Rho-kinase. Med Hypotheses, 56: 381–385.
3. Engeland van M, Kuijpers HJH, Ramaekers FCS, Reutelingsperger CPM, Schutte B (1997) Plasma membrane alterations and cytoskeletal changes in apoptosis. Exp Cell Res, 235: 421–430.
4. Evan AP, Connors BA (1996) Morphometric analysis of vascular smooth muscle cells by scanning electron microscopy. Int Rev Path, 36: 31–52.
5. Fucui R, Amakawa M, Hoshiga M, Shibata N, Kohbayashi E, Seto M, Sasaki Y, Ueno T, Negoro N, Nakakoji T, Li M, Nishiguchi F, Ishihara T, and Ohsawa N (2000) Increased migration in late G1 phase in cultured smooth muscle cells. Am J Physiol Cell Physiol, 279: C999–C1007.
6. Hegele RA (1996) The pathogenesis of atherosclerosis. Clin Chim Acta, 146: 21–38.
7. Kockx MM, Knaapen MW (2000) The role of apoptosis in vascular disease. J Pathol, 190: 267–280.
8. Krizmanich WJ (1997) Correlation of vascular smooth muscle cell morphology observed by scanning electron microscopy with transmission electron microscopy. Exp Mol Pathol, 64: 157–172.
9. Mayr M, Xu Q (2001) Smooth muscle cell apoptosis in arteriosclerosis. Exp Gerontol, 36: 968–987.

10. Neumüller J (1990) Transmission and scanning electron microscope preparation of whole cultured cells. In: Pollard JW and Walker JM (ed.). Methods in molecular biology, Vol. 5, Animal cell culture. Humana Press, Clifton, pp. 447–471.
11. Ping Ting-Beall H, Zhelev DV and Hochmuth RM (1995) Comparison of different drying procedures for scanning electron microscopy using human leukocytes. Microsc Res and Techniq, 32: 357–361.
12. Shanahan CM, Weissberg PL (1998) Smooth muscle Cell Heterogeneity. Ath Thr Vasc Biol, 18: 333–338.
13. Thyberg J, Hedin U, Sjölund Mm Palmberg L, Bottger BA (1990) Regulation of differentiated properties and proliferation of arterial smooth muscle cells. Arteriosclerosis, 10: 966–990.
14. Tukaj C, Kubasik-Juraniec J, Kraszpulski M (2000) Morphological changes of aortal smooth muscle cells exposed to calcitriol in culture. Med Sci Monit 6: 668––674.
15. Tukaj C, Wrzołkowa T (1996) Effects of vitamin D on aortic smooth muscle cells in culture. Toxicol *in vitro*, 10: 701–711.