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THE ROLE OF CELL-MATRIX INTERACTIONS IN ATHEROSCLEROSIS

Michael Thie and Horst Robenek
Institute for Arteriosclerosis Research, University of Munster, Munster, Germany

ABSTRACT

• *Atherosclerosis is a complex disorder of the arterial wall characterized by prominent changes in the extracellular matrix and in smooth muscle cells (SMC), which are the predominant resident cells in the vessel wall. During atherogenesis SMC modulate their phenotype from the contractile state to the synthetic state.*

The synthetic state SMC excessively synthesize extracellular matrix molecules, in particular collagens. In vitro experiments have demonstrated that the extracellular matrix itself may influence the morphology and metabolic properties ofmesenchymal cells. To get insight into the mutual interaction between the extracellular matrix molecules and functional properties of SMC we investigated the effects of a three-dimensional matrix of type I collagen on the behaviour of synthetic state SMC. Our results show that cultivation within a collagen lattice keeps SMC in a viable, growth-inhibited state with marked suppression of total protein synthesis and collagen synthesis. These data suggest that SMC in the synthetic state are able to respond to an altered extracellular environment and to modify their metabolic activities accordingly without any change in their ultrastructural character. Moreover, the findings suggest that the collagen lattice offers a promising model for further investigations of SMC responses and altered differentiation in the pathogenesis of atherosclerosis.

CHANGES IN THE VESSEL WALL DURING ATHEROSCLEROSIS

• Atherosclerosis is a complex disorder of the arterial wall characterized by prominent changes in the extracellular matrix and in smooth muscle cells (SMC), which are the predominant resident cells in the arterial wall (1). These cells are responsible for contraction and relaxation of the vessel wall as well as for integrity of the tissue by proliferation and synthesis of extracellular matrix components such as elastin, proteoglycans, glycoproteins and various types of collagens (2-5).

During atherogenesis, integrity of the vascular tissue becomes disrupted. As a result, SMC modulate their phenotype from the contractile state of the normal vessel to the synthetic state of the atherosclerotic vessel. Cells in a synthetic state show an abundant rough endoplasmic reticulum, Golgi apparatus and free ribosomes. In contrast, SMC in a contractile state show a cytoplasm filled with myofilament bundles (6).

The synthetic state SMC exceccively synthesize extracellular matrix molecules. It is well established that collagens represent the major extracellular product in the atherosclerotic arterial wall. These molecules represent one-third of the dry weight and up to 60% of the total protein content of the plaque (7). Type I and type III collagens together constitute the major proportion, probably 80-90 % of the total collagen present in this tissue. Compared to control tissue, an alteration in the relative amounts of the different collagen types is evident. Cells of atherosclerotic plaques synthesize type I collagen in preference to type III collagen unlike cells of the intact vessel wall (8, 9). This shift in the quantities and molecular compositions of collagens might be correlated with the pathogenesis of atherosclerosis (10) since collagens play several roles in vessel wall function. They are involved in the attachment of endothelial cells to the subendothelial matrix and in the activation of platelets. They may serve as a thrombogenic agent and a carrier molecule for other macromolecules of the matrix such as proteoglycans and growth factors.

CONCEPT OF MUTUAL INTERACTION BETWEEN EXTRA- CELLULAR MATRIX AND SMC

• In vitro experiments have demonstrated that the extracellular matrix influences the morphology and metabolic properties of mesenchymal cells. Collagen may speciffically inhibit collagen synthesis in fibroblasts (11). Other matrix molecules such as fibronectin have been described to accelerate the morphological modulation of SMC from a contractile state to a synthetic state (12), whereas laminin may have the opposite effect and helps to maintain the contractile state of SMC (13). Mechanical confinement by the extracellular surrounding itself may have regulating effects on the activity of fibroblasts (14). Thus a cell might create an enviroment which then acts back on the cell itself and perhaps also on its neighbours. We investigated the mutual interaction between the changing composition of the extracellular matrix and the functional properties of SMC. We compared the effects of a three-dimensional matrix of type I collagen on the behavior of vascular SMC, which had changed their morphology from the contractile state of the normal vessel to the synthetic state of the atherosclerotic vessel. ,

COLLAGEN LATTICES AS VESSEL WALL EQUIVALENTS

• Studies on the metabolic properties of SMC have mostly been done in two-dimensional cultures. Three-dimensional culture systems employing substrates to which all surfaces of the cell are exposed are supposed to simulate natural conditions more closely than do two-dimensional systems, i. e. conventional monolayer cultures. When mesenchymal cells are embedded into a matrix of collagen fibrils, the cells reorganize the randomly orientated fibrils causing the collagen lattice to shrink. Three-dimensional cell-collagen systems are formed which represent living tissue equivalents in vitro (15). Using this system, cells can be investigated with respect to synthesis, deposition and metabolism of extracellular material while in close contact with collagen fibrils (16).

Aortic SMC from swine were used for the experiments. Immediately after bleeding the thoracic aorta was dessected out. The vessel was opened longitudinally and the tunica intima was mechanically scrapped off. The tunica media thus exposed was gently peeled off and cut into pieces. SMC were released from these media pieces by collagenase digestion (17). The digest was passed through a filter and the freed cells were recovered by centrifugation. Cells were resuspended, seeded out in plastic flasks and kept in a moist atmosphere *of 5* % CO_2 and 95 % air at 37 °C. At confluency, the cells were removed from the flasks by trypsinization and used for lattice culture.

Hydrated collagen lattices from type I collagen were prepared as follows (15): 250000 cells in 150 |al of medium were added to 1350 iol of a neutralized type I collagen solution, i. e. 690 |il of a 1.76-fold concentrated medium containing antibiotics, 450 \r\ of type I collagen solution (3.3 mg/ml in 0.1 % acetic acid), 75 ul of 0.1 N NaOH and 135 |il of fetal calf serum; type I collagen was extracted from calf skin and purified by fractionated salt precipitation (18). The cell-collagen mixture was poured into a 35 mm tissue culture dish. The collagen formed a lattice within some minutes when the culture dish was placed at 37 °C. Immediately after its formation the collagen lattice was lifted off the bottom of the dish, thus floating in the medium. The lattice was supplied with culture medium and kept in a moist atmosphere as described above. After the experiment collagen lattices were incubated in collagenase and freed cells were counted.

PHEMOTYPIC APPEARANCE OF LATTICE CULTURED SMC

• For electron microscopy, lattices were fixed with Karnovsky's reagent and embedded in Epon 812. Ultrathin sections were mounted on copper grids, stained with uranyl acetate and lead citrate and examined with a Philips EM 410 (14).

The ultrastructure of lattice cultured cells was broadly comparable to that of the monolayer cultured cells. They developed a prominent Golgi apparatus and rough endoplasmic reticulum as well as abundant free ribosomes, but intracytoplasmic filaments were sparse. Nevertheless, the cells were characterized by a combination of several features. When the cells were examined directly after the collagen lattice had been formed, they were spherical in appearance. With prolonged culture time, the cells spread out and grew cytoplasmic extensions. Cells cultured within the lattice for a longer period appeared mostly spindle-shaped. The nucleus was rounded up or elongated, lacking invaginations typical for tissue resident SMC. The cytoplasm was largely filled by elements of the endoplasmic reticulum, Golgi complexes and a high number of mitochondria. Myofilament bundles were completely lacking. Small and large electron-dense, oval granules as well as bodies containing whorled osmiophilic membranes were present. The cells were completely surrounded by a matrix of collagen fibrils, which were in close contact with the cell membrane. A basal lamina as well as depositions of extracellular material other than collagen fibrils were lacking.

GROWTH ACTIVITY OF LATTICE CULTURED SMC

• Compared with SMC cultivated on plastic dishes, proliferation was strongly supressed. One day following lattice formation the cell number increased, reached a

plateau with 2.3-fold of seeding density around day 3 of culture and decreased slowly with prolonged culture time, while SMC on plastic still proliferated (8.1-fold of seeding density on day 6 in monolayer culture).

SMC which had been grown in collagen lattices over a longer period did not lose their capacity to proliferate. This was shown by the following experiment. Cells were cultivated within lattices for up to 3, 6, and 10 days and suspended by dissolving the lattice in collagenase solution. Subsequently, they were plated on tissue culture dishes. Approximately 70-90 % of the cells attached within 1 day, flattened on the surface and proliferated as did control cells.

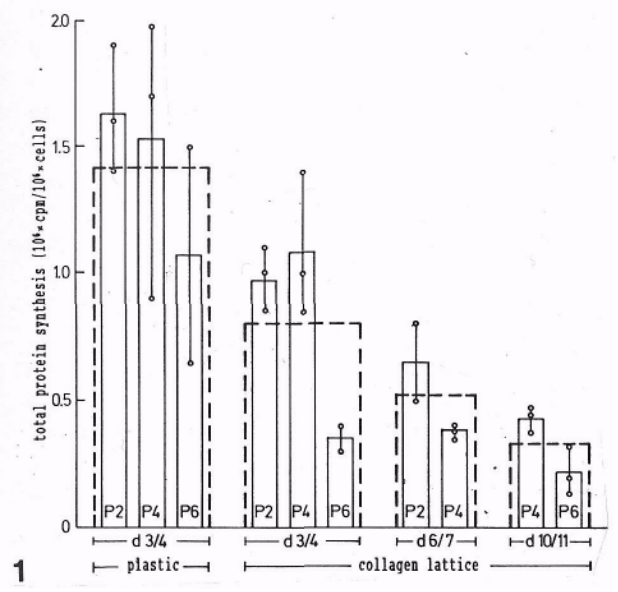

FIGURE l. Total protein synthesis state SMC of passage 2 (P2), 4 (P4) and 6 (P6) cultured as monolayer on plastic (plastic) or within collagen lattices (collagen lattice). After 3 (d 3/4), 6 (d 6/7) and 10 (d 10/11) days of culture protein synthesis was determined by measuring radioactivity. Circles represent data of one experiment, columns represent the average of three experiments, and dotted columns represent the average of all values.

and 95 % air at 37 °C the medium and collagen lattices were collected. Samples were dialyzed against acetic acid, hydrolyzed in HC1 and subjected to ion exchange chromatography for separation of proline and hydroxyproline. Collagen synthesis was calculated according to Krieget al. (19). Total protein synthesis was calculated by determining the amount of labelled proline and hydroxyproline. In general, SMC cultured within collagen lattices synthesized less total protein than did cells cultivated as monolayers. When SMC were cultivated within collagen lattices for a longer period, the level of total protein synthesis dropped continuously (Figure 1). SMC cultivated within collagen lattices showed lower values for synthesized collagen than SMC cultured as monolayers. Moreover, the

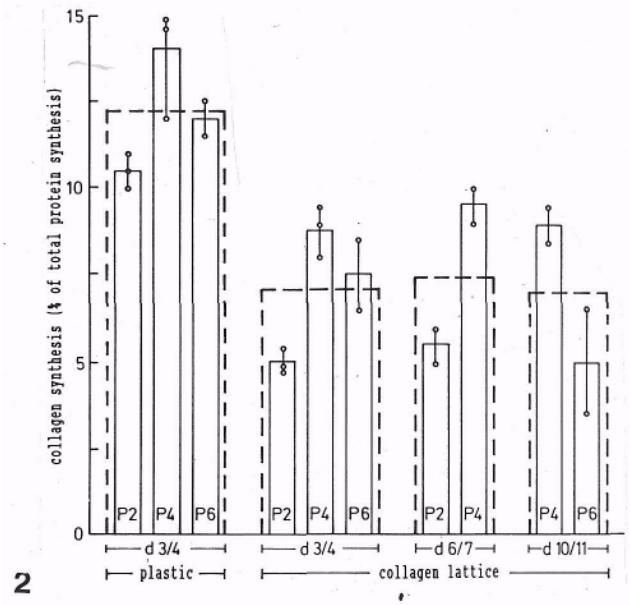

FIGURE 1. Collagen synthesis of synthetic state SMC expressed as percent of total protein synthesized in cells cultured as monolayer on plastic (plastic) or within collagen lattices (collagen lattice). Experimental conditions were the same as in figure 2.

SYNTHESIS OF EXTRACELLURAR MATRIX

• Collagen and total protein synthesis were measured after preparation of the cultures according to the following procedure (14). Growth medium of collagen lattices was changed to labeling medium, i. e. 1 ml of fresh medium, containing 50 ng/ml of ascorbic acid and 0.37 MBq/ml of ^{14}C-proline. After 24 h in a moist atmosphere of 5 % CO_2 level of synthesized collagen by lattice cultured SMC remained constant during the entire culture period (Figure 2).

HYPOTHESIS

• On the basis of the above mentioned results we suggest that extracellular matrix influences the behaviour of SMC. When SMC are removed from their physiological

matrix environment, they pass through characteristic morphological and biochemical changes. Electron microscopic studies on cells cultured as monolayers revealed nuclear enlargement, dispersion of the chromatin and formation of large nuclei, disappearance of myofilament bundles and appearance of an extensive rough endoplasmic reticulum and a large Goldi complex (20 - 23). Biochemical studies indicate the enhanced synthesis of both type I and type III collagen with preponderance of type I collagen (24, 25). When SMC are re-integrated into a matrix environment similar to that of tissue, i. e. a three-dimensional collagen lattice, the above mentioned biochemical changes in contrast to the morphological ones seem to be reversible.

The morphological data indicated the cells to be still in the synthetic state. Re-integration into type I collagen thus failed to cause synthetic state SMC to revert to the contractile state. The synthetic state is typical for metabolically highly active cells as found in early fetal life and in postnatal development (26), in atherosclerosis (20), and in monolayer cultures (21). Reversible changes of ultrastructure are, however, possible. When SMC are grown in primary culture reaching confluency within not more than 5 cumulative populatoin doublings, spontaneous reversion to the contractile state can be observed (21). In addition, reversibility of modulation is reported in the rabbit carotid artery after endothelial denudation by balloon catheter injury (27).

With regard to biochemical features, cultivation of SMC within a three-dimensional collagen lattice revealed prominent peculiarities. The amount of total protein and collagen synthesized by entrapped cells was reduced as compared to monolayer cultures. This indicates that SMC in the synthetic state are able to sense the macromolecular composition of the extracellular matrix and to modifiy their production of matrix components accordingly. Similar potency has been demonstrated for fibronectin, a glycoprotein component of the extracellular matrix enviroment (12).

Sensitivity towards extracellular matrix is a common feature of fibroblasts. However, when looking at the course of protein synthesis as well as of collagen levels, SMC acted quite differently from these cells. Lattice cultured fibroblasts kept up constant levels of protein synthesis all along, but showed a continuous decrease of proportion of collagen (14, 16, 28). Thus, altered SMC seem to be subjected to a regulatory system being different to that of fibroblasts. The marked suppression of biosynthetic activity of cultured SMC occured although cells were cultured in 10 % whole blood serum. This is an interesting effect of the collagen lattice culture in view of the data of other groups who reported stimulation of collagen synthesis in SMC grown in whole blood serum (29).

It is evident that three-dimensional lattices of pre-existing type I collagen fibrils cause SMC to reduce collagen synthesis, however, the mechanisms still remain unclear. As demonstrated for fibroblasts, there might be a correlation btween the extent of contact between cell and matrix material and the level of collagen synthesis (11). There is also evidence that the mechanical properties of matrices in which cells are grown themselves represent a regulatory factor of collagen synthesis (14). Moreover, we cannot exclude the possibility that the properties of procollagen molecules might be involved in the feedback regulation of collagen synthesis (30, 31). In the case of monolayer cultured bovine SMC, it had been demonstrated that expression of the genes for type I and type III collagens might be influenced by the proliferative state of the cells, i. e. proliferating cells contain low levels of collagen mRNA, whereas quiescent, growth-arrested cells contain high levels (32). However, our data are in contrast to these data. Changes in growth activity do not influence the synthesis of collagens. Examining the growth activity of SMC from a more general point of view we found that proliferation was strongly suppressed when cells were re-integrated into an extracellular matrix environment. Although this phenomenon is described (33) the reasons for the regulation of the proliferation within the collagen lattice remain unknown. It is possible that cell density within the lattice might be a limiting factor (34) and(or type I collagen fibrils themselves (35). In the case of fibroblasts it is speculated that the organisation of the extracellular matrix and its influence on the cell shape might play a role in growth control (36).

To sum up, our results show that cultivation in a three-dimensional matrix of type I collagen keeps aortic SMC in a viable, growth-inhibited state with marked suppression of total protein synthesis and collagen synthesis. These results suggest that smooth muscle cells in the synthetic state are able to respond to an altered extracellular environment and to modify their metabolic activities accordingly without any change in their ultrastructural character. With regard to a markedly inhibited cell growth and reduced total protein synthesis, SMC exhibit features of quiescent cultures (29). Overall our present findings suggest that the three-dimensional collagen lattice system offers a promising model for further investigations of SMC responses and altered differentiation in the pathogenesis of atherosclerosis.

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Address for corespondence:
Vniv.-Prof. Dr. Horst Robenek
Institute for Arteriosclerosis Researcli, HeadofDept. of Cell Biology and Ultrastructure Research, University of Minister, Domagkstr. 3, D-4400 Minister, Germany