Biochemical Effects of Collagen-Cultured Mesenchymal Stem Cells on Isolated Vascular Smooth Muscle Cells

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The present studies aimed the biochemical effects (apoptosis) induced on isolated rat aorta vascular smooth muscle cells by the cultured medium of rat mesenchymal stem cells grown on collagen fibers and stimulated by apelin, ghrelin, leptin, adiponectin, 9-cis retinoic acid, and cytosporone B. Flow cytometry results showed that the combined culture medium, including 10% of rat mesenchymal stem cells culture medium for 24 hours, is inducing apoptosis of isolated rat vascular smooth muscle cells in various degrees, as follows: 10 μM apelin > 10 μM ghrelin > 10 μM cytosporone B ≅ 10 μM 9-cis retinoic acid. Moreover, 10 μM leptin- and 10 μM adiponectin-conditioned medium of mesenchymal stem cells induced no apoptotic effects on isolated rat vascular smooth muscle cells. We need to deeply explore the intimate mechanisms involved in such effects, remaining as subjects for future studies.

Keywords: mesenchymal stem cell, vascular smooth mucle cells, apelin, ghrelin, cytosporone B

All the body cells, including vascular smooth muscle cells, gain a characteristic epigenetic pattern during differentiation, meaning specific histones repertory and DNA changes. The result of such alterations is the so-called euchromatin (open state), comprehending specific biomarker expressing genes and proteins as Acta 2 and Myh 11, and also housekeeping genes with general expression. Lack of transcription is found for so-called heterochromatin (closed state) in which several genes are prevented from being expressed in differentiated (adult) vascular smooth muscle cells. The epigenetic control represents a modern target for the development of new therapeutic strategies for important pathologies as atherosclerosis, hypertension, and their associations with diabetes mellitus [1].

Anomalous blood vessels are a characteristic of brain tumors named glioblastoma, associating extremely high malignancy. Glioblastoma stem-like cells are challenged by angiogenesis-inducing factor YKL-40/CHI3L1 to lead the forming of two types of vasculature through physiological angiogenic mechanisms and through vascularization mimetism mechanisms. These types of stem cells are pluripotent ones, able to be transdifferentiated into two types of adult cells: vascular pericytes and smooth muscle cells. Glioblastoma stem-like cells are coordinating with existing adult endothelial cells for neoangiogenesis induction or form auto-assemblies as channels to allow the flow of blood (vascularization mimetism). These aspects are really important since the glioblastoma growth could not be blocked by anti-tumoral therapies targeting angiogenesis processes. Maybe targeting of YKL-40/CHI3L1 protein could reduce the aggressive behaviour of glioblastoma and assure real benefits to patients with glioblastoma [2].

The use of mesenchymal stem cells is considered to become a promising therapeutic approach when discussing the repairing of myocardial tissues. One such approach is represented by the administration of mesenchymal stem cells modified to express thioredoxin-1 encoded by an adenovector and some experiments were developed in rats. Thioredoxin-1 associate antioxidant properties, is an important growth factor, an important regulator of transcriptional processes as well as a cofactor. The modified mesenchymal stem cells by thioredoxin-1-adenovector developed an augmented proliferative ability, kept multipotency and were able to transform into cardiomyocytes, smooth muscle cells, as well as into endothelial cells. They were capable to secret VEGF, HO-1, and CXCR4. Thus, the clear conclusion is that engineered mesenchymal stem cells with an adenovector forcing them to express thioredoxin-1 could represent a future therapeutic successful approach in the case of cardiac failure [3].

Another important trend is the need to differentiate mesenchymal stem cells toward bladder smooth muscle cells, especially when autografts are not to be used or are not available. Quantitative real time-polymerase chain reaction detected the expression of smooth muscle cells specific molecules as ACTA 2, TAGLN, CNN1, and MYH 11 in myogenic type-differentiated cells. The sodium and calcium ionic currents were also augmented and smooth muscle-transformed mesenchymal stem cells contracted when potassium induced their depolarization. Such a pattern of contraction resembles that of bladder intermediate-to-late smooth muscle cells and, thus, mesenchymal stem-derived cells could be useful in regeneration medical treatments [4-6].

There are several attempts to measure the capacity of external RNA to modify embryonic bodies and embryonic stem cells. For example, the administration of external transfer RNA, total cellular RNA and external ribosomal RNA significantly augmented the CD31-positive vessel constructions. Such an effect was not evident for DNA administration. The obtained molecular parameters showed a trending for the differentiation of embryonic bodies toward vasculogenesis and leukopoiesis patterns (VEGFR2+, CD31+, VE-cadhein+, as well as CD18+, CD45+, and CD68+ cells). The resulted embryonic bodies presented also augmented mRNA and, consecutively, protein expressed (e.g., HIF-1α, VEGF-165, NRP1, PI3K phosphorylation, VEGFR 2, α-SMA). The clear conclusion of the study is that vasculogenesis and leukopoiesis of

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embryonic stem cells (embryonic bodies) could be induced by external transfer RNA through the generation of superoxide/hydrogen peroxide by NADPH oxidase functioning stimulation and the same time activation of VEGFR2 as well as PI3K [7-9].

Our studies aimed the biochemical effects (apoptosis) induced on isolated rat aorta vascular smooth muscle cells by the cultured medium of rat mesenchymal stem cells grown on collagen fibers and stimulated by apelin, ghrelin, leptin, adiponectin, 9-cis retinoic acid, and cytosporone B.

Experimental part

Femural and tibial bone marrow from 180-200 g Wistar male rats (Baneasa source) were used to isolate mesenchymal stemm cells, using an adapted method [10]. Mesenchymal stem cells were grown in α-MEM medium (Sigma-Aldrich) with 10% fetal bovine serum (FBS, Sigma-Aldrich). The appropriate cells, the nucleated ones, were multiplied in Petri culture dishes through incubation at 37°C for 6 consecutive days. After 2 harvestings with 0.25% trypsin-EDTA (Sigma-Aldrich) and consecutive re-suspension in the above complete medium, the cells were further cultured for 3 days. The obtained monolayers were equated with rat mesenchymal stem cells [8]. The phenotype of resulted cells was analyzed using fluorescein isothiocyanate (FITC)-conjugated primary antibodies against CD34 and CD44. The secondary necessary antibodies were represented by goat anti-rat antibody (Sigma-Aldrich). The analysius was performed using flow cytometry using a FACS caliber flow cytometer (Becton Dickinson Immunocytometry Systems).

Obtained rat mesenchymal stem cells were further cultured on collagen from rat tail, prepared and applied on culture Petri dishes in agreement with producer instructions (Sigma-Aldrich).

The cultured rat mesenchymal stem cells were divided in several series and cultured at a density of 10.000/mL. Separate series were stimulated with 10 μM apelin, ghrelin, leptin, adiponectin, 9-cis retinoic acid, and cytosporone B.

The culture medium of rat mesenchymal stem cells was further used in a ratio of 10% together with culture medium for isolated rat vascular smooth muscle cells to stimulate the last ones for 24 h.

The isolated rat aorta vascular smooth muscle cells were isolated, characterized and cultured in accordance with a previous described and adapted technique [9].

The apoptosis and viability of isolated rat aorta vascular smooth muscle cells were also assessed in accordance with the adapted technique previously described [10-13].

The protocols involving Wistar rats were previously approved by the Ethics Committee of the Grigore T. Popa University of Medicine and Pharmacy from Iasi.

Results and discussions

Flow cytometry results showed that the combined culture medium, including 10% of rat mesenchymal stem cells culture medium for 24 h, is inducing apoptosis of isolated rat vascular smooth muscle cells in various degrees, as follows: 10 μM apelin (fig. 1) > 10 μM ghrelin (fig. 2) > 10μM cytosporone B (fig. 6) ≅ 10 μM 9-cis retinoic acid (fig. 5). Moreover, 10 μM leptin (fig. 3) and 10 μM adiponectin (fig. 4) - conditioned medium of mesenchymal stem cells induced no apoptotic effects on isolated rat vascular smooth muscle cells.

We didn't deeply explore the intimate mechanisms involved in such effects, remaining as subjects for future studies.

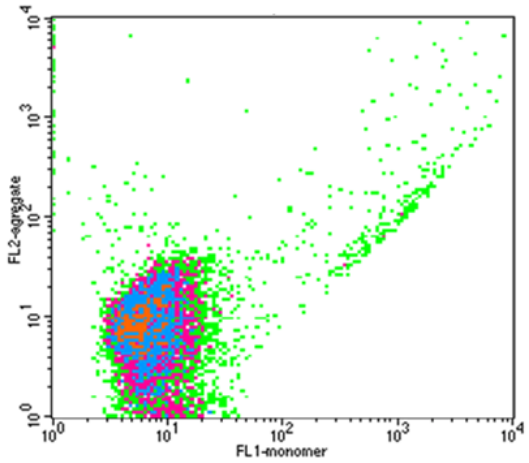

Fig. 1. Representative flow cytometry of apoptotic isolated rat vascular smooth muscle cells (81% on average) treated with conditioned medium of mesenchymal stem cells, initially stimulated with 10 μM apelin for 24 h

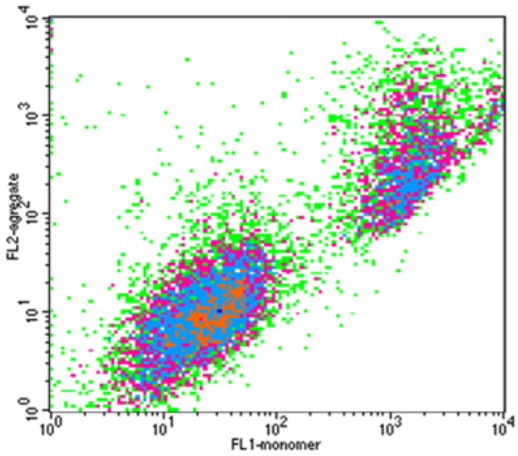

Fig. 2. Obtained culture medium of mesenchymal stem cells (10%), stimulated for 24 h with 10 μM ghrelin induced apoptosis of isolated rat vascular smooth muscle cells in a proportion of 65% (representative flow cytometry)

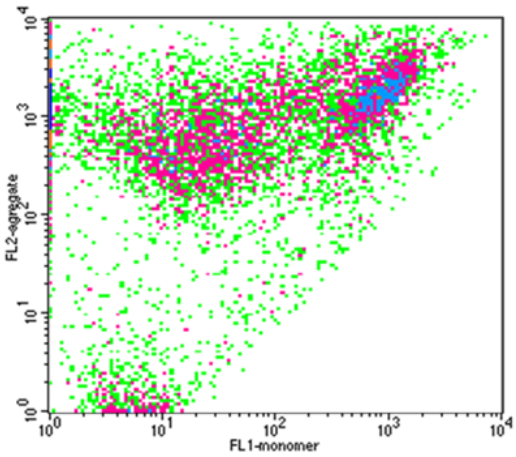

Fig. 3. Representative flow cytometry of apoptotic isolated rat vascular smooth muscle cells (32% on average) treated with conditioned medium of mesenchymal stem cells, initially stimulated with 10 μM leptin for 24 h

It was demonstrated that microRNA-34a is a key factor which controls the stem cells differentiation toward vascular smooth muscle phenotype both *in vivo* and *in vitro*. Which are the genes targeted by microRNA-34a? A variety of pathologic conditions could alter microRNA-34a expression in vascular smooth muscle cells through its transcription regulation and the binding site of p53 protein represents an important mediator of such alterations. When microRNA-34a is overexpressed and serum starvation is

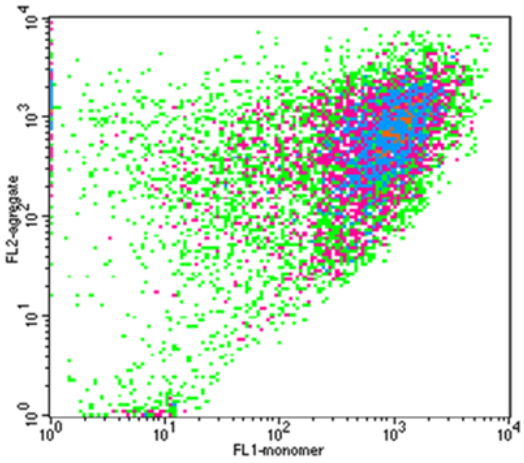

Fig. 4. Obtained culture medium of mesenchymal stem cells (10%), stimulated for 24 hours with 10 μM adiponectin induced apoptosis of isolated rat vascular smooth muscle cells in a proportion of 26% (representative flow cytometry)

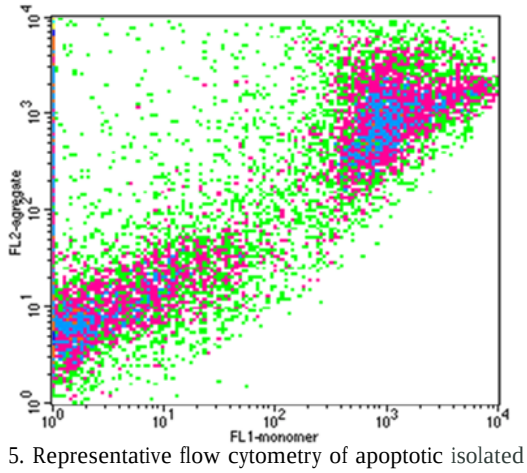

Fig. 5. Representative flow cytometry of apoptotic isolated rat vascular smooth muscle cells (49% on average) treated with conditioned medium of mesenchymal stem cells, initially stimulated with 10 μM 9-cis retinoic acid for 24 h

applied, the proliferative and migration capacities of vascular smooth muscle cells are really reduced. On the other hand, microRNA-34a knockdown amplified the proliferative and migration capacities of vascular smooth muscle cells. The microRNA-34a repressed Notch 1 gene expression, the encoded protein being considered an extremely important controller of vascular smooth muscle cells functioning and re-modeling of arterial beds. When injured arterial vessels were evaluated, the expressed amount of microRNA-34a was reduced. Moreover, overexpressed microRNA-34a by perivascular structures decreased as a result the expressed Notch 1, the proliferative capacity of vascular smooth muscle cells as well as the forming of neointimal tissue [14].

Ischemic tissues could beneficiate from the VEGF-therapeutically overexpressed by transformed cellular progenitors through induction of angiogenic processes. The risk involved by such therapeutic approaches is represented by induced tumors of angioma type. Normal angiogenesis was demonstrated to be the only one induced after VEGF released in the environment by adipose mesenchymal stromal cells transformation is injected in non-ischemic tissues. The limitations of such therapeutic approaches are represented by really poor survival of transduced cells, as well as the reduced area for the cells to be administered through injections. Maybe the implantation strategies involving cells cultured using three dimensional scaffolds could remove such disadvantages. For example, the

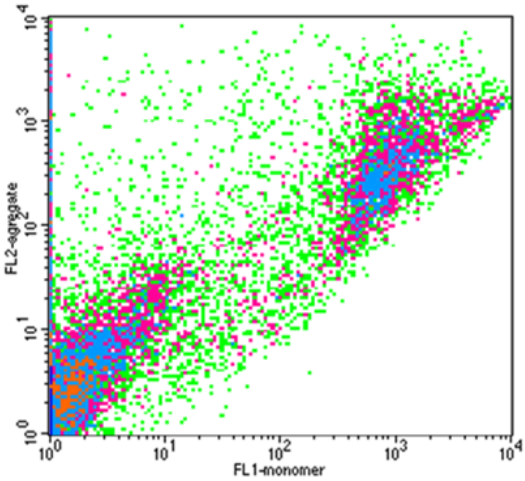

Fig. 6. Obtained culture medium of mesenchymal stem cells (10%), stimulated for 24 h with 10 μM cytosporone B induced apoptosis of isolated rat vascular smooth muscle cells in a proportion of 55% (representative flow cytometry)

scaffolds constructed from collagen, acellular, could provide control and reproducible conditions in the view of investigating the angiogenic subcutaneous models in mice. Overexpression of VEGF by transformed adipose mesenchymal stromal cells augmented the capillary-type vessels production, the implanted cells being characterized by a prolongation of survival life. Beside the above mentioned advantage, another important one is represented by the uniform vascularization in the interior and exterior of three dimensional scaffold constructs [15].

Paracrine secretion of mesenchymal stem cells could modulate the cellular micro-environments and could also modulate other types of cells exposed to the factors contained by the medium [16].

A lot of effort was invested in designing extremely complex systems, including engineered ones necessary to explore the pathophysiological mechanisms that arise in the interior of microvascular environments. Development and modeling of such systems are entirely dependent on the complex interactions between endothelial cells, supportive cells, different specific cells for various organs and also the interactions with extracellular matrix [17].

The assessment of alveolar bone repairing processes was attempted using allogeneic mesenchymal stem cells and assemblies based on hydroxyapatite/tricalcium phosphate. The most important fact is that such stem cells are able to induce calcification of tisues, including bone and vascular tissues [18].

The use of benzopyran derivatives as KL-1492 and KL-1507, which could alter the functioning of ATP-dependent potassium (KATP) channels, is to be studied also from the point of view of stem cells at the level vascular functions and dependencies on perivascular tissues [19].

At the same time, the involvement of the angiotensin-aldosterone system and oxidative stress in the functioning of stem cells systems is to be extensively studied, since angiotensin II for example is a very important mediator of vascular functions and inducer of vascular dysfunctions. Angiotensin II and other derivatives of angiotensin-aldosterone system are powerful inducers of free radicals of oxygen by the endothelial cells [20].

Conclusions

Our studies aimed the biochemical effects (apoptosis) induced on isolated rat aorta vascular smooth muscle cells by the cultured medium of rat mesenchymal stem cells

grown on collagen fibers and stimulated by apelin, ghrelin, leptin, adiponectin, 9-cis retinoic acid, and cytosporone B.

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Acknowledgements:The studies were partially supported from a grant 218/2014, Sisteme inovative de eliberare controlatã sub formã de particule pentru þintirea segmentului posterior ocular din cadrul programului PN II – Parteneriate in Domeniile Prioritare – Proiecte Colaborative de Cercetare Aplicativa – Competitia 2013, Cod proiect: PN-II-PT-PCCA-2013-4-1570, Ministerul Educatiei Nationale si Cercetarii Stiintifiuce, Romania.

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Manuscript received:19.03.2016