Biochemical Effects of Biological Supports-Included Stem Cells on Eye Cells Development

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Mesenchymal stem cells are considered a promising tool to help the repairing of ocular surface injuries, although it is not well established their possibility to be derived toward corneal epithelial cells. The pathways through which they communicate with or influence the turnover of other cell types are not well known. The aim of our studies was represented by the influences exerted by growth conditions on rat mesenchymal stem cell abilities to modulate the development of rat corneal epithelium cells. From this point of view, we compared the mesenchymal stem cells grown on a 3D polystyrene scaffold and those grown on classic culture dishes. The clear results are that the mesenchymal stem cells grown on a three-dimensional structure are more potent in influencing the development of rat corneal epithelium cells in vitro. Thus, the establishment of these last cells as a monolayer and their capacity to proliferate for many passages as well as the fine mechanisms of achieving these abilities are to be established by further studies.

Keywords: mesenchymal stem cell, corneal epithelial cell, rat, three-dimensional culture, in vitro

Incurable blinding conditions are under evaluation for retinal gene and cell therapies. There is an increased optimism that gene transfer and cellular transplantation might stop or reverse the modern blinding retinal pathologies. The actual technologies are allowing the safe and stable delivery of cloned genes also in humans in recessive diseases. Such delivery to certain retinal cells may improve visual function for a long time. In the case of autosomal dominant conditions, the delivering of biologically active proteins-encoded genes, targeting specific pathophysiologic well known pathways, represents an important therapeutic approach. Furthermore, the rendering of other retinal cells, beside altered photoreceptors, might help the healing processes. In the United States, the actual trials in patients with retinal degeneration involving the mutated RPE65 are a real success from the point of view of visual function. The same time, nowadays we are able to use safe stem cell-derived therapies with terminal differentiation, e.g. well established retinal pigment epithelium (RPE) cells [1].

The degenerative diseases affecting retinal tissues could have the origin in the backstage, involving retinal pigment epithelium. This one represents the basic unit of retinal homeostasis, sustaining the vital functions of photoreceptors on one side and of choriocapillaris on the other side. The decreased and loosed vision found in aged patients with macular degeneration is associated with an increased alteration of retinal pigment epithelium functioning. Fortunately, the derived retinal pigment epithelium from induced pluripotent stem cells (iPSC), specific to patients, represents a promising approach to treat or halt the evolution of such debilitating pathologic conditions. The genetically transformed cells are intensively tested for the replacement therapy in the patients with retinal pigment epithelium atrophy. Meanwhile, the retinal pigment epithelium cells derived from induced pluripotent stem cells are improving the modeling of the disease. Furthermore, they build the basis for the development of a model of retinal homeostatic unit in its integrality and efficiency [2].

For glaucoma, the accepted therapeutic approaches include pharmacologic, laser-based and surgical procedures targeting the lowering of intraocular pressure. Although, in many cases, the efficaciousness is exhibited, such approaches are not able to restore the already lost or reduced vision. The acquired knowledge in the field of stem cell biology could be applied in the therapeutics of glaucoma and other forms of neuropathies involving optic nerves. A wide range of human ocular cell types might be developed starting from stem or adult cells through differentiation in certain conditions. The flag trend of human trials is the production of retinal pigment epithelial cells, derived from induced pluripotent stem cells, in turn genetically transformed from adult cells. Specifically speaking about glaucoma domain, the recent achievements are involving the established protocols for stem cells to be differentiated into trabecular meshwork and retinal ganglion cells. The most challenging approach is represented by the attempt to generate cells derived from stem cells which are able to associate high specific secretion of neuroprotective factors [3].

Production and application of human pluripotent stem cells is increasing and refining, although challenging many strengths and weaknesses closely related to technique origins. The capability of pluripotent stem cells, also human ones, to differentiate is noteworthy, allowing comprehensive study of development pathways and the establishment of functioning models. The same time, such transformed cells are suitable for use in replacement therapies. Nonetheless, there exist many limitations and challenges in the process of their production on large scale [4], considering the time for their maturation, the culture environment and interactions with native tissue and organ cells [5].

Using Eiraku differentiation technique it was possible to induce in simple culture a structure like ciliary epithelium, double-layered, starting from limbal stem cells. This was done in the coxtext of establishing a protocol for 3D self-structuring of optic cup morphogenesis. The differentiation

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of the double layer organization was induced by an inhibitor glycogen synthase kinase 3 beta (GSK-3β) and was prevented by the pre-addition of a tankyrase inhibitor. Both cell layers expressed cyclinD2, zic1, tgfb2, aldh1a3, wfdc1, otx1, BMP4, and BMP7 (ciliary marker genes), as well as an increased accumulation of nuclear and cytoplasmic beta-catenin. The derived murinic induced pluripotent stem cells exhibited a physiologic functioning very similar to ciliary epithelium *in vivo*. Thus, the pathologic ocular hypotension could beneficiate of an efficient treatment in the nearest future [6].

Every corneal epithelial insult needs the activation of the repair processes through the limbus structure, more precisely the limbal stem cells. The alteration of the limbal stem cells microenvironment will have as consequence their deficiency. Further, the reepithelialization is disrupted, exaggerated, uncontrolled, development of neo-vascularization is triggered and chronic inflammation becomes certitude. When limbal stem cells deficiency is partial the transplantation of amniotic membrane associated with conjunctival epitheliectomy is able to reconstruct the healthy cell layers. On the other hand, in severe destructions or deficiencies the best therapeutic option is represented by stem cells transplantation, e.g. limbal stem cells harvested from the contralateral healthy eye. The final target of such unconventional treatments is the improvement of vision as well as of the quality-of-life [7].

Immune disorders benefit from the i.v. administration of mesenchymal stem/stromal cells (MSCs), although their engrafting is solely transitional. Such effects, of long-term benefits despite short presence, are related to the immune tolerance induced in the recipients. This would be due to the conditioning of immune cells, e.g. monocytes/macrophages, to adopt a regulatory phenotype. This mentioned processes might be dependent on TNF-alpha-activated gene/protein (TSG)-6. In described system, the mice protection was obtained in two models of ocular inflammation: allo (corneal allotransplantation) and autoimmune (experimental autoimmune uveitis). The conclusion of the study is that the mesenchymal stem/stromal cells i.v. administration induces the modulation of immune system through monocytes/macrophages populations, which activate the innate immune tolerance [8].

Mesenchymal stem cells associate increased therapeutic potential in allograft transplantation. Actual promising achievements are obvious for corneal diseases. Corneal epithelial cells were obtained from mesenchymal stem cells *in vitro*, as well as keratynocytes both *in vitro* and *in vivo*. But it is not clear if mesenchymal stem cells could differentiate toward epithelial cells *in vivo*. Further studies are asked to establish standards for mesenchymal stem cells isolation and characterization [9].

Our studies aimed the biochemical effects of biological supports-developed mesenchymal stem cells conditioning medium on rat corneal epithelial cells *in vitro*.

Experimental part

For our experiments we used the cells derived from bone marrow (femural and tibial Wistar male rats), methods adapted from Seo et al., 2009 [10]. Separation and proliferation of mesenchymal stem cells was achieved in α-minimum essential medium (α-MEM) with 10% fetal bovine serum (FBS, Sigma-Aldrich). The obtained nucleated cells were harvested and seeded onto Petri culture dishes and incubated at 37ºC for 6 days. After 2 consecutive treatments with 0.25% trypsin-EDTA, Sigma-Aldrich) and their resuspension in complete medium, the cells were cultured for 3 days. The monolayers resulted were considered as rat mesenchymal stem cells (rMSCs). These cells were further proliferated onto Petri culture dishes or Alvetex 3D polystyrene scaffold (gift from ReproCELL) [10]. To analyze these cells phenotype we used primary antibodies to CD34 and CD44, fluorescein isothiocyanate (FITC)-conjugated with goat anti-rat antibody (Sigma-Aldrich) and flow cytometry using a FACScaliber Flow cytometer (Becton Dickinson Immunocytometry Systems).

The mesenchymal stem cells culture medium (harvested each 3 days) was used in combination (10%) with corneal epithelial cells culture medium to follow its effects on the development and survival of last ones.

Corneal epithelial cells were also harvested from Wistar male rats, the same time with tibias and femurs, and were cultured in accordance with adapted methods from Kobayashi et al., 2009 [11]. Briefly, the enucleated eyes were incubated in DMEM/F12 (1:1 ratio) and dispase II, sorbitol, and antibiotic-antimycotic (Sigma-Aldrich) for 18 h at 4ºC. Further, the epithelial cells were isolated using trypsin and soybean trypsin inhibitor, as well as the pipetting. The epithelial cells were grown on type-I collagen coated Petri plastic dishes. The medium was replaced every 2 to 3 days.

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

The microscopic setup for phase contrast observation included an inverted Nikon Eclipse TE-300 microscope, contrast phase objectives (40X), as well as the Nikon software (NK Remote v.2.2.3.). The collected images were analyzed using free ImageJ (National Health Institute, U.S.A.). The collected images (having a standard imposed resolution of 1280 × 1024) were best analyzed after hematoxylin eosin staining [12-15].

Results and discussions

The conditioning medium obtained from rat mesenchymal stem cells cultured onto Alvetex 3D polystyrene scaffold (fig. 1) is inducing higher proliferative effects on rat corneal epithelial cells (fig. 2). At the same time, the absolute number of divisions is enhanced by the conditioning medium harvested from cells cultured onto 3D structures.

The obtained results proved evidence that the conditioning medium harvested from rat mesenchymal stem cells cultured onto Alvetex 3D polystyrene scaffold is

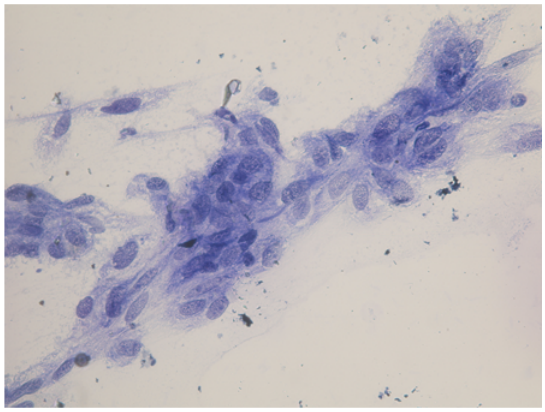

Fig. 1. Phase contrast photomicrographs of rat corneal epithelial cells developed in the presence of 10% conditioning medium harvested from rat mesenchymal stem cells cultured onto Alvetex 3D polystyrene scaffold (60X)

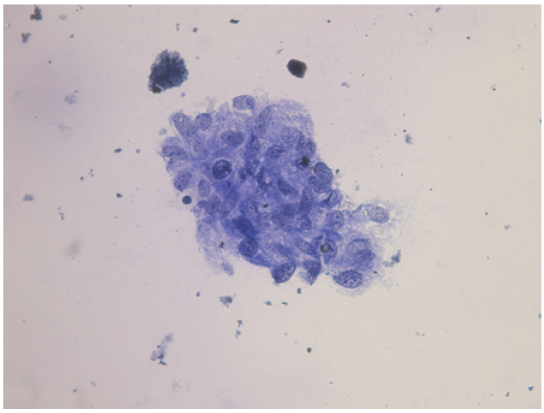

Fig. 2. The effects on proliferation and numbers of proliferations are reduced when rat corneal epithelial cells developed in the presence of 10% conditioning medium harvested from rat mesenchymal stem cells cultured onto normal plastic Petri dishes (60X)

proliferative effects on corneal epithelial cells.

Our findings could be in agreement with very recent discoveries involving the paracrine way of action of mesenchymal stem cells, although the intimate mechanisms are far away of being known. One of such way could be represented by exosomes, a kind of microvesicles delivered in the medium, containing proteins or RNAs. It was demonstrated that the intravitreal injection of mesenchymal stem cells (from mouse adipose tissue and human umbilical cord) and exosomes has the capacity to decreased damage, apoptosis and inflammation when injury of retina was induced by laser in mouse. These effects would require at least the partial down-regulation of MCP-1 [16].

Mesenchymal stem cells show multiple and special functions of extremely importance in regenerative medicine, being also able to be cell and tissues rescuers through transferring (providing) functional mitochondria [17].

Altered functioning of mitochondria is found in degenerative and hyperproliferative conditions as hypertriglyceridemia, nonalcoholic fatty liver disease, and metabolic syndrome [18].

Reactive oxygen species are largely released by mitochondria, inducing apoptosis, when photosensitizers are activated in tumor cells [19].

Betulinic acid, an anti-tumoral agent with anti-inflammatory, antiangiogenic, and immunomodulatory effects is enhancing the respiratory function (OXPHOS state) in isolated liver mitochondria from mice with murine melanoma [20].

Some biological processes are beneficial in some organs and tissues as well as destructive in other cases. For example, the artery diseases and ischemic heart diseases benefit from neoangiogenesis. On the other side, it associates inflammation and destroy the so-called *angiogenesis privilege* of avascular cornea, allowing the appearance and evolution of ocular surface pathologic conditions and decreased vision. Mesenchymal stem cells could be able to suppress the inflammatory processes, as well as to modulate the cells of immune system. It is not clear if they could transform in corneal cells, but for sure they might be of help for healing. All the recent studies suggest that mesenchymal stem cells are transforming in a sharp instrument of healing when speaking about disorders of ocular surface. The control of angiogenesis might also be involved in their beneficial actions [21].

Mesenchymal stem cells are pluripotent in their nature and could be derived in osteoblasts, chondrocytes, and adipocytes. These cell types are amazing in terms of their ability to escape the recipient immune rejection. They were efficient when administered to treat pathological conditions as lumican-null (Lum) and mucopolysaccharidosis (Gusb) in mice. Ocular surface injuries (corneal tissue) may benefit from their special immunosuppressive capabilities. Their xenograft transplantation survival (demonstrated e,g, for human umbilical mesenchymal stem cells) is due to the capacity to modulate the immune response, altering adhesive, invasive, polarization, apoptotic and maturation patterns of macrophages and T-regulatory cells. The intimate mechanisms of such abilities involve synthesizing of an important extracellular glycocalyx which includes hyaluronan, inter-α-trypsin inhibitor (heavy chains), versican, and pentraxin-3. The glycocalyx regulate the response of inflammatory cells and necessitates CD44 surface molecule for this goal to be achieved [22].

Although the therapies starting from stem cells could replace the classic corneal transplantation, there exist many obstacles taken into account the uniqueness of each of the corneal cellular layers. There were described several types of stem or progenitor cells in cornea: epithelial and stromal stem cells, and endothelial cell progenitors. Autografts of corneal limbus, including epithelial stem cells, were used successfully for transplantation in human recipients for around 20 years now. Current research concerns are related to *in vitro* cultures and other cells lineages to be used for corneal transplantation. Interestingly, some cells of corneal endothelium are equipped with the ability of self-renewal, although they are not proliferating *in vivo*. Their using for transplantation is hampered by the lack of established culturing protocols and the difficulty of posterior cornea delivering *in vivo* [23].

Conclusions

The obtained data clearly showed that the three-dimensional growth conditions of rat bone-marrow derived mesenchymal stem cells are influencing their capacities to influence the corneal epithelial cells, enhancing their establishment as a monolayer and their capacity to proliferate.

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