CORNEAL CELL REGENERATIVE BIOLOGY, A NEW THERAPEUTIC PERSPECTIVE OF EDEMATOUS KERATOPATHY

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Abstract: *As alternatives to the limited availability of donor cornea from cadaver to perform perforating or just endothelial keratoplasty, the same as the complications that consist of graft rejection, currently, new therapeutic alternatives are sought to consist in the regeneration or replacement of endothelial cells in view of recovering eyesight. The end result shows that regenerative cell therapy is limited, but attempts continue and hopes are that, in the future, the results will be favourable. That is why, presently, isolation and cultivation are sought through genetic engineering of cells which should play a vital role in the treatment of endothelial dysfunction.*

Corneal endothelium, a misnomer used because the corneal endothelium here is bathing in the aqueous humor, not in blood or lymph, and, also, it has origin and function different from other endotheliums, it is basically a simple squamous epithelium. Its cells, derived from neural crest cells (later mesenchymal cells), migrate from the optical cup, this migration being triggered by the separation of the optic vesicle from the ectoderm surface, and then it sets apart during the cornea development phases. The neural crest stem cells are also the origin of periocular mesenchymal cells leading to the development of corneal epithelium and to the synthesis of the primary stroma. When these cells keep migrating to the periphery of the optical cup between lens and corneal epithelium, the corneal endothelium and the trabecular meshwork will be created, wherefrom, subsequently, in the second phase of development, the corneal keratocytes from the neural crest cells from the level of the primary stroma shall differentiate. Human corneal endothelial cells are displayed in a single layer, although they originally were in a double layer and their shape is hexagonal. They are flattened and the basal lamina or the Descemet membrane represents their basement. This is the point where the apical basal and apical tight junctions polarize, these existing constantly throughout the adult life.

The approximately 300 000-450 000 endothelial cells on cornea which represent total postnatal endothelium corneal cellularity are achieved in the second trimester of gestation, subsequently, the density of endothelial cells (but not the absolute number of cells) dropping rapidly, in direct proportion to the increase of the fetal cornea surface. In newborns, the endothelial density is approximately of 4 000-5 000 cells/mm^2 (but they are mitotically inactive), in adult, it reaches 2 500 cells/mm^2, while in senior persons, it reaches 2 000 cells/mm^2. We can thus see that there is an inverse relationship between age and density of the endothelial cells, according to specialized studies, this cell density is very high from birth until the first year of life, then the density decreases rapidly during the first part of childhood, this being associated with the increase in size of the cornea (relative to a normal eye), being 3 500 cells/mm^2 at the age of five years old, following also a fast decrease until the age of 10 years old, and, from now on, the rapid decrease rate of endothelial cells slows down, reaching a gradual decline, which according to specialized studies, this decline in cellularity is of 0.6% per annum until the most advanced ages. The fact that this type of cells is unable to recover in number, being, therefore, non-regenerative, and the cornea seems to remain unchanged in size throughout life, explain those listed above. The initial rapid decrease is non-linear, but then the slow decrease is linear.(1)

Over the years (2), there have been reports on the existence of corneal endothelial progenitor cells with peripheral cornea location, but they were not later confirmed; by performing endotheliometry on normal human cornea was thought to be an advantage their uneven distribution in the central, paracentral and peripheral regions in the sense that their density is higher at the paracentral and peripheral level as compared to center and the level was consistently higher in the upper peripheral region of the endothelium.

This increase in endothelium density in the upper side, located near the Schwalbe's line, gave rise to the idea, at the time, that it would actually represent a storage population, cell backup in view of endothelium regeneration in case of compromised corneas, this idea was actually contradicted by other studies performed on human corneas in tissue bank, which showed an annual decrease of the endothelial cells, less in the periphery as compared to the central and paracentral level. The explanation for this unequal distribution is that the cornea is thicker in the periphery.

So far, the only predictable source of human corneal endothelial cells is the human corneal tissue, because the uniform and, concurrently, functional generation of the endothelial monolayer of corneal stem cells or other cell types is not known.

Although this human tissue is the only source, it also has significant disadvantages as it has a very limited mitotic

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capacity and specific morphology loss occurs in vivo. Since the availability of the donor corneal tissue is limited due to the increasing number of patients with such needs, it explains the urge to find other saving solutions. The end result is that the regenerative cell therapy is limited, but attempts continue and hopes are that others are results are achieved in the future.

Manipulation of human corneal endothelial cell oncogenes was attempted such as transforming viral antigen oncogenes SV40 T and HPV E6 / E7 or overexpression of the CDK4 mutant. Biologists' studies focused on these viral oncogenes with interfering action, namely they interfere with the p 53 pathway which they repeal and, also, with the stress-enhanced apoptosis leading to corneal endothelial disorders.

Under the action of the CDK4 mutant, the corneal morphology was affected, namely that led to the formation of intercellular connections, but the human telomerase reverse transcriptase (hTERT) has adverse effect, its action extending cell life and the impact in terms of corneal physiology and its differentiation is minimum.(3) The use of telomerase has been attempted in order to obtain cell cultures to compensate for the self-limiting lifetime of endothelial cells.

The telomerase used resulted in obtaining stable and functionally competent mitotic cell populations.

Since endothelial cells' in vitro disadvantage is that they have phenotypes, a cell self-renewing was obtained by using this telomerase, as well as CDK4 and D cyclins, while, by the excess addition of human telomerase reverse transcriptase, cells were obtained with a higher standard of their mitotic capacity and also with functional and morphological phenotypic features similar to the in vivo cells.

The corneal endothelial cycle takes place in close interrelation with cyclins and cyclin-dependent kinase inhibitors (eg. $p16^{INK4}$ and $p21^{Cip1}$), which it regulates.

Cyclins are a family of proteins that are involved in cell cycle regulation. Along with cyclin-dependent kinase enzymes, cyclins form complexes (CDKs), in the end, these complexes activating the kinase function.

During one cell cycle, cyclin concentration may vary; in case of low concentrations, the function corresponding to the cyclin-dependent kinase is also inhibited.

Cyclin D is a protein involved in the cellular cycle. During the early G1 phase, cyclin-dependent kinases 4 (CDK4) connect to this cyclin D, and the resulting complex enables progression to the late G1 phase and, afterwards, progression to the S phase.

The D-CDK4 cyclin complex disarms a strong inhibitor of cell cycle progression; it forms the pRB proteins and inactive transcription factors.

The fact that the telomerase extends the life of cells is explained by the fact that it does not induce chromosomal abnormalities, but its action depends on that tissue and on its mitotic competence.

In the presence of the latter (mitotic competence) in somatic cells, such as epithelial cells and vascular endothelial cells, their sensitivity to human telomerase reverse transcriptase was noted which leads to the development of phenotype- specific proliferative cells, but the reverse is also true, namely in case of incompetent mitotic somatic cells (e.g. nerve cells, glial cells, muscle cells).

As a result of the growth of $p16^{INK4}$ and $p21^{CIP1}$ and activation of the p53 target genes, a process which limits cell division is generated and corneal endothelial cellular senescence is, thus, produced. That is why, in vitro, for instance on donor corneas, their replicative senescence generated by donor's age is important, because the stage of cell cycle arrest and the high levels of $p16^{INK4}$ and $p21^{CIP1}$ are taken into account.(4)

Obtaining human corneal endothelial cells from donor corneas was based on their harvesting from the Descemet membrane along with the corneal endothelial layer, they being then subjected to the enzyme digestion process by means of collagenases, but, yet, the method has the disadvantage that, in the event of a lengthy manipulation of layer separation, a thin layer from the posterior corneal stroma may remain adherent to the Descemet membrane, which may lead to contamination with unwanted stromal keratocites in the plate count, this triggering rapid growth of stromal corneal fibroblasts, with concurrent implications on the pump function of the corneal endothelial cells, acting as a barrier to this function. The reason why human corneal endothelial cells show limited proliferative capacity is justified by the rapid emergence of cellular senescence and endothelial mesenchymal transition. The endothelial mesenchymal transition represents the physio-pathological mechanism that fibroblast-like are turned into and a loss of phenotype of the specific endothelial cell occurs. The presence of transition takes place in pathological conditions and in primary cultures of corneal endothelial cells.

Attempts continue by optimizing the plate count by supplementation with growth factors such as FGF2, EGF and NGF. To extend endothelial cells, attempts were made on bovine corneas to improve with the Y27632 Rhokinaza inhibitor and ascorbic acid phosphate. Or, the use of the extracellular matrix and immortalization of endothelial cells with SV40T antigen. The consequences of these experiments consisting in achieving a certain cell expansion.(5)

Studies based on cellular biology and immunology have been made in order to minimize the contamination of the corneal fibroblast cells; according to them, cells were purified or separated based on the technique of magnetic cell separation using magnetic particles.

As a latest development, transplantation of primary human corneal endothelial cell suspension is intended to be made on human cornea in the future. The result depends on the cell isolation and cultivation techniques used before transplantation. When using immortalized cell lines, cell densities were obtained when transplanting human corneal endothelial cell suspensions on an in vitro deendotalized cornea.(6)

The incorporation of superparamagnetic microspheres in human corneal endothelial cells is a new therapeutic strategy. By placing the magnet in front of the cornea and by injecting cells as suspension in the anterior chamber, they generated an endothelial monolayer.

Additionally, attempts are made to create an equivalent human cornea by assembling cell layers. The layers contain immortalized human corneal endothelial cells, fibroblasts (native stromal cells) or immortalized corneal epithelial cells.

Another strategic plan is the reconstruction of pathological corneal layers with biomaterials: cultivations of biological membranes, biological polymers, biosynthetic and even synthetic materials.

The lyophilized human amniotic membrane would be a natural biological material: the biological polymers are gelatin gels coated with type IV gelatin, type I ceramic - collagen-type gels; synthetic materials: collagen and chondroitin sulfate reticulated with glutaraldehyde, porcine type I collagen recombined with type I and III human collagen; synthetic biological polymers were originally hydrogel contact lenses and biodegradable polymers are currently used. These material grafts should be transplanted

together with the cells because they are an integrant part.

Conclusions:

The reason for seeking saving solutions to recover sight in the edematous keratopathy by the regeneration or replacement of endothelial cells stands in the disadvantage that these cells are non-regenerative in vivo.

The additional material which is interposed by these genetic engineering techniques between endothelial cells and stroma (growth factors, cytokines) disturbs cornea's hydration balance, which can lead to immune, inflammatory reactions.

In the future, we seek to overcome the drawbacks underlying failures, specifically the fact that only primary human donor cells can be used for clinical application and, secondly, the surgical challenge for the transfer of the cell monolayer to the anterior chamber and firmly fixing it to the posterior cornea needs to be overcome. The success of this regenerative alternative strategy is due to the fact that the disruption of the intercellular connection no longer takes place. Thus, we can obtain mono-layers of human corneal endothelial cells that can be transplanted. A particularity is that they have normal cellular density and phenotype.(7)

REFERENCES

1. Moller-Pedersen T. Keratocyte reflectivity and corneal haze,Experimental eye research. 2004 March;78(3):553-560.
2. Amann J, Holley G, Lee SB, Edelhauser HF. Increased endothelial cell density in the paracentral and periphera lregions of the human cornea, American Journal of Ophthalmology. 2003;135(5):584-590.
3. Schmedt T, Chen Y, Nguyen TT, Li S, Bonanno JA, Jurkunas UV. Telomerase immortalization of human corneal endothelial cells yields functional hexagonal monolayers, PLOS ONE, ISSN 1932-6203, Published:December 21; 2012.
4. Adams PD. Healing and hurting: molecular mechanisms,functions and pathologies of cellular senescence,Molecular Cellular Biology Journal. 2009;36(1):2-14.
5. Rachelle NL, Palchesco L, Lathrop L. Funderburgh A. Feinberg, In vitro expansion of corneal endothelial cells on biomimetic substrates, Scientific Reports 5, Article Number 7955, ISSN 2045-2322 (online), Published 22 January; 2015.
6. Teichmann J, Valtink M, NitschkeM, Gramm S, Funk HW, Engelmann K, Werner C.Tissue Engineering of the corneal endothelium:a review of carrier materials, Journal of Functional Biomaterials. 2013;4(4):178-208.
7. Zhu YY, Li F, Han B, Tighe S, Zhang S, Chen SY, Liu X, Scheffer CG. Tseng SC. Activation of RhoA-ROCK-BMP signalling reprograms adult human corneal endothelial cells, The Journal of cellular biology. 2014;206(6):799-811.