Allogenic Corneal Stem Cell Transplantation in Rabbits

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Background: This is the first study regarding corneal stem cells cultivation in Romania. Our objective is to find a way to cultivate corneal stem cell into tissue that can be used to repair ocular surface.
Material and methods: We have conducted a study using an animal model (rabbit). Corneal fragments were cultivated on amniotic membrane substrate (intact or denuded).
Results: Cultures using denuded AM substrate showed high replication rates, especially after week 2, whereas cultures using intact AM showed little progression. After 1 month, 8 mm fragments trephined from cultivated tissues were used as allografts and transplanted on 8 rabbit eyes. All grafts integrated well, but with loss of transparency and corneal vascularization.
Conclusions: We have demonstrated the technique of cultivating limbal stem cells in vitro, on amniotic membrane substrate. We have also proved that surgical technique of transplantation is straightforward. Allograft use of cultivated stem cells was not efficient in this study.

Keywords: stem cells, cornea, amniotic membrane, transplantation, allograft

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Introduction

Cornea is a transparent tissue located at the anterior pole of the eye, functioning as a lens. Histologically, it consists of three layers, epithelium on the surface, stroma and endothelium. Ocular surface homeostasis is highly dependent on the health of corneal epithelium and surrounding conjunctiva. The healing of the corneal epithelium is done through proliferation of the basal epithelial layer towards the surface and centripetally.

In the last decade strong evidence emerged regarding the presence of stem cells located in the corneal periphery (at corneal limbus). These stem cells are essential in the healing process, functioning as epithelial progenitor deposits. The adult type stem cell is activated to transient activated cell, that will move centripetally towards the center to become a differentiated epithelial cell.

There are a number of diseases that can destroy limbal epithelial cells. Corneal epithelium will disappear without stem cells, being replaced by cells with conjunctival origin. This will result in loss of transparency of the cornea and low vision, even blindness. The most common factors are chemical (alkali) burns, but also Stevens Johnson syndrome, aniridia, trachoma, ocular pemfigoid. There is no efficient treatment so far, and corneal transplant is not efficient in this cases.

Over the last ten years the research on corneal stem cells brought new hope, investigating the possibility of replicating these cells in vitro and transplanting the resulting tissue on diseased cornea. When the number of stem cells is decreased or there are sectors of cornea without stem cells, there is a new possibility to expand the few remaining cells in vitro, using tissue cultivation techniques.

This is the first study regarding corneal stem cells cultivation in Romania, to our knowledge. Our objective is to find a way to cultivate corneal stem cell into tissue that can be used to repair ocular surface (someway similar to epidermal cultivation).

Material and methods

We used an animal model in this experiment. Rabbit eye is quite similar to the human eye. We harvested half of corneal limbus from one rabbit (2 mm wide). The fragment was further divided into small pieces (about 1 mm) and cultivated on amniotic membrane (AM) substrate. Amniotic membrane was harvested from cesarean section, under written consent. Five tissue samples were cultivated on AM with intact epithelium and 18 samples were cultivated on AM with denuded epithelium (the epithelium was removed with trypsin digestion). Limbal stem cells were maintained in a co-culture transwell system with feeder layer of murine fibroblasts cells and incubated at 37°C under 5% CO_2 atmosphere for 1 month. Weekly photographs documented cell growth.

After 1 month the tissue samples were trephined and transplanted on 8 rabbit eyes. Eight mm diameter trephination at corneal limbus and over perilimbic conjunctiva at 12 o'clock was employed on host side, performing superficial keratectomy and removal of the corresponding conjunctiva. The allograft matching the host defect size was sutured with 8.0 Vicryl resorbable sutures (8 for each graft) by the same surgeon. Photographs of rabbit eyes were taken weekly for up to 1 month, when sutures were removed.

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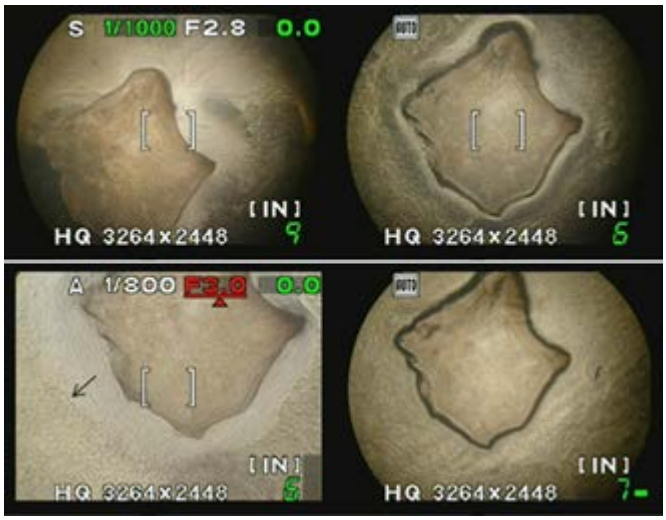

Fig. 1. In vitro stem cell expansion at 1, 2, 3 and 4 weeks (arrow – bounderiers of cultivated tissue)

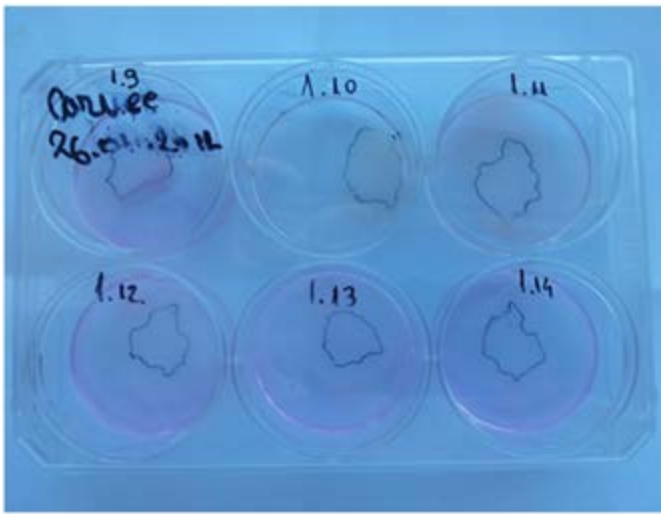

Fig. 2. Cell expansion highlighted with marker at week 4 (six samples out of the eighteen)

Results

The cultures using intact AM developed slowly without cell confluence and were discarded after 3 months. On the other hand, cultures using denuded AM substrate showed high replication rates after week 2. The first week showed only isolated cells on AM near the explants, and at week 2 important tissue growth (Figure 1). At week 2 the explant almost doubled the surface and at week 4 about 2 cm wide tissue was obtained. The same growth pattern was noticed on all 18 cultures (Figure 2). Eight samples were harvested using trephination (resulting discs of tissues of 8 mm diameter). The tissues were transplanted on 8 rabbit eyes as allografts (Figure 3). All the grafts integrated well on host site. At 1 month the grafts lacked transparency though and on 6 of 8 eyes heavy vascularization at graft site was noted. The remaining 2, although more transparent, showed minimal amounts of vascularization.

Discussions

Unilateral total stem cell defficiency finds modern treatment with the use of cell culture techniques [1]. The contralateral healthy eye is a safe donor in this case, because very small amounts of tissue have to be harvested. When the disease is bilateral, allografts still can be used.

We showed that a 1 mm sample containing stem cells is enough for cultivation, being able to obtain a 2 cm tissue in one month. In this study the explants grew slowly in the first week, the first cells adjacent to the explants being noticed in the second week. The last 2 weeks demonstrated high rates of proliferation. The same growth pattern was observed in other studies [2].

We have used the cultured tissues as allografts. There are a few reports that stretched out the possibility of successful use of ex vivo cultivated stem cells allografts [3,4,5]. These studies were performed in humans and showed that visual acuity improved after transplantation and the grafts improved the quality of corneal surface. We did not observe a favorable outcome in any of the allografts. The presence of vascularization and opacity of the graft make us affirm that the AM was covered by conjunctiva epithelium and no corneal epithelium survived. It is possible that in the cited studies the use of AM as a substrate can be enough to improve visual acuity. AM could function by its own as a promoter of healing.

Different cultivation substrates can be used, including AM or collagen and laminin extracellular matrix substrate. Adult limbal stem cells are mitotically quiescent in vivo, but they can be activated to proliferate in vitro. Without fibroblasts feeder layer they differentiate and loose stemness. The authors are investigating different substrates and culture mediums to keep the limbal progenitor in an immature state. Amniotic membrane, especially denuded amniotic membrane, has proven to be more amenable for the cultivation of corneal epithelial cells than other substrates [6,7]. Intact amniotic membrane is colonized by corneal cells much slower than denuded amniotic membrane (in our study we haven't seen any proliferation after 3 months in the group cultivated on intact AM).

Fig. 3. Allograft transplant on rabbit eye, juxtalimbic, at 12 o'clock – at surgery and 1 month after surgery (opalescent graft and cornea vascurazization)

Conclusions

We have demonstrated the technique of cultivating limbal stem cells in vitro, on amniotic membrane substrate. In one month it is possible to grow enough epithelial tissue with preserved proliferative state that can be transplanted on diseased cornea. We have also proved that surgical technique

of transplantation is straightforward. The animal model can be easily replicated in humans. We have used the tissues as allograft, but the final outcome is not favorable, with vascularization and conjunctival invasion of cornea.

Acknowledgement

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