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Culture morphology of the autologous cultivated corneal epithelium

Morfologia hodowli autologicznego nabłonka rogówki

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Summary:

Purpose: Ocular surgery based on cultivated corneal epithelium has become a very promising procedure eligible to restore the ocular surface. Analysis of morphologic features and the phenotype of cultivated epithelial cells determines their quality and eligibility of transplantation.

Material and methods: Corneal epithelial cultures were carried out in 25 patients suffering from limbal deficiency after chemical or thermal burns. Fellow healthy eyes were the source of limbal epithelium for the culture. Limbal cells from a 2 mm^2 biopsy were seeded on an amniotic membrane after enzymatic pretreatment. Cultures were carried in standard conditions in a supplemented DMEM HAM/F12 medium in the presence of 3T3 fibroblasts. Light microscopy was used to analyze the regularity of the cultivated epithelial layer, histologic examination was used to establish number of epithelial layers, and immunohistochemistry for epithelial and proliferation markers was applied to confirm cell origin and proliferative potential. Staining for cytokeratin 3, 12, 19, connexin 43, and protein p63 was performed.

Results: In 25 donors, 27 cultures of the epithelium were performed. In 2 cases, plates were contaminated. Both cultures were repeated. In 84% of the cultures, regular stratified growth of the epithelium with complete covering of amniotic membrane was observed. In 16% of cultures, growth was not regular, showing differences in the number of cell layers. Staining for cytokeratin 3/12 confirmed the corneal origin of cultivated epithelia. The number of epithelial layers ranged from 3 to 9; the average was 5.3 $\pm$ 1.9 layers.

Conclusion: Cultures of limbal epithelial cells are a valuable source of tissue for restoration of the corneal epithelium.

Key words: Cultivated corneal epithelium, epithelial morphology.

Streszczenie:

Cel: chirurgia, której podstawą jest hodowany nabłonek rogówki, to obiecująca metoda rekonstrukcji powierzchni oka. Celem badania jest ocena morfologii i cech fenotypowych hodowli pod kątem jej przydatności do przeszczepu.

Materiał i metody: hodowlę nabłonka prowadzono u 25 pacjentów z niewydolnością rąbka spowodowaną oparzeniem chemicznym lub termicznym. Rąbek drugiego oka stanowił źródło komórek rąbka rogówki do hodowli nabłonka rogówkowego. Komórki nabłonka rąbkowego z 2 mm^2 biopsji rąbka po trypsynizacji wysiewane były na podłoże z błony owodniowej i hodowane w standardowych warunkach w zmodyfikowanym DMEM/HAM F12 w obecności fibroblastów linii 3T3. W mikroskopii świetlnej oceniano regularność nabłonka, a techniką histologiczną – liczbę warstw komórek. Barwienie immunocytochemiczne stosowano w celu oceny pochodzenia nabłonka i jego potencjału proliferacyjnego. Barwienia wykonano w kierunku cytokeratyn 3., 12., 19., koneksyny 43. i białka p63.

Wyniki: przeprowadzono 27 hodowli nabłonka u 25 dawców. Dwie hodowle zdyskwalifikowano z powodu infekcji, a następnie powtórzono. W 84% przypadkach uzyskano regularny, warstwowy wzrost nabłonka rogówkowego. W 16% hodowli wzrost był nieregularny ze zmienną liczbą warstw komórek. We wszystkich hodowlach barwienie w kierunku cytokeratyn 3. i 12. potwierdziło rogówkowe pochodzenie nabłonka. Liczba warstw komórek wyniosła od 3 do 9, średnio 5,3 $\pm$ 1,9.

Wniosek: hodowla nabłonka rogówki jest wartościowym źródłem tkanki dla odtworzenia nabłonka rogówkowego.

Słowa kluczowe: hodowany nabłonek rogówki, morfologia nabłonka.

Concept of culture of the corneal epithelium raised from features of cells covering the cornea (1). The great ability of the limbal cells to restore the corneal epithelium provided the impulse to attempts to receive cultured epithelial sheets potentially applicable in clinical practice (2). Several markers characteristic of low differentiated cells in the limbus are known; however, scientists still have not had the opportunity to identify particular stem cells. Stem cells are located in the deep layers

of the limbal epithelium, a microenvironmental niche that is very difficult to explore. The real marker of this cell is not known; therefore, we now analyze daughters of stem cells, which begin the differentiation process.

Loss of the corneal epithelial stem cells leads to a secondary pathologic process in which the cornea is covered by conjuctival tissue, a condition called limbal stem cell deficiency (LSCD). For many years the concept of limbal disease management concentrated on limbal transplantation (3). Indeed, transfer of the healthy limbus to the diseased ocular surface led to epithelium restoration, improvement of vision, and decrease of ocular discomfort. This idea caused the development of many surgical techniques of autologous and allogenic limbal transplantations. The surgical approach was radical. All damaged tissues were removed, followed by transplantation of the healthy limbus. Surgeries transferred to the ocular surface not only the limbus, but also the conjuctiva and sclera. Many patients required immunosupression, amniotic membrane bandages, and autologous serum drops. Treatment in such cases was very complex and difficult. However, for severe burns these techniques were unique and highly effective.

In mild cases, a less radical approach was sought. The answer was found in culture techniques, which enabled less invasive surgery to be performed on the ocular surface. As is well known, the limbus in eye bank organ cultures maintains its proliferative potential (4). Corneal rings after penetrating keretoplasty were used for limbal allograft, because they still presented proliferative potential (5). Lindberg showed that this is possible (6). Another advantage was the small amount of limbal tissue necessary for culture. Tsai et al. performed a successful surgery using a piece of limbal tissue to propagate epithelial growth (7). Koizumi et al. applied cell suspension to propagate cell seeding on the corneal surface (8).

Discovery of cultured epithelia applicable in LSCD caused the publication of a number of papers describing different culture and surgical techniques. These studies proposed different carriers for epithelial cell expansion. Whereas Pellegrini et al. preferred fibrin scaffolds as a carrier (9), Koizumi et al. proposed denuded amniotic membrane – a carrier containing basement membrane (10). Results showed that the latter method was promising and could be very effective (11).

Aim

The purpose of our study to analyze the morphologic features and phenotype of cultivated epithelial cells to determine their quality and eligibility for clinical application.

Material and methods

Culture media and chemicals were purchased from Sigma (Germany) and reagents for immunostaining from Santa Cruz Biotechnology Inc. (USA).

All parts of the experiment were conducted under the tenets of the Declaration of Helsinki. The Ethics Committee of Silesian Medical University agreement no NN-6501-184/I/05/06 was achieved before the described procedure was applied.

Donation procedure

Donors' fellow eyes were healthy and eligible as limbal epithelium sources. Before donation, each eye was carefully examined to detect ocular surface pathology which could case potential risk of future visual acuity decrease. All patients were informed about the procedure and signed the agreement form. Limbal epithelium was collected in the operating theatre under topical anesthesia with local decontamination with 10% solution of povidone-iodine for skin or 5% povidone-iodine for the conjuctiva. The decontamination agent was washed out with buffered salt solution (BSS). With a crescent knife, a 2 mm^2 specimen of limbal tissue from the top of the Vogt palisades was gently cut. Biopsies were taken from the superior limbus, and no sutures were left. The tissue was transferred to a corneal storage medium at 4°C. It was immediately taken to the laboratory after donation. The tissue specimen was tripsinized with 1% tripsin with 0.01% EDTA for 10 min to obtain cell suspension. Cells were gently scraped with the micro scraper. Cellular suspension with a density of 1-4 x 10^4 cells per 1 ml were settled in the culture medium (Cell counter, Coulter Z1, Miami, USA) (Fig. 1).

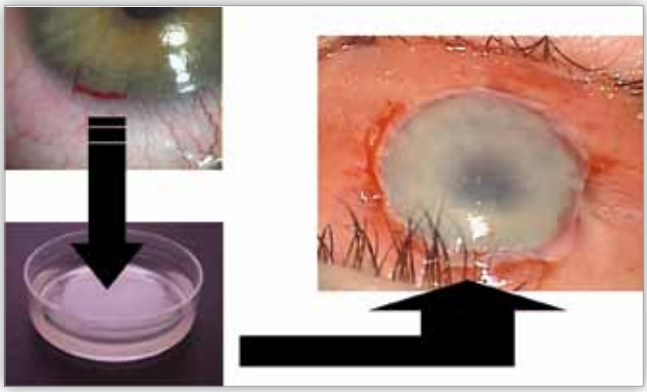

Fig. 1. Cultivation schema from limbal biopsy, cell expansion on the amniotic membrane and 2 weeks culture epitelium transferred on the diseased corneal surface.

Ryc. 1. Schemat hodowli nabłonka rogówki od biopsji rąbka, przez umieszczenie zawiesiny komórek na podłożu błony owodniowej, do umieszczenia dwutygodniowej hodowli na powierzchni oka chorego.

Culture procedure

Six-well culture dishes (Becton Dickinson, USA) for planed epithelial culture were covered with 3T3 (ATCC, USA) fibroblasts a week before epithelial cell seeding. Cells were cultivated in Dulbecco Modified Eagle's Medium (DMEM) with 10% bovine serum and 100 μg/ml penicillin and streptomycin mixture. A monolayer of fibroblasts (5-7 days culture) was inactivated by incubation in a medium containing 2 μg/ml of mitomicin C for 2 hours. The entire epithelial culture was carried out in the presence of 3T3 fibroblasts, a source of growth factors.

The carrier for transplantation of the epithelial sheet was an amniotic membrane located over the fibroblasts. Amniotic membrane slides were delivered by Homograft Tissue Bank in Zabrze. The amniotic membrane samples ware washed out from crioprotective medium with phosphate buffered saline (PBS), and the amniotic epithelium was gently scraped with a culture scraper. Amniotic membrane slides with the dimensions of 15 x 15 mm on nitrocellulose paper were put on culture plates over the layer of fibroblasts. One of them was qualified for transplantation, the others for histopathologic evaluation.

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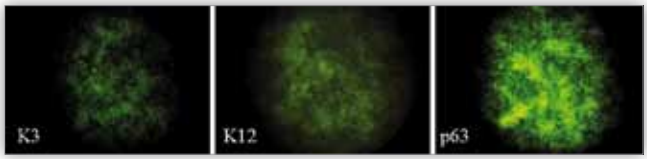

Fig. 2. Immunostainig positive for cytokeratin 3, 12, protein p63 in autologous cultivated epithelium.
Ryc. 2. Barwienie w kierunku cytokeratyn 3., 12. i białka p63 autologicznej hodowli nabłonka.

On another plate epithelial cells were seeded directly on the fibroblast layer. Two days before planed transplantation immunostaining for cytokeratin 3, cytokeratin 12, cytokeratin 19, protein p63, and connexin 43 was performed to confirm the origin of the epithelium and the presence of low differentiated cells (Fig. 2) .

Single cells were seeded on a denuded amniotic membrane spread on nitrocellulose paper. Cultures were carried in standard conditions at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was supplemented by a DMEM/HAM F12 mixture with 10% bovine serum, 0.5% dimethyl sulfoxide (DMSO), 10 ng/ml mouse spidermal growth factor (EGF), 5 μg/ml bovine insulin, 0.1 nM cholera toxin, 0.18 mM adenine, 2 nM trijodothyronine, 4 mM L-glutamine, 0.4 mg/ml hydrocortisone, and 100 μg/ml penicillin and streptomycin mixture. Culture medium was changed every 48 hours. On the 10th day of culture, plates were evaluated under the light microscope for evaluation of epithelial growth. At the same time air lifting was done. Cells were cultivated with a minimal amount of culture medium for 1 hour.

The amniotic membrane with regular epithelial cover was qualified for grafting. The corneal epithelium was then immunostained as described above with final qualification for transplantation. After 2 weeks, cultures were transferred in the transport medium to the operating theatre for grafting.

Histologic evaluation focused on epithelial layer regularity and the number of cell layers. For analysis, Hematoxilin-Eosin staining was performed.

Results

In 25 donors, 27 cultures of the epithelium were performed. In 2 cases, plate contamination disqualified the culture on the 8th and 12th day of cultivation. The procedure was repeated in both donors.

In 84% of the cultures, there was regular stratified growth of the epithelium with covering of the entire surface of amniotic membrane. In 16%, growth was not regular, showing differences in the number of cell layers (1 to 6 layers on the same specimen) and local areas covered by epithelial colonies. For these cultures, cultivation was carried further with reexamination before surgery. On the 14th day, the majority of the amniotic surface was covered by the epithelial layer, qualifying these cultures as eligible for grafting.

In each donor, the number of epithelial layers differed from 3 to 9 layers; the average was 5.3 $\pm$ 1.9 layers. On plates with more than 7 layers, we observed loss of outer epithelial layers in histologic specimens, which confirmed the intensive growth of epithelial cells.

Immunostaining for cytokeratin 3 and cytokeratin 12 was positive in all cases. It confirmed the presence of low differentiated cells; protein p63 and connexin 43 were found in all cases. In 24% of cases, conjuctival cells were cytokeratin 19 positive. The presence of these cells did not disqualify the cultures, because the majority of cultivated cells were of corneal origin.

Discussion

Zagórski experimented on rabbit epithelium and endothelium cultures in the 1970s (12). Using culture methods, Pellegrini et al. discovered that the corneal limbus has the highest potential to create epithelial colonies in the culture plates (13). These studies confirmed that the limbal area is responsible for corneal epithelium renewal. They also showed that it is possible to selectively culture corneal epithelium and determine its proliferative potential during the analysis of colony features.

Many years before Pellegrini et al.'s study, the limbus was suspected as a location of the stem cells of the corneal epithelium (14). Experimental papers confirmed that damage to the peripheral cornea causes severe ocular surface disorders and lack of epithelial renewal (15). Recent research on epithelial cultures has shown that this treatment method is an alternative procedure for limbal transplantations.

The corneal epithelium forms colonies in the first days of culture. It is clearly visible when epithelial cells are seeded directly on the feeder layer of fibroblasts. To reach confluence, epithelium needs at least 8 days of culture. Differences depend on the proliferative potential of the limbal specimen taken to the culture. De Paiva et al. described 3 to 5 layers of epithelial cells in culture on the 10th-14th days of cultivation (16). In our observations, on the 10th day the majority of culture plates were covered by stratified epithelium. In cases with single colonies or irregular epithelial layers, it is worthwhile to consider extending the time of cultivation to achieve regular and complete epithelium on the carrier. Many authors have suggested performing air lifting to promote differentiation of the outer layers of the epithelium with tight junctions between cells. Doing so stabilizes the epithelium and stratifies the cells into basal to superficial levels.

The amniotic membrane is a very important substrate for organ culture. The amniotic tissue has a basement membrane for its epithelium, which is useful for corneal epithelium multilayer growth. The first experiments showed that both intact and denuded amniotic membranes are valuable for cell culture (10). The effectiveness of the clinical application has been improved (17). Koizumi et al. demonstrated that denuded amniotic membrane can receive multilayered epithelia with 5 or more layers of cells. Intact amniotic membrane also allows the expansion of epithelial cells; however, epithelial growth is less effective with a decreased number of epithelial layers in cultivated sheets. Additionally, outer layers, which are crucial for forming intercellular connections among epithelial cells, are usually lost during air lifting. Air lifting does not interfere with the structure of the cultivated epithelium (18), but makes it more stable and integrated.

The amniotic membrane is not the only the substrate for epithelial cell expansion. Another substrate is fibrin scaffolds (19). The effectiveness of this variant of treatment is well improved. Long term observations showed that treatment of ocular surface disease with corneal epithelium cultivated on fibrin

gel maintains its postoperative stability for years. Rama et al. described stable corneal cultivated epithelium after transplantation in over ¾ of patients with follow-up up to 10 years (20).

In our cultures, we observed an increased number of epithelial layers, a phenomenon connected with culture conditions. The cultivated epithelium in histologic specimens showed that overgrown layers are poorly adherent compared with basally located cells. This condition is observed in cultures with fast growth and does not have any further implications.

Immunostaining for protein p63 (21) and connexin 43 (22) seems to be the best choice for surgical qualification. Both markers indicate the proliferative potential of suprabasal cells taken from the limbus.

Support of epithelial growth in culture requires the application of animal derived products such as feeder layer 3T3 fibroblasts or fetal bovine serum (FBS), but these applications carry additional risks for the recipient (23). They can generate the potential risk of xenogenic microchomerism, xenoantigenicity, transmission of virus, or prion related diseases (24,25). Therefore, the future use of serum- and feeder-free culture media should be considered as well sufficient as traditional culture media (26).

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