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ALPHA-ENOLASE IMMUNOHISTOCHEMICAL STUDY OF STEM CELLS IN LIMBAL AUTOGRAFT TISSUE

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The purpose was to detect the presence of stem cells in the limbus and cornea following limbal autotransplantation for chemical eye injury.

Fifteen New Zealand white rabbits were used in the study. The first group (n=5) served as healthy controls, in group 2 (n=5) one eye of each rabbit was burnt by 1 N NaOH and in the last group (n=5), the same chemical injury was followed by limbal autotransplantation from the contralateral eye. The eyes were examined clinically and studied by immunohistochemistry after the enucleation. A monoclonal antibody against alpha-enolase - a biochemical marker for stem cells, was used for immunohistochemistry. The density of cells in both limbus and the cornea was calculated per mm^2. The Tukey-Kramer parametric test was used for statistical evaluation.

Healthy limbus and basal corneal layers showed numerous α-enolase positive cells. The injured group showed marked depletion of these cells ($p<0.0001$). Compared to this group the treated group, exibited a statistically significant ($p<0.0001$) increase in α-enolase positive cells.

This study provides evidence that limbal autograft transplantation transfers stem cells to the chemically injured area with depletion of these cells. To demonstrate this we used a monoclonal antibody against alpha-enolase for immunohistochemical analysis of the presence of stem cells in transplant tissue and its surrounding milieu. We showed that the transplanted limbal graft was the source of corneal epithelial stem cells.

Key words: alpha-enolase, immunohistochemistry, stem cells, limbal autotransplantation, chemical eye injury

INTRODUCTION

The past two decades have witnessed several major advances in the field of stem cell medicine. Although present in different tissues and organ systems, stem

cells have certain unique characteristics such as a high capacity for proliferation and self-renewal, a long life span and the capacity of asymmetric cell division. Stem cells are a small subpopulation of the total tissue and together with transient amplifying cells and terminally differentiated cells represent a dynamic compartment responsible for self-renewal of tissues (Potten 1990).

In the human eye, the stem cells responsible for renewal of damaged or desquamated corneal epithelium have been localized in the limbus, an intermediate between the corneal and conjunctival epithelium (Costarelis 1989, Davanger 1971, Schermer 1986). Histologically, the limbal epithelium consists of more than 10 layers and is the thickest among the three anatomical areas. Damage to this area may result in depletion of stem cells which may be associated clinically with persistent epithelial defects, corneal ulceration, vascularization of the cornea, conjunctival epithelial ingrowth on the cornea and corneal scarring (Huang 1991). Many recent reports add to the growing body of evidence that limbal transplantation is effective in replenishing the stem cell pool and improving the clinical picture in limbal deficiency states (Kenyon 1989, Kozák 2002, Morgan 1996, Tan 1996, Tsai 1994, Tsubota 1999, Xu 1999).

The objective of this study was to demonstrate that transplantation of limbal tissue is associated with transfer of stem cells to a previously injured limbal area and that these cells survive in the new locus and carry on their function as if they were in their original place. This was accomplished by immunohistochemical analysis of corneal stem cells using a monoclonal antibody against alpha-enolase, which is a biochemical marker of stem cells.

MATERIALS AND METHODS

Animals and injury

Fifteen white New Zealand male rabbits weighing 2.5 - 3.5 kg were used. The experiment was approved by the Institutional Ethical Committee of the University of Veterinary Medicine in Košice, Slovak Republic. All rabbits were handled in compliance with the Association for Research and Vision in Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic Research. Prior to acceptance for use, all rabbits underwent a baseline ocular examination to rule out preexisting anterior segment abnormalities. The rabbits were divided into three groups. The first group consisted of five rabbits that served as healthy (negative) controls (group 1). The second group consisted of five rabbits in which one eye of each was chemically injured and no treatment applied (injured/positive controls, group 2). These rabbits were euthanised on the third day after injury and the globes were taken for immunohistochemical analysis. The last group consisted of five rabbits in which one eye of each (by random selection) sustained the same injury as in the previous group followed by treatment with a limbal autotransplant from the contralateral eye (treated group, group 3).

The chemical injuries and all surgical procedures were preceded by general anaesthesia. Premedication was intramuscular atropine 0.05/kg (Atropin inj. 0.5 mg/ml, Spofa, Czech republic). General anaesthesia was induced with xylazine

hydrochloride (Rometar 2%, a.u.v., Spofa, Czech Republic) 5mg/kg and ketamine hydrochloride (Narkamon 5%, a.u.v., Spofa, Czech Republic) 40 mg/kg. One eye of each rabbit in groups 2 and 3 received a drop of tetracaine for local anaesthesia after which 1. N NaOH solution was spread over the ocular surface and limbal area with a cotton tipped applicator and left for 10 seconds. The injured area was then continually washed for 5 minutes with balanced salt solution. Surgery in each rabbit in group 3 was performed on day 4 after the chemical injury.

Surgery and post-operative care

Limbal grafts of 2.5 - 3 mm width, 1 mm depth and from an 11-2 o´clock position were acquired from each contralateral uninjured eye in group 3. The donor groove was covered by adjacent conjunctiva using a non-resorbable suture (Ethilon 10.0 with microfilament polyamide fiber and atraumatic 6 mm "micro-point" needle with 3/8 curvature). The same single sutures were used to attach the transplant to the corresponding area of the injured eye as taken from the healthy fellow eye.

In the post-operative period the treated eyes received Infectoflam® (gentamycin + fluorometholone, NOVARTIS) eyedrops five times a day for the first two weeks and then three times a day for another two weeks after which the eyedrops were discontinued. Each rabbit in group 3 was examined clinically at 2 weeks, 4 weeks, and finally at 3 months after surgery. The extent of oedema, vascularization, blepharospasm, conjunctival hyperemia, limbal ischaemia and signs of uveitis was measured using biomicroscopy. Fluorescein was used to test for erosion and tear film stability. Limbal graft assessment was performed by slit lamp examination. At the end of the follow-up period the rabbits were euthanised and the treated eyes were taken for immunohistochemical examination.

Immunohistochemistry

Microscopic sections were taken from the cornea and from the limbus at the 12 o´clock position, where, in group 3, the limbal transplants were located. Tissue sections of 5 ìm thickness for immunohistochemical examination were placed on coated slides and deparaffinized in xylene (2 x 10 min), 96% benzyl alcohol (2 x 10 min) and 70% ethyl alcohol (5 min). After inhibition of the endogenous peroxidase activity in 3% H_2O_2, the sections were washed in running and distilled water and then in phosphate buffered saline (PBS) for 3 minutes. Sections were kept at 37°C for 30 min and washed in distilled water. Sections were incubated with mouse monoclonal antibody against alpha-enolase 4G10.3 (kindly provided by Dr. James Zieske, Schepens Eye Research Institute, Boston, MA, U.S.A.) for 16 h at 4°C. Analysis was conducted on frozen tissue sections using the biotin-streptavidin multilink staining kit (Biogenex, St. Ramon, CA, U.S.A.). After washing with PBS, the sections were reacted with biotinylated antiimmunoglobulins in PBS for 30 min, washed again with PBS, incubated with streptavidin-peroxidase conjugate for 30 min and subsequently stained with a substrate solution containing DAB (3,3-diaminobenzidine, FlukaChemie AG, Buchs, Switzerland) for 5 min. The sections were counterstained with haematoxylin and studied under different mag-

nifications. The number of -enolase positive cells was counted from the whole area and then, using a simple planimetric method, calculated per standardized unit (mm^2) separately for the cornea and the limbus. The mean numbers for particular groups were compared for statistical significance using the Tukey-Kramer test at a probability of $p<0.05$.

RESULTS

The alkali injury left eyes in groups 2 and 3 with a white, ischaemic limbus devoid of blood vessels and damaged corneal epithelium. In group 3 limbal transplantation was followed by hyperaemia and blepharospasm at two weeks in all eyes. At four weeks examination these were less prominent and blood vessels were noted in the vicinity of the limbal graft. At the last follow-up exam before euthanasia the eyes were quiet, corneas had intact epithelium and the areas around the limbal transplant were slightly vascularized.

In the cornea, -enolase positive cells were located mostly in the basal layers in all groups. In healthy corneas they had a columnar shape and slightly differed in intensity of staining (Fig. 1). In group 2 they were small and almost extinct (Fig. 2). In group 3 they could be seen again, in a less uniform pattern, shape and also in the suprabasal layers (Fig. 3).

Figure 1. The presence of alpha-enolase positive cells (arrows) in the rabbit corneal epithelium in a healthy non-treated control (E-epithelium, S-stroma, magnification 40x, bar 5 m).

In the limbus, these cells had round shape and stained more intensely than in the cornea. In group 1, they were numerous and located in the proximity of

blood vessels (Fig. 4). In group 2 they were scarce among damaged adjacent cells (Fig. 5). Group 3 showed the healed portion of transplant tissue and repopulation of stem cells (Fig. 6). In all cases, binding was cytoplasmic excluding the nucleus and adjacent to cell membranes.

Figure 2. The depletion of alpha-enolase positive cells (arrow) in the rabbit corneal epithelium after chemical injury. Notice markedly thin epithelial layer (E-epithelium, S-stroma, magnification 40x, bar 5 m).

Figure 3. The replenishment of alpha-enolase positive cells (arrows) in the rabbit corneal epithelium after limbal autotransplantation (E-epithelium, S-stroma, magnification 40x, bar 5 m).

Figure 4. The presence of alpha-enolase positive cells (arrows) in the rabbit limbus in a healthy non-treated control (E-epithelium, S-stroma, magnification 20x, bar 5 m).

Figure 5. Reduced number of alpha-enolase positive cells (arrows) in the rabbit limbus after chemical injury. Notice regenerated epithelium (E) and stroma (S); magnification 20x, bar 5 m.

Figure 6. The replenishment of alpha-enolase positive cells (arrows) in the rabbit limbus after limbal autotransplantation (E-epithelium, L-limbus, magnification 40x, bar 5 m).

Table 1 shows the mean number of -enolase positive cells with respective standard deviations after calculation per standardized area unit (mm^2). The numbers are shown separately for the cornea and the limbus in groups 1 (healthy controls), 2 (injured controls) and 3 (treated eyes). Statistical comparison showed high significance ($p<0.01$, 95% CI) between the healthy cornea (group 1_{CORNEA}) and injured cornea (group 2_C). There was also a statistically significant difference ($p<0.01$, 95% CI) between group 2_C and the corneal count in the group of treated eyes (group 3_C). Similarly, there was a significant difference ($p<0.01$, 95% CI) between the cell count in the healthy (group 1_{LIMBUS}) and injured limbus (group 2_L) and between group 2_L and the limbus cell count in the treated group 3_L ($p<0.05$, 95% CI).

Table 1. The number of -enolase positive cells with standard deviation found per mm^2 in the cornea and limbus in groups 1, 2 and 3.

Cells/mm^2 (SD)	control eyes (1)	injured eyes (2)	treated eyes (3)
Cornea	4.68 (0.19)	0.62 (0.17)	3.27 (0.29)
Limbus	12.75 (2.72)	5.10 (1.82)	9.30 (2.45)

DISCUSSION

It is becoming a standard practice that patients with stem cell deficiency affecting the whole corneal surface require limbal transplantation (LT) to restore the

corneal surface (Tseng 1997). Limbal transplantation procedures vary, depending on the carrier tissue used for the transfer of the limbal stem cells. Carrier tissue is needed in LT because it is not possible to transfer limbal stem cells alone. Limbal transplants have included either conjunctiva, the cornea, or both as carrier tissue for limbal stem cells (Holland 1996, Sundmacher 1997).

The reason for success of LT is replenishment of lost or damaged stem cells in the limbus. Since the stem cell compartment represents a dynamic system of progenitor cells, transient amplifying cells, post-mitotic and terminally-differentiated epithelial cells, direct histological evidence of stem cells does not exist and all evidence of these cells is indirect. Currently, it is possible to identify potential stem cells useing antibodies against cytokeratins K3, K12 and K19 (Costarelis 1989, Schermer 1986), monoclonal antibody against alpha-enolase (Zieske 1992), 5-bromo-deoxyuridine (Beebe 1996), glycoprotein p63 (Yang 1999) or by autoradiography after ^{3}H-thymidine incorporation (Beebe 1996, Bickenbach 1986, Costarelis 1989).

In this study we provide evidence of transfer of stem cells by limbal autograft transplantation to an injured area depleted of these cells. For this demonstration we used a monoclonal antibody against alpha-enolase for immunohistochemical analysis of the presence of stem cells in transplant tissue and its surrounding milieu.

Alpha-enolase is a glycolytic enzyme that catalyzes conversion of 2-phosphoglyceric acid to phosphoenolpyruvic acid. Migration and differentiation of epithelial cells are associated with increased rates of glycolysis and glucose transport (Burger 1994, Kuwabara 1976). The monoclonal antibody against -enolase (4G10.3), developed by Zieske *et al.* (1992a), binds to limbal basal cells in rat, rabbit, and human corneas (1992b). This antibody, after central epithelial debridement, bound to an increased number of cells, which extended towards the central cornea. Therefore, it can be regarded as a biochemical marker for corneal epithelial stem cells and daughter cells that still carry enhanced levels of -enolase (Zieske 1992s).

We have found that chemical injury to the corneal epithelium and limbus causes marked depletion of -enolase positive cells in these areas. This depletion in both the cornea and the limbus, compared to healthy tissue, was statistically highly significant ($p<0.01$). Thus, subsequent limbal autotransplantation successfully transfers -enolase positive cells to the previously damaged area. The repopulation of these cells was statistically significant in both corneal ($p<0.01$) and limbal ($p<0.05$) areas when the injured versus treated groups were compared.

Clinical examination at three months after the LT showed healed transplant tissue and an intact corneal surface. Because the life span of transient amplifying cells (TAC, cells already present in the corneal epithelium and committed to epithelial differentiation) is believed to be less than three months (Kinoshita 1981), maintenance of a normal corneal epithelium at the end of our follow-up suggests sustained viability of the corneal stem cell grafts. No immunosuppression was used as the grafts were autologous.

Similarly to Chung and coworkers (1995), we noted the presence of stem cells in the vicinity of limbal blood vessels. In the healthy limbus the -enolase positive cells were relatively small and stained intensely. In the peripheral cornea they changed shape from cuboidal to columnar in the central cornea, all restricted to basal cell layers. In group 2 the stem cells in the limbus were scarce and almost extinct in the cornea. The TAC in the cornea stained faintly, which reflected their low metabolic activity, probably as a consequence of severe injury. In group 3, the -enolase positive cells were not necessarily located adjacent to limbal blood vessels but were scattered throughout the transplant tissue. The intensity of staining was comparable to limbal portions of group 1. Both peripheral and central corneas showed medium intensity staining of round to cuboidal cells, together with a few central columnar cells, situated in the basal and suprabasal corneal layers. Chung *et al*. (1995) reported that the -enolase positive cuboidal cells in the mid-cornea correspond to late TAC, while the columnar cells with little staining in the central cornea correspond to terminally differentiated cells. In our study we identified both types in the corneas in group 3. Their source was from the transplanted limbal graft, which explains why corneal epithelization is successful after limbal transplantation.

CONCLUSIONS

In this study we provide evidence that limbal autograft transplantation transfers stem cells to the chemically injured area depleted of these cells. For this demonstration we used a monoclonal antibody against alpha-enolase for immunohistochemical analysis of the presence of stem cells in transplant tissue and its surrounding milieu. We showed that the transplanted limbal graft was the source of corneal epithelial stem cells.

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IMUNOHEMIJSKA STUDIJA STEM ĆELIJA ALFA ENOLAZOM U IVIČNOM AUTOGRAFTU ROŽNJAČE

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SADRŽAJ

Cilj ove studije je bio da se dokaže prisustvo stem ćelija u limbusnom delu i u rožnjači posle hemijskog oštećenja oka i ivične autotransplantacije. Za ova ispitivanja je korišćeno 15 Novo – Zelandskih kunića podeljenih u tri jednake grupe. U prvoj oglednoj grupi izazvano je oštećenje oka pomoću 1 N Na OH a u drugoj je nakon izazivanja iste lezije vršena autotransplantacija sa kontralateralnog oka. Treća grupa je služila kao kontrola. Posle perioda oporavka, vršena je enukleacija i histološki preparati su obeležavani monoklonskim antitelima koja se vezuju za alfa enolazu a ona je specifični marker za stem ćelije limbusa. Kod zdravog oka stem ćelije su u limbusnom delu i bazalnom sloju rožnjače zastupljene u velikoj meri što nije bio slučaj kod povređenog oka. Nakon autotransplantacije, kao i kod zdravog oka, bilo je moguće uočiti ćelije koje sadrže alfa enolazu. Na ovaj način je dokazano da je ivični graft izvor kornealnih epitelijalnih stem ćelija.