Intrastromal Corneal Ring Segment Implantation (Keraring 355°) in Patients with Central Keratoconus: 6-Month Follow-Up

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Brief Note

Isolation, culture, characterization and optimization of human corneal stem cells

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Key words: limbal stem cells**,** limbal stem cells deffiency (LSCD), human EGF, mouse EGF.

ABSTRACT: The effects of human versus mouse EGF on cell growth and culture duration were studied to optimize a human limbal stem cells culture method for therapeutical autologous transplantation. Limbal cells were obtained by trypsin digestion and transferred to a culture medium. The time needed to reach full confluence in culture was determined. Specific antibodies to corneal stem cell marker (P63) versus corneal epithelial differentiation marker (K3) were used for histochemical determinations. A high proportion of P63 positive cells (85± 4.6%), and a correspondingly low proportion K3 positive cells (15 ± 3.8%) indicated that most cultured cells remained undifferentiated and were considered as stem cells (mean ± SE, *n*=10). Cultures reached full confluency after 17.3 ± 1.2 days when the medium was supplemented with human EGF, while 21.7 ± 1.5 days were needed when the medium was supplemented with mouse EGF. The results showed that limbal stem cells proliferate more easily and reach to full confluency in a shorter time if the medium is supplemented with hEGF rather than with mEGF.

The corneal epithelium is a transparent, non-keratinized epithelium covering the entire cornea which has a high regeneration potential and is constantly being renewed from corneal stem cells (Dua and Azuara-Blanco, 2000a), located at the limbus (the corneo-scleral junction) (Schermer *et al.*, 1986). Pathologic conditions such as chemical and thermal injuries, and inflammatory disorders such as the Stevens–Johnson syndrome, may cause partial or total destruction of the limbal epithelium leading to corneal scars (Dua *et al.*, 2000).

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Complete limbal stem cells deficiency (LSCD) leads to re-epithelialisation of corneal surface by bulbar conjunctival cells. Unilateral limbal stem cells defficiency can be successfully treated by the autologous transplantation of limbal grafts taken from the healthy eye. However, this therapeutic procedure requires a sizeable limbal explant to be removed from the healthy eye which may be dangerous.

Ex vivo expansion of corneal limbal stem cells (Tseng, 1989; 1996) is a new therapeutic option for patients suffering from unilateral limbal stem cell deficiency (LSCD) (Pellegrini *et al*., 1997; Tsubota *et al*., 1999; Dua and Azuara-Blanco, 2000b; Tsai *et al*., 2000). Since this therapeutical approach has been employed (Koizumi *et al*., 2001; Griffith *et al.*, 2002), there is

great interest in optimizing the culture of limbal stem cells (Lavker *et al.*, 2004).

The present study was aimed to compare the effects human versus mouse EGF activity on cell confluency and culture duration, since it has previously shown that the affinities of mEGF for human low affinity receptors and high affinity receptors are much lower than those of hEGF (Connolly and Rose, 1987). Also we have quantified the effect of human EGF on the expression of corneal stem cell marker (P63) (Pellegrini *et al*., 2001) versus corneal epithelial differentiation marker (K3) (Rodriguez *et al*., 1987) in order to define optimal culture conditions to obtain high quantities of undifferentiated cells for autologous transplantation.

Limbal tissue biopsies (1 mm^2) were obtained from normal donors from whom an informed written consent was obtained. The limbal biopsy was dissected from the limbal margin by lamellar keratectomy, minced and treated with 0.025% EDTA/trypsin (Gibco) for 30 minutes in 37ºC and was transferred to DMEM/F12 medium (Gibco), supplemented with recombinant hEGF (40ng/ml- Roche), bovine insulin (10mg/ml- Gibco), cholera toxin (40ng/ml- Fluka) and 10 % fetal calf serum (Gibco) at 37ºC with 5% CO_2 for 3 weeks.

The presence of limbal stem cells was confirmed by assessing the colony-forming efficiency of representative cultures, assuming that only stem cells can establish colonies of >50 cells from an individual founder cell (Schwab *et al.*, 2000). For this purpose, hematoxylin-eosin staining was used (Fig. 1. AI-II).

Cultured cells were immunohistochemically stained using monoclonal anti-human K3 (CBL218, Millipore; dilution 1:250) and P63 antibodies (P3362, Sigma; dilution 1:250) and the percent of positive cells were determined by counting in 10 random high power fields (100x objective) per plate.

For immunoblotting, cells proteins were extracted, separated on 12% SDS-PAGE, transferred to PVDF membrane and detected using mAb-P63. Molecular weight standards of 39 and 66 kDa were used for comparison.

To evaluate the effects of EGFs, cells were transferred to culture dishes containing medium enriched with either mouse or human EGFs (40ng/ml). Percent

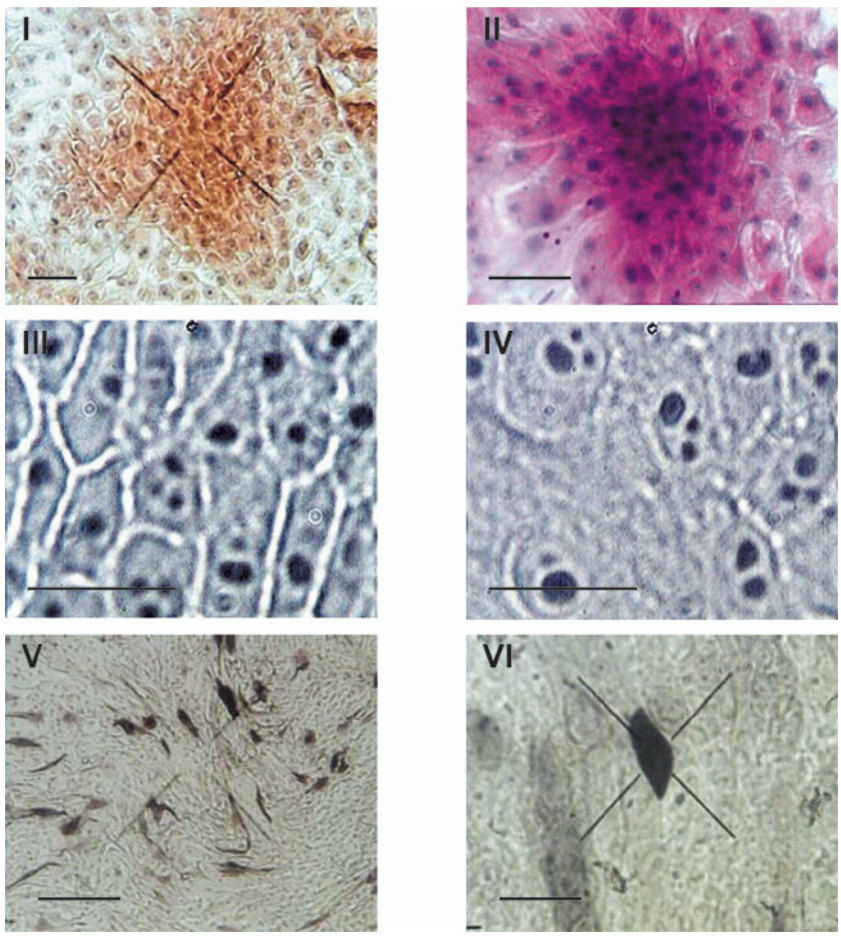

FIGURE1. Characterization of human limbal stem cells cultured in medium supplemented with human EGF.
I-II. Stem cells colonies formed in culture from limbal explants (hematoxylin-eosin). III-IV. Nuclear immunochemical staining for P63 shows the presence of limbal stem cells after reaching confluency in culture. V-VI. Cytoplasmic immunochemical staining for K3 keratin shows a low proportion of differentiated corneal epithelial cells after reaching confluency in culture. Scale bars are 100µm.

of confluency (field area) was determined quantitatively using a plate grid during culture period (n=10).

Positive nuclear staining for P63 confirmed the presence of corneal stem cells (Fig. 1. III-IV) in cultures grown on hEGF supplemented medium. The percent of P63 positive cells when reaching full confluency was 85 ± 4.6% (mean ± SEM, n =10) indicating the presence of corneal stem cells as the majority of the cells which was also confirmed by Western blotting using P63 antibody. Meanwhile, only 15 ± 3.8% (n =10) of cultured cells were positive for the differentiation marker, K3 keratin (Fig. 1. V-VI). Also, Western blot analysis of the protein extract from cells grown on hEGF supplemented medium showed a band of approximately 63 kDa.

The percent area of confluency was significantly higher (P<0.05 or less; Student's t test) from 10-18 days of culture when the cells were grown in medium supplemented with hEGF as compared with mEGF.

The culture duration for full confluency in cells treated with hEGF, was 17.3 ± 1.2 days, which was significantly lower (P<0.01, Student's t test) than 21.7 ± 1.5 days for mEGF treated cells (mean ± SEM, n= 10).

This study has shown that limbal stem cells can proliferate in a shorter time when grown in a medium supplemented with hEGF, as compared with mEGF, Also, a high proportion of undifferentiated cells was thus obtained. These results will be relevant to optimize a method for autologous corneal stem cell transplantation in humans.

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