OPTIMIZATION OF HUMAN LIMBAL EPITHELIAL STEM CELL EXPANSION UNDER CHEMICALLY DEFINED CULTURE CONDITIONS

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Limbal epithelial stem cells (LESC's) are responsible for corneal epithelium regeneration due to corneal damage, or LESC deficiency. One of the major challenges of LESC's culture is their optimal derivation and expansion in a chemically defined culture media; with the purpose to generate enough amounts of cells with the correct genotype and phenotype. Chemically defined conditions would avoid cells that could compromise the security and efficiency of cell therapy. The need for alternative options to cadaveric corneas will continue to grow in importance as a result of increasing incidence of keratoplasties, hence if we investigate the use of different proteins as extracellular matrix (Laminin, Fibronectin, Collagen type I and Collagen Type IV) during the expansion of LESC's, we could optimize a "selective expansion of LESC's".

LESC's isolation was carried out from human sclera-corneal rims remaining after penetrating keratoplasty procedure. After disinfection, rims were treated by enzymatic digestion followed by a mechanical separation. We investigated different proteins (Laminin, Fibrin, Collagen type I and Collagen type IV) as extracellular matrix in order to culture the cells obtained from enzymatic digestion. Culture plates were pre-treated with 50μg•mL^{-1} of each protein for 24h prior to use. The absence of extracellular matrix was included as control. Cell culture and expansion was performed at standard culture conditions (5% CO_2, 37º C) using chemically defined culture media Epilife$_{10}$ (Invitrogen).

The presence of Keratin 3 and P63-α proteins, were used to identify epithelial cells and limbal stem cells (respectively) by Immunofluorescence analyses; and mRNA detection by PCR. Cell expansion was assessed by cell count, viability and flow cytometry identifying limbal stem cells (P63-α+) and epithelial cells (CK3+). Once we identified the protein that allows an optimal expansion of LESC, we compared two different culture media (chemical defined Epilife$_{10}$ medium and medium SHEM).

The use of collagen Type I as extracellular matrix, allowed the "selective expansion" of LESC, obtaining 90% of cells expressing p63-α+ ($p=0.0023$). Meanwhile Epithelial cells (CK3+) did not show a selective expansion with the use of any protein as extracellular matrix. Previous reports suggest the use of collagen Type I as an "adhesion gradient" by culturing cells only for 24 h post enzymatic digestions of sclera-corneal rims. However we found that culturing cells for the whole period (up to 15 days) of expansion under chemically defined conditions, allows the expansion exclusively of LESC. Growth kinetics showed that the exponential phase of LESC expansion is between 216h to 260h (9 – 12 days). When comparing two different culture media, the non-defined medium SHEM results in expansion of LESC and fibroblast cells; meanwhile the use of chemically defined Epilife$_{10}$ (Invitrogen) media avoids the presence of fibroblast cell phenotype.
Our results suggest that Collagen Type I can be used as extracellular matrix for the selective expansion of limbal epithelial stem cells, reaching the maximum cell number between 216 h and 260 h of culture. Using this strategy, it is possible to obtain up to 1,760,000 cells per rim after 576 hours of culture. This expansion procedure, delivers enough amount of LESC in order to obtain at least 3 corneal substitutes (through 3D culture strategy) from sclera-corneal rims derived and cultured under chemically defined conditions.

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