Apoptosis Gene Expression Profiling of Ex Vivo Expanded Human Limbal Epithelial Cells

Sten Raeder, Tor Paaske Ultem, Oygunn Aas Ultem, Savita Prabhakar, Yiqqal Cai, Borghild Roald, Kristiane Hauge, Anders Kvalheim, Edvard Berger Messelt, Hewen Zhang, Liv Drolsum, Bjorn Nicolaissen, Torstein Lyberg, Center for Eye Research, University of Oslo, Department of Ophthalmology, Ulleval University Hospital, Norway, Department of Oral Biology, University of Oslo, Department of Pathology, Ulleval University Hospital, University of Oslo, Norway. Center for Clinical Research, University of Oslo, Center for Eye research Ulleval University Kirkeveien, Oslo, 0407, Norway, SuperArray Bioscience Corporation, 7320 Executive Way, Suite 101, Frederick MD, 21704.

Introduction

Transplantation of ex vivo expanded human limbal epithelial cells (HLEC) is used as a therapy for limbal stem cell deficiency (LSCD). HLEC may be cultured ex vivo by a variety of expansion protocols. Although these protocols have shown good clinical outcomes, limbal epithelial stem cell therapy still faces challenges regarding surgery logistics, tissue sterility, tissue transportation, and availability of tissue. Our laboratory was the first to report a method for short-term organ culture (OC) eye bank storage of cultured HLEC which may be beneficial in limbal epithelial stem cell therapy. This study was conducted to investigate whether conventional OC storage and Optisol-GS storage were applicable to cultured HLEC and to evaluate the extent of cell death due to apoptosis after eye bank storage of cultured HLEC. HLEC were expanded on intact amniotic membranes for 21 days and stored for one week at 31°C and 23°C in OC medium or at 5°C in Optisol-GS. Cultures were fixed in formaldehyde and embedded in paraffin. Labeling indices were observed under different storage conditions as measured by immunostaining with caspase-3 (range 0.9%-1.2%) and a DNA fragmentation assay (TUNEL, range 0.9%-2.3%). Cellular results were confirmed at the gene expression level using the Human Apoptosis RT2 Profiler™ PCR Array from SuperArray Bioscience, which profiles the expression of key apoptosis genes. The results showed that pro-apoptotic genes were significantly down-regulated and anti-apoptotic genes were significantly up-regulated in all three storage conditions. This study demonstrates that eye bank storage of cultured HLEC is associated with minor cell death due to apoptosis at both the cellular and the gene expression level. This work was supported in part by the Eastern Norway Regional Health Authority, the Norwegian Association of the Blind and Partially Sighted and the Blindness IL

Figure 1: Eye Bank Storage of Cultured Human Limbal Epithelial Cells (HLEC)

Figure 2: Organ Culture Storage of Cultured HLEC at Ambient Temperature is Superior to OC Storage at 31°C and Optisol-GS Storage at 5°C

Histology and Immunostaining

HLEC cultures were fixed in neutral-buffered 4% formaldehyde and embedded in paraffin. Serial sections of 3 µm were stained with haematoxylin and eosin (H&E). Immunohistochemistry was performed with a panel of antibodies for markers of human ocular surface epithelia. Histological evaluation and semiquantitative immunohistochemical localization of the epithelial markers were carried out by two independent investigators using a microscope at a magnification of 400X. Results: The ultrastructure was preserved at 23°C, while storage at 31°C and 5°C was associated with enlarged intercellular spaces, separation of desmosomes, and detachment of epithelial cells. HLEC cultured remained undifferentiated under all storage conditions.

Figure 3: Quantification of Apoptotic Cells

Apoptosis is minimal following eye bank storage of cultured HLEC, though multi-gene profiling revealed interesting alterations in gene expression in cultured HLEC.

Figure 4: Anti- and Pro-Apoptotic Genes with Greater than Three-Fold Change in Expression (p<0.05) in Cultured Human Limbal Epithelial Cells following 1-week Storage at Three Different Temperatures

Experimental Design

We hypothesized that OC storage at 31°C and Optisol-GS hypothermic storage may preserve the characteristics of cultured HLEC. Accordingly, we compared these conventional storage methods with the novel storage method. Furthermore, because cell death due to apoptosis has been reported in human corneal epithelia after OC culture storage and hypothermic storage, we studied expression of apoptosis-regulatory genes and examined apoptosis markers in cultured HLEC following eye bank storage.

Stem Cells: 3-week HLEC (n=48) cultures were either organ-cultured at 31°C (n=12) or 23°C (n=12) or stored in Optisol-GS at 5°C (n=12) in a closed container for one week. Figure 1 explains in brief as to how the human limbal tissue from human donor eyes were cultured on intact amniotic membranes and stored in the eye bank.

Figure 3A demonstrates H&E staining (top), cleaved caspase-3 immunohistochemistry (middle), and TUNEL staining (bottom) of cultured human limbal epithelial cells after one week’s organ culture storage at 23°C. H&E staining demonstrates an apoptotic epithelial cell with circular nuclear fragments (arrow). Cleaved caspase-3 positive surface cells have cytoplasmic immunoreactivity and well-defined nuclear membranes (arrowheads). A TUNEL positive cell (arrowhead) is also observed. (Original magnification: 400X) Figure 3B contains a histogram demonstrating the H&E apoptotic index, caspase-3 labeling index, and TUNEL labeling index in cultured human limbal epithelial cells after three weeks’ culture and one week’s storage at the three different temperatures. Results are expressed as mean percent of the apoptotic or labeling index in the individual experimental groups. Error bars denote 1 SE. Results: No significant increase in cleaved caspase-3 or TUNEL staining was observed in response to eye bank storage of any of the three storage conditions.

Figure 4. RNA was isolated from the formalin-fixed paraffin-embedded (FFPE) tissue using SuperArray’s ArrayGrade™ FFPE RNA Isolation Kit (GA-023). RNA (40 ng) was amplified and reverse transcribed using a modified version of the TaqMan® one-step RT-PCR kit (GA-150) and the RT2™ PCR Array First Strand Kit (C-012) from SuperArray Bioscience. The RT2™ Profiler™ Human Apoptosis PCR Array (APHS-012) from SuperArray Bioscience was used to analyze the mRNA levels of 84 key genes involved in apoptosis, in a 384-well format. Three biological replications were included in each experimental group. Relative changes in gene expression were calculated using the ΔΔCt method. Results: Following storage at 23°C and 5°C, down-regulation of BCL2A1 and BIRC1, and reduced expression of TNF receptor signaling components (TNF and TRADD) was revealed suggesting a reduction in nuclear factor-κB activity. Under all storage conditions, expression of BNP2 was up-regulated, whereas MCL1 expression was down-regulated.

Conclusions

Our results indicate that OC storage of cultured HLEC at ambient temperature is superior to OC storage at 31°C and Optisol-GS storage at 5°C as the original tested storage conditions of cultured HLEC is preserved at 23°C storage.

Apoptosis is minimal following eye bank storage of cultured HLEC, though multi-gene profiling revealed interesting alterations in gene expression in cultured HLEC.

Eye bank storage of cultured HLEC may provide a reliable source of tissue for treating limbal stem cell deficiency, although its feasibility for clinical use has to be evaluated further.

References