EXTENDED REPORT

Epithelial proliferative potential of organ cultured corneoscleral rims; implications for allo-limbal transplantation and eye banking

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Aims: To determine the epithelial proliferative capacity of organ cultured limbal tissue and correlate this with various donor and eye banking factors.

Methods: 24 corneoscleral limbal (CSL) rims left over from penetrating keratoplasty were split in half and set up as in vitro explant cultures. Corneal epithelial proliferative potential (CEPP) was assessed by the number of "cycles" of growth achieved before explants underwent exhaustion and failure to generate an epithelium to subconfluence. The dependence of CEPP on the age of the donor, time of death to enucleation, time of enucleation to organ culture, and time in organ culture in the eye bank was determined.

Results: CSL rims were capable of up to four cycles of culture with a wide variation between tissue samples. Of the various factors examined, death to enucleation time was the only statistically significant factor affecting the CEPP (regression coefficient: -0.062 (cycles/hour), CI -0.119 to -0.004 , $p = 0.037$). Time in organ culture had little effect on CEPP.

Conclusions: Preselected organ cultured CSL rims from eye banks may offer a viable alternative tissue source for use in allo-limbal transplantation.

Putative stem cell deficiency is a well recognised pathology in several blinding ocular surface diseases.¹ The transplantation of stem cells in the form of allo-limbal transplants is now a well established treatment option.² However, current protocols dictate that the corneoscleral limbal (CSL) tissue used should be procured from donors under the age of 50 and used within 72 hours of donation (that is, death of the donor).³ This is because of the questionable viability of stem cells retrieved outside the above parameters. Such a protocol often necessitates local retrieval of tissue and conducting screening tests for HIV and hepatitis on a one-off basis, at times requiring on-call emergency staff. These constraints lead to severe logistical problems and considerable costs. In reality, this leads to unplanned surgery done on an ad hoc basis with considerable inconvenience to both the hospital and the patient.

In the United Kingdom and several European countries it is common practice to preserve donor corneal tissue in organ culture medium, whereby tissue can be stored for up to 4 weeks and be successfully used in corneal transplantation.^{4,5} The organ culture medium is based on an Eagle's minimum essential medium supplemented with fetal calf serum and antibiotics. Before issue the endothelium is assessed and it is transported in a dextran solution. This reverses any stromal swelling that has occurred during the organ culture period, thereby making the cornea easier to handle during surgery. It may be kept in dextran for up to 5 days. Advantages of the corneal organ culture method include the opportunity to plan surgery in advance, carry out tissue typing (if necessary), and perform microbiological tests on the storage medium before release of tissue for clinical use. If CSL tissue that has been preserved in organ culture could also be used in allo-limbal transplantation many of the logistical problems outlined above could be addressed. Limited work has been done on the possible use of eye bank material as a suitable alternative to "fresh" donor material.^{6,7} The purpose of this study was to determine whether organ

cultured CSL retains its epithelial proliferative potential to allow for such use and whether any factors could be identified that may influence this potential.

METHODS

Preparation of epithelial cell cultures

CSL rims remaining after penetrating keratoplasty from donors who had given consent for research as well as transplantation were obtained. All rims were processed for culture within 5 days following transfer to dextran containing organ culture medium. The excess sclera was trimmed and posterior stroma and endothelium stripped away. The rims were cut into two equal halves and set up as explants in tissue culture, with the epithelial side up, on Nunclon 35×10 mm culture plates (obtained from VWR international Ltd, Poole, UK), which were grid marked in order to measure areas of epithelial growth. A standard corneal epithelial culture medium was used and replaced twice weekly. This consisted of Dulbecco's modified Eagle's medium and HAMS F12 (1:1), fetal calf serum (5%, Life Technologies), cholera toxin (0.1 µg/ml Calbiochem-Novabiochem), insulin (5 µg/ml, Life Technologies), epidermal growth factor (10 ng/ml, R&D Systems), gentamicin (5 µg/ml), and dimethyl sulphoxide (0.5% Sigma).

Assessment of proliferative potential and statistical analysis

When the expanding epithelial sheet covered an area of 432 mm² or more (approximately 60% subconfluent) the explants were removed and set up again on new plates. This process was sequentially continued until the explants were unable to produce a viable epithelial sheet of at least 40 mm² over a period of 30 days. At this end point the experiment was terminated. Each transfer of explants that met the above

Abbreviations: CEC, corneal epithelial cells; CEPP, corneal epithelial proliferative potential; CSL, corneoscleral limbal

Table 1 Statistical analysis of various donor and eye banking factors (variables)

Variable	Regression coefficient	95% CI	r ²	p Value
Donor age (years)	-0.0077 (cycles/year)	-0.035 to 0.020	0.02	0.56
Death to enucleation (hours)	-0.062 (cycles/hour)	-0.119 to -0.004	0.18	0.037
Enucleation to culture medium (hours)	+0.027 (cycles/hour)	-0.054 to 0.108	0.02	0.50
Time in organ culture medium (days)	-0.0005 (cycles/day)	-0.135 to 0.134	<0.01	0.99

criteria was defined as one cycle, except the last, which was included in the cycle count only if the sheet area was 40 mm² or more. The corneal epithelial proliferative potential (CEPP) was defined as the mean number of cycles for the two halves of each rim. Explants that became contaminated or those that had floated within the first 3 days of being set up were excluded from the study. The relation between the CEPP and the following variables was examined: (i) age of donor, (ii) time lapsed between death and retrieval (enucleation), (iii) time of enucleation to time in organ culture in hours, (iv) time in days in organ culture before transfer to dextran. Univariate linear regression analysis was performed using Stata version 6 (Stata Corp, College Station, TX, USA) to assess the relation between the CEPP and these four variables.

Immunohistochemistry of epithelial outgrowth

The epithelial phenotype of the cultured cells was confirmed by immunostaining for cytokeratins 3 and 19. Samples of cells from random plates were obtained by trypsin/EDTA treatment and processed for cytospin preparation on glass slides. Mouse anti-human antibodies to cytokeratin 3 (Clone AE5, Biogenesis, UK) and cytokeratin 19 (Clone b170, Novacastra, UK) at dilution of one in 50 and one in 100, respectively, were used as primary antibodies in an indirect three step alkaline phosphatase anti-alkaline phosphatase

reaction with fast red chromagen. Non-specific mouse IgG was used as a negative control. Membrane integrity of cells was determined by the exclusion of trypan blue from a test sample.

RESULTS

Dependence of CEPP on various donor tissue variables (table 1)

Donor age

No statistically significant association was found between the CEPP and the age of the donor (fig 1A).

Death to enucleation time

This was the time lapsed between the death of the donor and actual retrieval (enucleation) of the eye and transfer into a moist chamber (fig 1B). When the dependence of CEPP on this time was determined a statistically significant association was noted. The shorter the time the greater was the proliferative potential of the explants. This relation was consistent; however, there was one outlier in the study where the death to enucleating time was 27 hours but yielded four complete cycles of growth.

Enucleation to culture medium time

After transfer to the moist chamber, donated whole eyes were transported at 4°C to the UK eye banks (fig 1C). A courier

Figure 1 Graphs illustrating relation of CEPP with various eye banking factors: (A) age, (B) death to enucleation time, (C) enucleation to organ culture time, (D) time in organ culture.

Figure 2 Immunohistochemical staining of corneal epithelial cells (CEC) cultured from limbal explants. (A) Positive staining of some CEC with CK3. (B) Positive staining of some CEC with CK19. (C) Negative control with a mouse IgG primary antibody. Magnification $\times 100$.

service is used for this purpose. Upon arrival in the eye bank, the eyes are examined and processed for removal of the corneoscleral disc and transfer in to the organ culture medium. All this takes a variable period of time which could influence the viability of limbal (stem) cells. No statistically significant association was found between the CEPP and the time between enucleation and transfer to culture medium.

Time in organ culture medium

The duration for which CSL discs were maintained in organ culture medium ranged from 10 days to 27 days before transfer to dextran medium (fig 1D). This time had virtually no effect on the CEPP.

Immunohistochemistry

Immunostaining of cytopins of cells showed a mixed pattern of staining for CK3 and CK19 (fig 2) confirming the epithelial nature of the cell cultures.

DISCUSSION

The ability to identify organ cultured CSL rims that can be used in allo-limbal transplants would be a major step forward in the field of ocular surface reconstruction. This study attempted to address this question and the data demonstrated that most organ cultured CSL rims do have the potential to produce an epithelium under in vitro culture conditions. Such potential, however as tested by the number of growth cycles possible, is very variable and depends on a number of factors.

Several methods exist to assess epithelial growth such as clonal assays, colony forming efficiency and the number of cell passages of single cell suspensions obtained from the limbus.⁸ We chose to adopt a different approach by using explant cultures as this was more representative of the clinical situation in that it is the explants and not the cells in suspension that are used in allo-limbal transplants. This is of relevance as it is recognised that within mammalian epithelial tissue stem cells reside within a niche for their maintenance and function.⁹ Indeed, work from our laboratory indicates that within the palisades of Vogt there are certain discrete repositories of epithelial cells that form cord-like structures—the “limbal epithelial crypts”—and these have some immunophenotypical features that are consistent with a putative stem cell niche.¹⁰ We also chose to look at total epithelial growth rather than rate of growth as it indicates the overall proliferative potential and may better reflect the longevity of the limbal tissue, which is a major difference from previous work on this issue. The end point of 30 days was chosen as preliminary experiments with this culture system had indicated that CSL rims which had not reached subconfluence by 30 days would not do so if left for longer periods of time. The main limitation of this study was the lack of comparison with fresh rims. This is because of the scarcity of fresh tissue available purely for research. The few fresh CSL rims that we did assess generally grew well but also had a degree of variability (data not shown).

The existing protocols and surgical experience with fresh rims suggest that tissue from younger donors provides better epithelial coverage.³ However, in our study we were unable to confirm that CSL rims from younger donors had a greater proliferative potential, which was surprising as we expected this to be the most influential factor.

This study clearly demonstrated that the single most important variable determining proliferative potential was the death to enucleation time. The reasons why short death to enucleation time was the only statistically significant factor ($p < 0.05$) are not clear. It may be that swift placement of the eye into a moist chamber protects the limbal epithelium against desiccation and further damage. Furthermore, examination of the data suggested that 12 hours may be used as a cut-off point for any pre-selection of CSL rims. This was confirmed by further statistical analysis which revealed that eyes enucleated up to and including 12 hours from death yielded a mean CEPP of 2.3 cycles compared with 1.2 cycles for those retrieved after 12 hours (Mann-Whitney test; $p = 0.022$). Another important result was that the time in organ culture (up to 30 days) appears to have little detrimental effect on the epithelial proliferative potential. This indicates that the organ culture medium used supports the viability of the limbal epithelial tissue.

With allo-limbal transplantation now established as an important procedure in ocular surface reconstruction, it naturally follows that eye banks will have to play an important part in providing such tissue. The identification of predictive factors will be a crucial step if eye banks are to pre-select potential donor CSL tissue as a source for allo-limbal transplants. Most previous studies have examined the effects of eye banking procedures on corneal tissue but not the limbus. To our knowledge only two studies have looked at the limbus. Kim *et al*, using cold storage (4°C) in Optisol preserved tissue, identified death to enucleation and time in Optisol, but not donor age, as significant predictors of growth rates from limbus tissue.⁷ Another study, however, found age to be the only influencing variable.⁶ This study had several confounding factors as they had used tissue preserved in four different conditions including fresh eyes. These included the cold storage in Optisol, in Dulbecco's modified Eagle's medium, organ culture medium with dextran (as in our study), and fresh eyes. Any growth within 7 days was taken by them as an indicator of “successful growth.” The results were analysed for the combined data and any difference related to the culture conditions was not analysed. Our study is therefore important because it deals with tissue placed initially in organ culture followed by culture medium containing dextran which is the standard preservation medium in the major European eye banks.^{4,5} It compares favourably with Kim's study, indicating that Optisol preservation too can be an option with the limitation that the latter technique can be used to store tissue for only 2 weeks compared to 4 weeks by the organ culture method.

Clearly there are a multitude of other intrinsic biological factors that could influence the capacity for cell proliferation.

For example, Gaudreault *et al* identified the expression of a series of transcription factors known as specificity protein 1 and 3 (Sp1/Sp3) as good predictive indicators of corneal epithelial cell proliferation.¹¹ While such molecular approaches are useful and give an insight into the mechanisms that govern corneal epithelial cell proliferation and migration they are of limited value in the practical setting of an eye bank.

There have been advances in the treatment of stem cell deficiency, notably the expansion of corneal epithelial (stem) cells on amniotic membrane and more recently cultured autologous oral mucosal transplants (for bilateral stem cell deficiency).¹⁰⁻¹² These composite tissue constructs are then transplanted onto the eye with favourable results thus offering an alternative to allo-limbal transplantation.¹³ However, it is unlikely that such constructs provide the full "stem cell" niche. Moreover the stringent laboratory conditions and costs required to develop such constructs limit their availability.¹⁴

In the long term, allo-limbal transplants are known to undergo exhaustion or failure because of chronic rejection.¹⁵⁻¹⁶ The use of organ cultured rims for stem cell transplantation provides an opportunity for HLA typing and matching that may help to reduce rejection related attrition. A further consideration is that there will be a depletion of Langerhans antigen presenting cells during organ culture storage and this too may help address the rejection issue.¹⁷

In conclusion, although fresh tissue may remain the accepted standard for allo-limbal transplants, this work supports the idea that preselected CSL rims from eye banks may act as an alternative source for limbal transplantation. This study shows that death to enucleation time will be a key factor in setting such pre-selection criteria. This study also provides the important information that such tissue can be held in organ culture for up to 30 days without affecting its proliferative potential. The ability to identify good limbal tissue and hold it in an eye bank until required for transplantation will enable widespread use of the tissue in a planned manner.

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