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

V A Shanmuganathan, A P Rotchford, A B Tullo, A Joseph, I Zambrano, H S Dua

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.
criteria was defined as one cycle, except the last, which was included in the cycle count only if the sheet area was 40 mm$^2$ 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)**

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

**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 are 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.
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 cytospins 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. 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. 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. 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. 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 eyebanking 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. Another study, however, found age to be the only influencing variable. 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.

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. \(^{34}\) 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. \(^{15}\) 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. \(^{13}\) 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.

ACKNOWLEDGEMENTS
We thank the departments of ophthalmology at the HM Stanley Hospital, St Asaph, Wales, and the Leicester Royal Infirmary for the provision of tissue used in this study.

Authors’ affiliations
V A Shanmuganathan, A P Rotchford, A Joseph, H S Dua, The Larry Donoso Laboratory, Division of Ophthalmology and Visual Sciences, University of Nottingham, UK
A B Tullo, I Zambrano, David Lucas Eye Bank, Manchester Royal Eye Hospital, Manchester, UK

This work has been supported by grants from the Henry Smith Charity and the Charles Hayward Foundation.

This work was an oral presentation at the European Eye Bank Association (EEBA) meeting, January 2004 in Barcelona and at the 8th Nottingham Eye Symposium and Research Meeting January 2004.

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

V A Shanmuganathan, A P Rotchford, A B Tullo, A Joseph, I Zambrano and H S Dua

Br J Ophthalmol 2006 90: 55-58
doi: 10.1136/bjo.2005.071910

Updated information and services can be found at:
http://bjo.bmj.com/content/90/1/55

These include:

References
This article cites 17 articles, 5 of which you can access for free at:
http://bjo.bmj.com/content/90/1/55#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
Ophthalmologic surgical procedures (1223)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/