Bahrain Medical Bulletin, Vol. 29, No.4, December 2007

Immune Activation Markers in Cadaver Limbal Tissue and Ex-vivo Expanded Limbal Epithelium

Lakshmi Kiran Chelluri, Ph.D* Kamaraju Suguna Ratnakar, M.D** Namita Gupta, M.S** Vasantha A, M.Sc**

Background: It is hypothesized that certain amount of antigen presenting cells are present in the normal limbus and cornea interfering in the allograft survival.

Objective: Differential expression of normal limbus and ex-vivo expanded limbal epithelial cells on human amniotic membrane needs to be evaluated in order to understand the immune status of the allograft. The evaluation of the allograft prior to limbal stem cell transplantation would enhance the prognostic value in formulating the immunosuppressive therapy protocol for the patients.

Design: Prospective study.

Setting: Stem Cell Department, Global Hospitals, Hyderabad.

Method: It was a prospective study design, where the cadaver limbal tissues were tested for the presence of immunoregulatory markers. Markers under study included CD4, CD8, CD25, CD68, HLA-DR (MHC-II) before and after ex-vivo expansion of the limbal epithelium by immunofluorescence technique.

Result: The study was carried out on 10 subjects, and 3 lymph nodes were treated as positive control. Corneoscleral rims were analyzed in three cases. Markers of immune activation such as CD4, CD8 were negative in normal and ex vivo expanded limbal epithelium, while CD25, CD68 were present occasionally. The cultivated limbal epithelium remained predominantly negative for these markers.

Conclusion: The cultivation of limbal epithelium may be a safer proposition for limbal stem cell transplantation as against the original allografting. The culture system alleviates the chance of graft rejection by way of removing the trigger factors.

Bahrain Med Bull 2007; 29(4):

Immunological rejection by far remains the most common cause of rejection in corneal allotransplantation despite being immune-privileged status[1]. The limbus is an entry zone for Langerhans cells and leukocytes to invade the diseased cornea[2].

* Head, Department of Transplant Biology & Stem Cell
** Stem Cell &Transplant Immunology Laboratory,
Global Hospitals, Hyderabad
India

It is reported that in limbal transplantation, there is mild response of CD4 (+) and CD8 (+) T cells surrounding the amniotic graft post transplantation[3]. HLA-DR positive dendritic cells were reported to be seen in corneal epithelium in normal human conjunctiva, corneal limbal epithelium, and in limbal vascular endothelium[4]. No studies have been reported so far, to determine the role of these inflammatory markers in human limbal epithelium, stroma, endothelium at basal, suprabasal and superficial zones. In view of this, it is premised that the study on markers of immune activation in limbal tissue and cultivated limbal epithelium may help in better management of allografts. Furthermore, CD25 antigen is expressed predominantly on activated T-cell (marker for IL-2 receptor) and activated macrophage and to a lesser extent on activated B cell. Our study would evaluate the hypothesis that there may be certain amount of antigen presenting cells persisting in normal limbus and cornea which may be involved in the rejection process by evaluating the cadaver limbal tissue. We would also evaluate if they will undergo any change during or after ex-vivo expansion of limbal epithelial cells on human amniotic membrane. The results of the study would therefore help enhance our understanding of the immune status of the allograft and would guide the clinician in formulating the immunosuppressive therapy protocol for the patients. Further studies on the evaluation of PK graft would help understand the persistence of these immune markers.

METHOD

Immunohistochemistry and Antibodies

Paraffin embedded specimens and ex-vivo cultivated limbal epithelial cells were analyzed for the following concentrations of monoclonal antibodies viz., 5.0 µg/mL CD4 (Dako, USA), 2.5 µg/mL CD8 (Dako, USA), 1.0 µg/mL HLA-DR (Dako, USA), CD25 (Dako, USA), CD68 (Dako, USA).The results were graded using fluorescence microscopy. Elite avidin-biotin conjugated system was optimized for each antibody and applied to the tissue for antigen localization. Positive controls were processed simultaneously on lymph node biopsy specimen.

Ex-vivo expansion of cadaver limbal epithelial cells[5]

The limbal cells were grown on de-epithelialized amniotic membranes (2.5 x 5 cm). For de-epithelialization, a small piece of glass slide was placed into a 55mm culture plate. One milliliter of trypsin-EDTA was added onto the surface and incubated for 30 minutes at 37^0C. After removal of the cell debris, the membrane was washed thoroughly with phosphate buffered saline, and viewed under the phase contrast microscope to ensure complete denudation, followed by secure tucking around the glass slide piece to obtain a uniform surface. The tissue bits were picked up with 24 gauge sterile needle and explanted onto the denuded amniotic membrane. After 20 minutes of explantation few drops of human corneal epithelial medium was added onto the explants, and kept in the CO_2 incubator for allowing the adherence of the tissue bits to the membrane for about 8-10 hours. Finally, the culture dish was flooded with 4-ml human corneal epithelial medium with 10% autologous serum. The medium was changed every alternate day and

growth of the cells monitored under phase contrast microscope and observed for confluency.

RESULT

The results of the immunohistological analysis on the test limbal sections are summarized in Table 1.

Table 1: Immune Responses to the Immune Activation Markers before and after Cultivation of the Cadaver Limbal Tissues

Antibody	Limbus (Before) positive	negative	Limbus (After cultivation) positive	negative
CD4		**10**		**10**
CD8		**10**		**10**
CD25	**3**	**7**	**3**	**7**
CD68	**3**	**7**	**3**	**7**
HLA-DR (MHC-II)	**2**	**8**	**2**	**7**

All of the immunoregulatory markers under investigation were predominantly negative (CD4, CD8, CD25, CD68, HLA-DR) [Fig.1, 2, 3, 4, 5]. No inflammatory cells were seen and HLA class II molecules were not detected on the surface of epithelial, occasionally on stromal and none on basal or suprabasal levels. The prospective study is a case series presentation to demonstrate the differential expression of immunoregulatory markers, if any, for better clinical management.

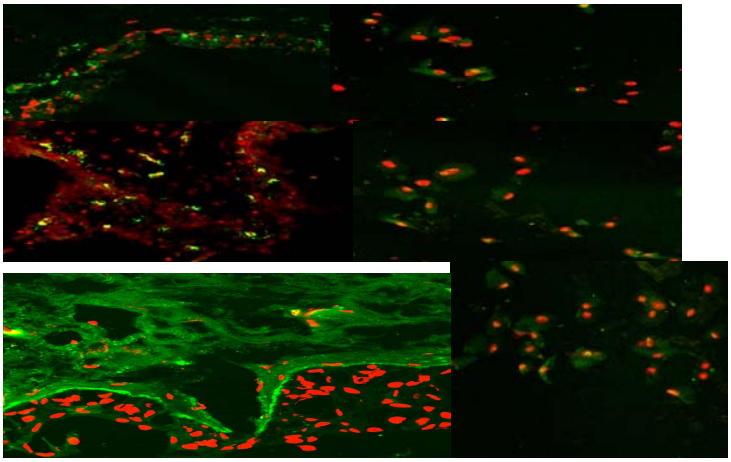

Figure 1: HLA-DR Is Moderately Present in Limbal Epithelium and Stroma in Normal and Cultivated Limbal Epithelium

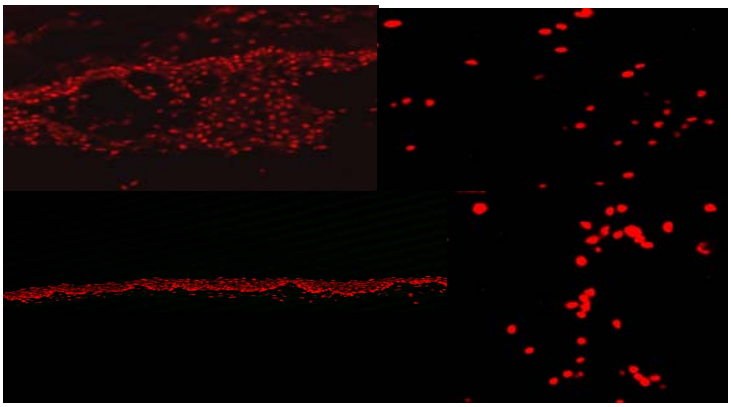

Figure 2: CD4 Is Absent in Normal Limbal and Cultivated Limbal Epithelium

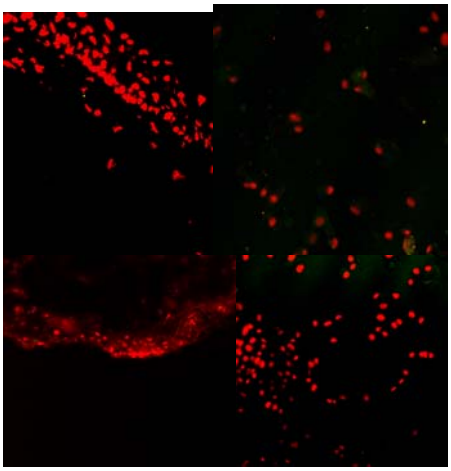

Figure 3: CD8 Is Absent in Normal and Cultivated Limbal Epithelium

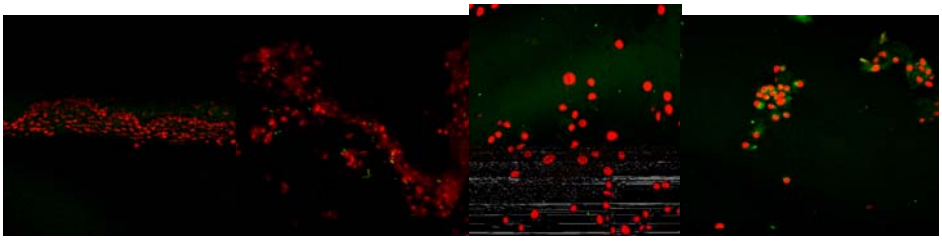

Figure 4: CD25 Is Absent in Normal and Cultivated Limbal Epithelium

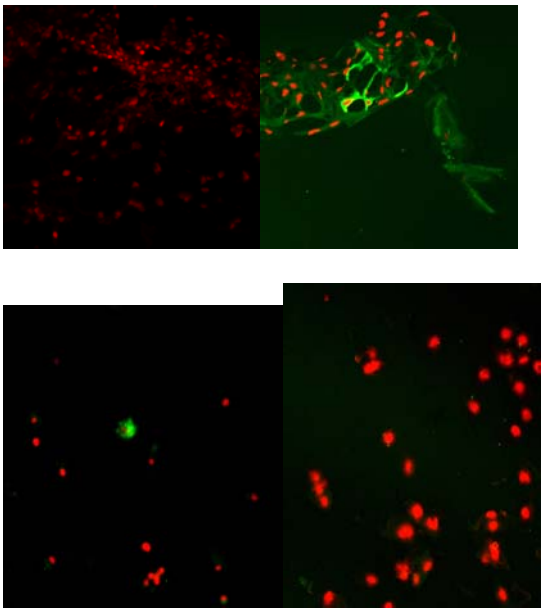

Figure 5: CD 6 Is Present Occasionally in Normal and Cultivated Limbal Epithelium

DISCUSSION

Immunological rejection remains the most common cause of corneal limbal allograft failure. The cascade of events that follows on external stimuli releasing proinflammatory cytokines leading to upregulation of MHC-II expression and Co-stimulatory markers are detrimental to allograft survival. For permanent survival of cultivated limbal epithelium sheets, it is important to suppress postoperative inflammation, as well as immunological rejection[6,7]. Donor derived antigen presenting cells (APCs), dendritic cells, ocular Langerhans cells may prove to be a major impediment in ocular surface reconstruction. Furthermore, APCs in the corneal limbus and corneal epithelial stem cells, when transplanted together may incite intense and frequent rejection. It is also reported by the other groups that the resident corneal Langerhans cells, DCs present in the anterior corneal stroma, both in humans and mice undergo maturation and function as APCs, during inflammation[8]. On the contrary, very few or almost no APCs were present in the cultivated sheets as demonstrated by the others[8,9]. The culture medium thus supports the growth of epithelial cells alone and not the bone marrow derived cells. Hence, the possible risk of donor derived APCs being transplanted to recipient may be minimized by the cultivated epithelial sheets[10].

Amniotic membrane, which has recently been shown to be an immune-privilege-like tissue, may possess anti-inflammatory properties, including a) immunoregulatory secretion factors, such as IL-1 receptor antagonist, and b) HLA-G and Fas ligand expression in the mesenchymal cells of amniotic stroma. Such properties may result in the reduction of corneal stromal inflammation and ulceration in HSV-1 keratitis models, and in the suppression of bFGF-induced corneal neovascularization by the supernatant of

amniotic membrane. These properties have an effect on surrounding cells, such as suppression of IL-1 alpha and IL-1 beta gene expression, but upregulation of IL-1 receptor antagonists, in the case of cultured human corneal and limbal epithelial cells on amniotic membrane[8,9].

The other immunoregulatory markers such as CD4, CD8, CD25 (IL-2 receptor) being negative before and after cultivation, amply demonstrates the safety profile in transplanting the cultivated limbal allograft without the risk of T-cell alloresponses. In conclusion, the presence of limbal stroma in direct limbal transplantation in allogenic cases could be an additional source of immunogenic stimulus, which is obviated in cultivated epithelial cells.

CONCLUSION

The present research aimed at studying the differential expression of immunoregulatory markers in normal cadaver limbus and ex-vivo expanded limbal epithelial cells on human amniotic membrane. The result showed that markers of immune activation such as CD4, CD8 were negative in normal and ex vivo expanded limbal epithelium, while CD25, CD68 were present occasionally. HLA-DR which encodes the MHC-II expression is present occasionally in the normal epithelium while moderately in the stroma. The cultivated limbal epithelium remained predominantly negative for these markers. This is in support of the earlier studies that the culture system alleviates the chance of graft rejection by way of removing the trigger factors. Therefore, the evaluation of the allograft prior to limbal stem cell transplantation would enhance the prognostic value in formulating the immunosuppressive therapy protocol for the patients.

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