CHAPTER 5

DISCUSSION

Cultivation of fresh limbal and oral mucosal tissues

Generation of cultivated epithelial sheet from either limbal or oral mucosal specimens, each laboratories have their own technique with the variation in culture methods. However, most techniques base on the same principal. In this study, the cultured limbal stem cells and oral mucosal epithelial cells were successfully grown on a denuded amniotic membrane by using explants culture which was a simple technique. The de-epithelialized HAM was used in this study because it allows the corneal epithelial cells to grow uniformly and better adhesive to the underlying membrane than HAM with intact epithelium (Koizumi et al., 2000b). HAM was used as a culture substrate because of its ability to reduce inflammation, vascularization, adhesions and scaring and also enhances wound healing and epithelialization. Furthermore, graft rejection after transplantation has not been occurred as the amniotic membrane expresses incomplete HLA-A, B, C, and DR antigens 2 (Akle et al., 1981; Kim and Tseng, 1995; Lee and Tseng, 1997; Tseng et al., 1998). Co-culture with MMC- treated 3T3 fibroblasts was performed to preserve the stem cell population in culture sheet (Balasubramanian et al., 2008). Moreover, air-lifting method has been used to promote the epithelial stratification and improve intercellular junctions (Ban et al., 2003).

Using previously described culture technique, the epithelial sheets were able to generate from both fresh and cryopreserved limbal and oral mucosal biopsies on denuded HAM with the use of MMC-treated 3T3 cell line. Cultured epithelial sheets generated from fresh limbal and oral mucosal tissues were no difference in morphology which had approximately two to five layers that was similar to previous studies (Koizumi et al., 2000a; Nakamura et al., 2003; Chen et al., 2009; Ma et al., 2009). Cuboidal basal cells attached well to the underlying HAM and the superficial cells were flat. Cultured oral mucosal epithelial sheet has no papillary structure under the epithelium unlike native oral mucosa character. Both limbal and oral mucosa cultures showed the phenotype of non-keratinized stratified squamous epithelium. No goblet cell was observed in all cultured sheets suggested that there were no conjunctival tissue grew in the cultivated epithelial sheet. The morphology of all cultures was also resembled to normal corneal epithelium.

Immunohistochemistry was performed to examine the stem cell population by studying the expression of p63, a transcription factor that found in nucleus. In this study, distribution pattern of p63 positive cells in culture sheets were found in the basal layer and some area in the intermediate layer but not in the superficial layer demonstrated that there were stem cells among epithelial cells. The presence of p63 positive cells in culture sheet might have benefit in long term graft survival since cells are able to proliferate after transplantation.

This study also examined the expression of differentiated corneal epithelial cell markers including K3 and Cx43. In all epithelial sheets, K3 was stained positive in cytoplasm of cells located in superficial and intermediate layer but not in basal layer. The inversely related result showed that the cells in basal layer and some area in

intermediated layer were p63 positive and K3 negative indicated that these cells were most likely the stem cells. This characteristic was also related to native limbal epithelium which the p63 protein is highly present in basal layer of limbal epithelium but not in corneal epithelium whereas K3 is found in all layers of the differentiated corneal epithelium but absent in the basal layer (Schermer et al., 1986; Pellegrini et al., 2001). The number of p63 and K3 positive cells in cultivated oral mucosal epithelia were lower than cultivated sheets from limbal tissues indicated that the proliferation and differentiation potential of oral mucosal was inferior to limbal cultivation. This result was consistent with the study of Hayashida and colleagues. They reported that the colony-forming efficiency of the primary oral epithelial cells was approximately 15% lower than that of the primary rabbit limbal stem cells (Hayashida et al., 2005). Moreover, the difference in thickness of these two epithelia might also influence; the oral mucosal epithelium is relative thicker than limbal epithelium resulting in the lower ability of resided stem cells to grow out from the explants edge.

The Cx43, a transmembrane protein in which six molecules of them forms the communicating cell to cell junction called gap junction, is a marker of the differentiated corneal epithelium (Kumar and Gilula, 1996). The result showed that Cx43 positive cells were scattered throughout the epithelial sheets indicated that there were communications between these cells via gap junction that played an important role in development and differentiation. However, cell population within the cultured epithelial sheet also contained the Cx43 negative cells demonstrated that these cells had no communications and consistent with the immunological finding of p63 positive cells in culture sheet. The absent of cell to cell communication reflected the

need of cell in creating the suitable environment of stem cells niche for maintaining their stemness that was the property of stem cells (Matic et al., 1997).

Although, some inferior characters were found in cultivated oral mucosal tissue but the cell morphology and immunohistological staining presented in similar pattern with cultivated limbal tissues. This study demonstrated that oral mucosa can be one of the possible choices in producing an autologous alternative epithelium for transplantation in patients with LSCD instead of using allogeneic limbal tissues.

Eye Enucleations

In this study, both cultivated limbal stem cell transplantation and cultivated oral mucosal epithelial transplantation were successful in ocular surface reconstruction of rabbit's eye with alkaline burn. The important factor that supported this successful grafting was the containing of stem cells in cultivated epithelial sheet. These stem cells were expected to proliferate after transplantation although the fate of expanded stem cells still unclear. Two months after transplantation, the eyes were obtained for histological and immunohistochemical examinations. Histology of all reconstructed eyes was most likely the native ocular epithelium though different characters were found. Few goblet cells were sparsely observed in limbal epithelium of rabbit No. 3 and 4 suggested that they still had some conjunctival epithelial tissue ingrowths or conjunctivalization into limbal area. Nevertheless, goblet cell was not found in all corneal epithelia. After COMET in rabbit No. 4, 5, 6, stromal vascularization was found in cornea of rabbit No. 4. Cultured oral epithelial cells were reported to have greater angiogenic potential than cultured corneal epithelial cells because these cells decreased expression of anti-angiogenic factors and showed capability in induction of

corneal neovascularization (Sekiyama et al., 2006; Kanayama et al., 2007). However, the eye of rabbit No. 5 and 6 were similar to normal corneal and limbal epithelia. Moreover, some areas of corneal epithelium showed increasing of epithelial stratification up to about 10 layers without underlying papillary structure which were similar to previously studies (Nakamura et al., 2007; Chen et al., 2009). Nakamura and colleagues described that the present of epithelial thickening affected by subepithelial environment including the stromal vascularization may affect the epithelial phenotype (Nakamura et al., 2007). In this study, stromal vascularization was found in rabbit No.4 which had the thickening of epithelium. Nonetheless, the expression patterns of p63, K3 and Cx43 in rabbit No. 4 were similar with cultured sheets of others rabbit. These results indicated that the difference in stratification between rabbits did not influence the marker expression.

Immunohistochemical staining was also performed to examine the expression of p63, K3 and Cx43 within limbal and corneal epithelia of enucleated eyes. In normal ocular epithelium, p63 was generally expressed in basal layer of limbal epithelium but not corneal epithelium. This protein also presented in native oral mucosal epithelium. In this study, the enucleated eyes showed that p63 positive cells were not only found in limbal region but also in corneal epithelium. Study of Chen and colleague reported similar result that the expression of p63 was presented in transplanted corneal specimens and not limited to the basal layer as well as in the thickening epithelium (Chen et al., 2009). With little exception, in this study, corneal epithelium of rabbit No. 5 and limbal epithelia of rabbit No. 4 and 6 showed p63 negative. In general, limbal stem cells are believed to reside in the basal limbal epithelium where undulating surface called the palisades of Vogt is presented (Davanger and Evenson,

1971). The deep undulating obtained the best niche for limbal stem cells to protect them from shearing forces (Gipson, 1989). Moreover, the palisades are predominantly found in the superior and inferior limbus where can be protected by the eye lids (Shortt et al., 2007). So the negative staining of p63 that found in this study might be due to the tissue did not section at this area. Even the negative staining of p63 were found, there was no eye that showed negative result in both limbal and corneal regions. This result indicated that in all transplanted ocular epithelia contained stem cells that can lead to the possible success of graft survival after transplantation. However, further study are needed to find the source of these stem cells whether they come from the cultured epithelial sheet, the remaining LSCs after alkaline burn or the hibernated cells in ectopic site. Kawasaki and colleagues reported that the expression of corneal specific keratin, K12, was found in clusters in bulbar conjunctival epithelium underlying with p63 positive cells that is the character of corneal epithelium (Kawasaki et al., 2006).

Cornea-specific keratin 3 or K3 is reliable and mostly used as the corneal differentiation marker. In native tissue, K3 was strongly expressed in corneal epithelium, weak expressed in suprabasal layer of limbal epithelium and in suprabasal layer of oral mucosal epithelium. In this study, the expression of K3 in limbus was weak or none staining indicated that the cells in this area were less differentiated. Whereas strong expression of K3 was found especially in suprabasal layer of corneal epithelium. Cx43 expression in normal tissue was in superficial corneal epithelium and suprabasal layer of oral mucosal epithelium whereas in limbal epithelium was Cx43 negative. Transplanted eye in this study showed that Cx43 expression was observed in corneal epithelium of all transplanted eye similar to normal cornea. In all

limbal regions showed weakly staining of Cx43 that presented in the superficial layers. From these results indicated that the expression pattern of both K3 and Cx43, the differentiated cell markers, was very similar to native ocular epithelium although there were different degrees of differentiation in superficial cells of limbal epithelium.

Cryopreservation by vitrification

This study examined the suitable CPA formula for usage in tissues and cells vitrifications. The first vitrification solution was opaque while the 2nd and 3rd solutions were transparent suggested that visible ice crystal formation occurred in the 1st solution. Therefore, the 1st solution was excluded because it may cause cell damage and affect cell growth. Although, 10% DMSO is the most widely used for maintaining the viability of various types of cells (Schwartz, 1986; Temmerman et al., 2008; Yeh et al., 2008; Qu et al., 2009), the ice crystals formation still decrease post thawing viability (Mazur, 1984; Pegg, 2002).

The tissue that cryopreserved by the 2nd formula showed no cell outgrowing even there was no visible ice crystal. Many factors affect the cell viability not only ice crystal formation but also permeability and toxicity of CPAs. Permeability of CPAs depends on theirs molecular weight that the higher molecular weight has the lower cell permeability. The 2nd and 3rd formulas composed of 25% glycerol, 25% propylene glycol and 25% DMSO, 25% propylene glycol respectively. Glycerol (MW 92.09) in the 2nd formula has higher molecular weight than DMSO (MW 78.13) in the 3rd formula. During cryopreservation, when the freezing medium is added, the osmotic gradient leads to rapid efflux of water and influx of CPA. If both of these processes were not balance due to the low permeability of the used CPA, the cells

would be shrinkage. The poor permeability of glycerol may be one of the important factors that lead to cell damaged and retardation of growth. This study indicated that DMSO is effective in vitrification of limbal and oral mucosal biopsies than glycerol with the same concentration of propylene glycol. In 1990, Rich and Armitage have studied the effect of propylene glycol on the structure and function of rabbit corneal endothelium. They suggested that 25% propylene glycol was a potential component of a vitrification solution for corneas (Rich and Armitage, 1990). From these reasons, the 3rd CPA formula was chosen.

After CPAs have been chosen, the another two parts of study were conducted. In the first part, limbal and oral mucosal tissues were immediately cryopreserved after biopsy. After two months of cryopreservation, the tissues were thawed and cultured. The epithelial cells expanded from all three limbal and three oral mucosal tissues were able to form epithelial sheets within 3 weeks. This results showed that the CPA, 25% DMSO, 25% propylene glycol in DMEM containing 20% FBS, has capacity in preserving limbal and oral mucosal biopsies in liquid nitrogen. Similarity of the cultured cells morphology and markers expression in fresh and cryopreserved tissue cultures was observed. The results suggested that the 3rd vitrification solution not only have capacity in preserving but also maintaining proliferation potential and phenotypes of the tissue biopsies.

In the later part, cell viability after vitrification was higher in oral mucosal epithelial cells (70.57 ± 14.71%) than corneal epithelial cells (59.99 ± 5.89%). This result suggested that this CPA formula might affect these two types of cells in difference way that the mechanism needed further study.

In vitrification process, the period of time between mixing the cell suspension with CPA and cooling process is called equilibration time. It is the time for allowing intracellular water to leave the cell and CPA penetration into the cell. Normally, the cell that has low intracellular water needs long equilibration time. For example, many studies used longer equilibration time in freezing sperm than oocyte due to water in sperm was less than oocyte (Nowshari et al., 1994; Iaffaldano et al., 2012). However, the equilibration time also depended on type and concentration of CPA so each researcher needed to find their own suitable condition. In this study, cultured cells from oral mucosa and limbal tissues were cryopreserved under the same condition. Therefore, the different intracellular water between these two types of cells might be the factor that influenced the cell viability. Corneal epithelial cells seem to have lower intracellular water due to a high degree of dehydration was found in the cornea so it held less intracellular water than it could (Agarwal, 1969). In this study, rapid cooling was also needed to reduce toxic effect of the high concentration of CPA so the equilibration time was only within 1 minute that this time might not long enough for corneal epithelial cells to complete equilibration and leading to the lower cell viability. Further study may need to find the suitable equilibration time for increasing cell viability in cryopreservation of corneal epithelial cells. Thawing process and CPA unloading process are also important in cryopreservation which entire CPA must remove from cells. Three steps thawing by washing 3 times with DMEM for CPA elimination from cells might not sufficient for oral mucosal epithelial cells so the remaining of CPA might cause cell toxicity and unable to generated the cultured sheet. To solve this problem, hypertonic sucrose were suggested to add in

extracellular solute at first step of thawing for complete efflux of CPA (Ozkavukcu and Erdemli, 2002).

Moreover, from this study, the cryopreserved tissues showed better result in cultivation after thawing than cryopreserved cells indicated that this CPA formula had potential in cryopreservation of tissues than cells. This result might be due to the cells in tissue were closer to each other than single cells that could promote the cell-to-cell communication when growth after thawing. However, cell signaling is a complex system and the survival after thawing is still crucial and need further study.