Use of PLGA scaffold for mucociliary epithelium transfer in airway reconstruction: a preliminary study

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ABSTRACT

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Conclusion. A PLGA biodegradable membrane can be used as a scaffold for mucociliary epithelium transfer. *Objectives.* The aim of this study was to examine the usefulness of the PLGA membrane as a biodegradable scaffold for mucociliary epithelium transfer in order for it to be used as a substitute for a skin graft for restoring mucosal defects in the airway. *Methods.* A PLGA biodegradable membrane was synthesized using the immersion precipitation method, and morphologic characterization was carried out using scanning electron microscopy (SEM). The degradation test was performed by soaking the PLGA membrane in a culture medium, and the morphological changes were observed by SEM. Human nasal basal epithelial (HNBE) cells were cultured on the newly synthesized PLGA membrane, and the morphological changes were analyzed using SEM. The MUC5AC and MUC8 mRNA levels were analyzed by RT-PCR. *Results.* The PLGA membrane for the mucociliary epithelium transfer was successfully fabricated. It had a 24 mm diameter,

a 50 μm thickness, and many pores with diameters of approximately 3 μm. The PLGA membrane began to degrade from 7 days after it was soaked in the culture medium. It rapidly degraded from 3 weeks and severe destruction of the pore structure was noted from 4 to 6 weeks of soaking. The HNBE cells were well differentiated into the mucociliary epithelium on the PLGA membrane both phenotypically and genotypically.

Key words : *cultured nasal epithelium, mucociliary epithelium, graft, PLGA, differentiation*

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I. INTRODUCTION

Some cases of upper respiratory tract malignancies, such as sinonasal, laryngeal and tracheal malignancies, require a complex reconstruction of the defects after the primary lesion has been removed. Such surgical defects have been successfully reconstructed using three-dimensional free-flaps.[1,2] The airway side of the free flap is usually grafted with skin. In addition, a skin graft or an epithelial equivalent[3] can be applied to the denuded area of the trachea after removing the lesion, particularly in some cases of tracheal stenosis. To date, skin has been used most widely to reconstruct the luminal side of the airway[4,5] because it is the most effective graft material.

However, there are well-known problems associated with the use of skin, especially when the squamous epithelium is used to as a substitute

for the mucociliary epithelium of the airway. These problems include mucus stagnation due to the absence of mucociliary clearance, graft constriction, desquamation, the re-growth of hair, and an unpleasant odor.[6,7] On the other hand, theoretically, these problems would not exist if the ciliated columnar epithelium were used as the graft material. From this point of view, it would be most physiological for mucociliary differentiation to cover the defect with the corresponding mucosa when reconstructing a mucosal defect. Nevertheless, finding an appropriate donor site from which a sufficient amount of mucociliary epithelium can be obtained to cover the defect is almost impossible.

Our laboratory has set up the normal human nasal epithelial cell culture systems.[8] However, it is difficult to obtain an epithelial cell sheet that is easy to handle, even if the mucociliary epithelial cells are fully differentiated. This is because the tight junction, desmosome, and the gap junction in the mucociliary epithelium are too weak to achieve the required mechanical strength to support the structural integrity.

One of the aims of this study was to use a poly-DL-lactic-co-glycolic acid (PLGA) polymer membrane, which is one of the most popular biodegradable membranes for transferring various tissues. To date, the PLGA biodegradable membrane has been used as a scaffold for various cell culture systems such as osteocytes[9] and chondrocytes.[10] PLGA is a polymer compound that has been used in many different experiments associated with tissue engineering owing to its ability to degrade when it is placed in contact with water. It was speculated that if human nasal basal epithelial (HNBE) cells (pluripotential stem cells) could be cultured

and differentiated into the mucociliary epithelium on a PLGA biodegradable membrane, it would be possible to develop a graft material as a substitute for a skin graft, which is easy to suture and manipulate by using the mechanical strength of PLGA.

This study also examined usefulness of a PLGA membrane as a biodegradable scaffold for mucociliary epithelium transfer. It is believed that this will be the first step in determining if autologously cultured nasal epithelial cells can be used as a skin substitute for restoring mucosal defects in the airway.

II. MATERIALS AND METHODS

Materials

The glycolide and D,L-lactide were purchased from Boehringer Ingelheim (Germany). They were recrystallized from ethyl acetate and dried under vacuum prior to use. Stannous octoate and poly-D,L-lactide-co-glycolic acid (PLGA) (molecular weight 90,000~120,000 kDa), whose copolymer ratio of D,L-lactide and glycolide is 75:25, were purchased from the Sigma Chemical Co. Methylene chloride, *N*,*N*-dimethylformamide (DMF) (Duksan Pure Chemicals, Ansan, Korea), *N*,*N*-dimethylacetamide (DMAc) and *N*-methyl-2-pyrrolidone (NMP) (Junsei Chemical Co., Japan) were used as solvents. Distilled water, methanol, and ethanol (Duksan Pure Chemicals) were used as the non-solvents. Distilled water was of Milli-Q quality (Millipore, Bedford, MD, USA). All organic solvents were either high performance liquid

chromatography (HPLC) grade or American Chemical Society analytical grade reagents.

Synthesis of biodegradable polymer

- Polymerization of PLGA

Ring opening polymerization was carried out in a Pyrex ampoule containing a Teflon-coated stirring bar. The D,L-lactide and glycolide monomer were placed in the Pyrex ampoule at a molar ratio of 75:25. Stannous octoate (dissolved in toluene) was added at a specified concentration. The ampoule was evacuated using a vacuum pump and was sealed by a torch. The ampoule was heated in an oil bath at 130°C for 12 h. The ampoule was broken after the reaction was complete. The resulting polymers were purified by dissolving the reaction mixture in methylene chloride and precipitated into excess methanol. The purified polymers were dried under vacuum.

- Preparation of biodegradable porous membrane by immersion precipitation method

The biodegradable porous membranes were prepared using the immersion precipitation method. Various polymer solutions were prepared by dissolving the synthesized and purchased PLGA in methylene chloride (co-solvent) and 10% v/v solvent such as *N*,*N*-dimethylformamide (DMF), *N*,*N*-dimethylacetamide (DMAc) and *N*-methyl-2-pyrrolidone (NMP) at a concentration of 8% w/v. This polymer solution was cast on a glass

mold, and then dried for 12 h at room temperature to evaporate co-solvent. The semi-solidified samples were immersed in a coagulation bath containing an excess amount of non-solvent such as DDI water, methanol, and ethanol for 5 h. The temperature of the coagulation bath containing the non-solvent was kept at approximately 22°C. The membranes were then dried for 10 h at room temperature.

Morphologic examination and degradation test of PLGA membrane in culture media

The PLGA membrane was observed using a scanning electron microscopy (SEM, H-800, Hitachi, Japan). The degradation test was performed using the PLGA membrane soaked with a culture medium in the incubator, and the SEM observations were performed after 0, 1, 2, 3, 4, and 6 weeks.

HNBE cells culture on PLGA membrane

The passage-2 HNBE cells were plated on a PLGA membrane (2 x 10^5 cells/well) placed on the six-well Transwell-clear (Costar Co., Cambridge, MA, USA) culture insert. The cells were grown using a submerged culture technique for 9 days and an air-liquid interface (ALI) was created. The SEM observations were performed 2, 7, 14, and 21 days after creating the ALI.

RT-PCR for MUC5AC and MUC8

The oligonucleotide primers for the mucous differentiation marker, the mucin gene 5AC (MUC5AC),[8] and the ciliary differentiation marker, mucin gene 8 (MUC8),[11] were designed according to the published sequences.[8] The oligonucleotide amplimers for β2 microglobulin (β2M; Clontech Laboratories Inc., Palo Alto, CA, USA) were used as the control gene for RT-PCR; they generated a 335 bp PCR fragment. RT-PCRs were performed using a Perkin Elmer Cetus DNA Thermal Cycler according to the manufacturer's recommendations. Annealing was performed for 1 min at 55°C for MUC8 (PCR product size = 239 bp), and at 60°C for MUC5AC (PCR product size = 680 bp) and β2M. Extension was performed at 72°C for 1 min. Comparative kinetic analysis was used to compare the mRNA levels for each gene in each set of culture conditions. The PCR products were separated by electrophoresis on a 2% Seakem agarose gel (FMC, Rockland, ME, USA) containing 50 ng/ml ethidium bromide and were photographed on Polaroid Type 55 film. The same experiments were repeated three times.

III. RESULTS

Synthesis of PLGA membrane and its degradation in culture media

The PLGA membrane for the mucociliary epithelium transfer had a 24 mm diameter and a 50 μm thickness (Figure 1A). This membrane was designed for fitting in a six-well Transwell-clear culture insert. SEM showed many pores in the PLGA membrane (Figure 1B), and their diameters were approximately 3 μm (Figure 1C).

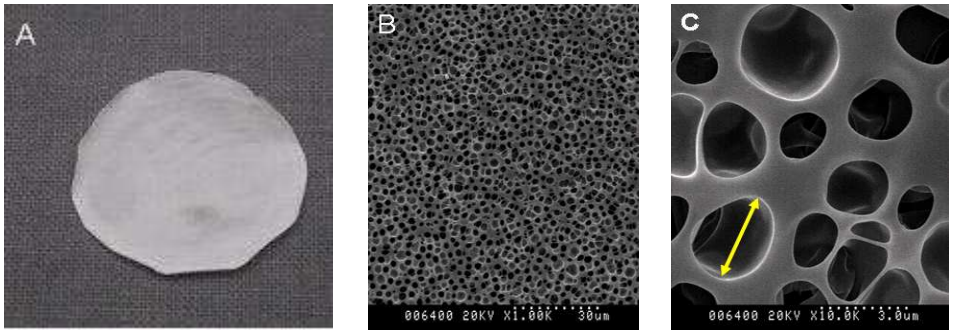

Figure 1. Morphological and SEM observations of the PLGA membrane. (A) A 24 mm diameter, 50 µm thickness PLGA membrane was designed to fit in an air-liquid interface culture insert. (B) Many pores were observed (x 1000) and (C) pore diameter was approximately 3 µm (arrow, x 10000).

In the degradation test (Figure 2), the PLGA membrane began to degrade
from 7 days (Figure 2B) after it was soaked in the culture medium. It rapidly degraded from 3 weeks (Figure 2D) and severe destruction of the pore structure was noted from 4 to 6 weeks of soaking (Figure 2E, F). In addition, it usually takes 3 weeks for the mucociliary differentiation of the airway basal epithelial cells in this culture system. These results suggest that the PLGA membrane fabricated in this study is suitable for airway epithelium transfer.

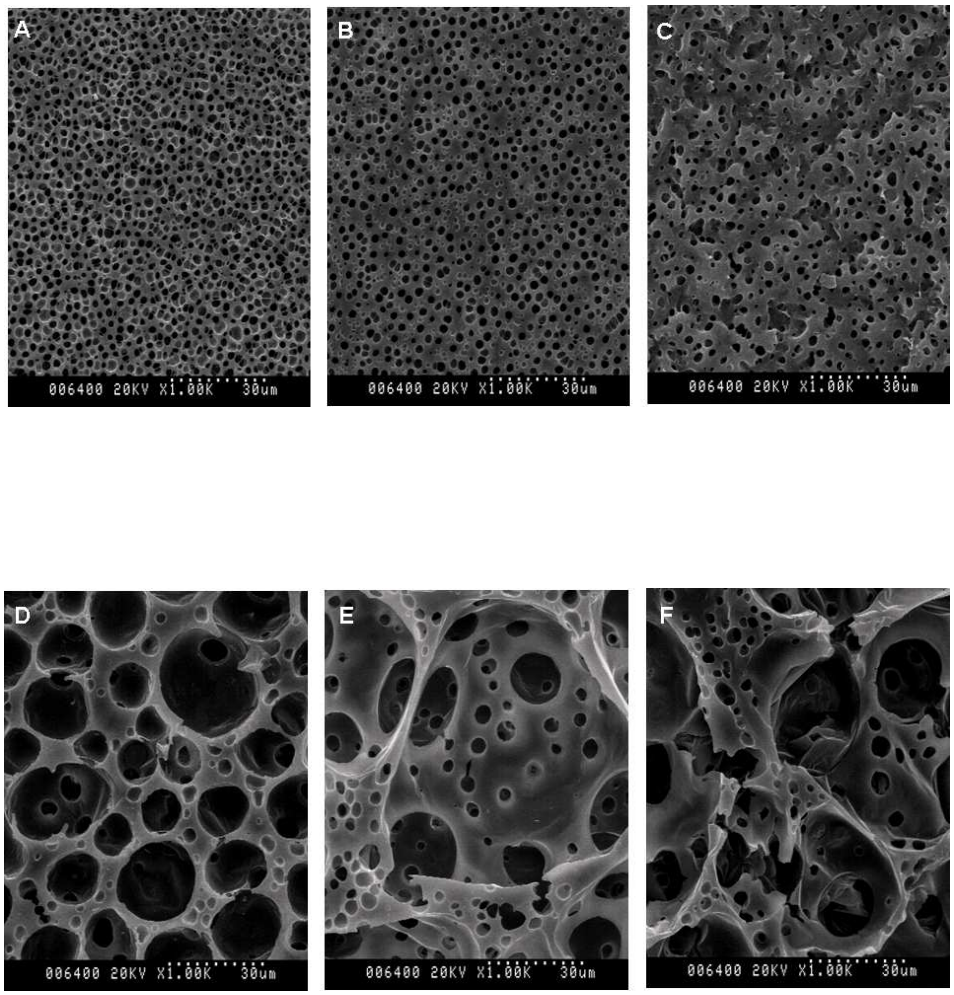

Figure 2. Degradation test for the PLGA biodegradable membrane. SEM observations of the PLGA membrane after soaking in a culture media for 0 (A), 1 (B), 2 (C), 3 (D), 4(E), or 6 weeks (F) (x 1000).

Differentiation of airway basal epithelial cells into mucociliary epithelium on PLGA membrane

Mucociliary differentiation was induced in the presence of $5x10^{-7}$ M

retinoic acid using the PLGA membrane and passage-2 HNBE cells. The HNBE cells were grown on the PLGA membrane for 3 weeks after they reached confluence. HNBE cells with microvilli on the surface were noted 2 days after confluence, and the cells were found to be loosely attached together (Figure 3A). On the 7th day after confluence, the cells were tightly adherent and the cell size was slightly enlarged (Figure 3B). Fourteen days after confluence, some of the cells differentiated into ciliated cells as the proliferation of the cells was decreased due to contact inhibition (Figure 3C). Twenty-one days after the cells reached confluence, most of the cells were of a similar size and differentiated into ciliated cells (Figure 3D). These results show that HNBE cells can differentiate into the mucociliary epithelium on the PLGA membrane.

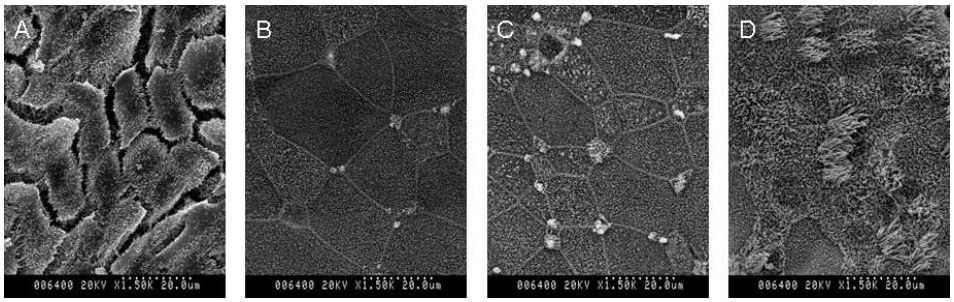

Figure 3. Human nasal basal epithelial (HNBE) cells cultured on a PLGA biodegradable membrane. Mucociliary differentiation of HNBE cells was observed 2 (A), 7 (B), 14 (C) and 21 days (D) after ALI creation (x 1500). Full differentiation was achieved 21 days after air-liquid interface (ALI) creation.

Expression of mucous and ciliary differentiation marker during mucociliary differentiation

The expression levels of the MUC5AC, which is a mucous differentiation marker, and MUC8, a ciliary differentiation marker, were investigated. The MUC5AC gene expression level increased from 7 days after ALI creation, which did not show significant increment up to 21 days. The MUC8 gene expression level increased as a function of differentiation (Figure 4). Mucous differentiation and ciliary differentiation of the basal epithelial cells occurred simultaneously on the PLGA membrane. It was confirmed that the cultured epithelium is the mucociliary epithelium at the genetic level.

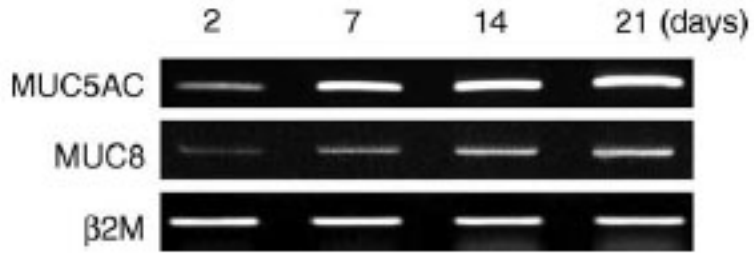

Figure 4. MUC5AC and MUC8 mRNA expression as a function of differentiation. The expression levels of MUC5AC, mucous differentiation marker, increased from 7 days after air-liquid interface (ALI) creation, which did not show significant increment up to 21 days. The expression levels of MUC8, ciliary differentiation marker, increased as a function of differentiation.

IV. DISCUSSION

Since it is believed to be physiologically proper to use a ciliated columnar epithelium for reconstructing the luminal side of the airway, we previously attempted to use cultured mucociliary epithelium directly for an airway luminal reconstruction. Using this concept, two different methods were attempted, but both failing. One involved seeding the dissociated epithelial cells after inducing the mucociliary epithelium directly onto the defect site of the airway mucosa. The problems encountered using this method were that it was difficult to retain the cellular polarity of the epithelial cells when seeding the cells and the seeded cells tended to easily exfoliate from the defect site. The other method involved inducing the fully-differentiated mucociliary epithelium with nasal basal epithelial cells in vitro and then attempting to graft it directly on the defect site of the airway mucosa. This effort was also unsuccessful. An epithelial cell sheet could not be made that could be manipulated easily by a surgeon because the mucociliary epithelium does not have a strong intercellular tight junction, which is unlike the squamous epithelium. Accordingly, it was believed that if an appropriate scaffold was used to support the mechanical strength of the fully differentiated mucociliary epithelium, cultured mucociliary epithelium might be used as a substitute for skin in an airway luminal reconstruction.

Therefore, this study examined the usefulness of a PLGA biodegradable membrane for mucociliary epithelium transfer. PLGA is commonly used

as an absorbable suture material[12] and in drug-delivery devices.[13] A PLGA biodegradable membrane has also been used as a scaffold for various cell culture systems, such as fibroblasts,[14,15] uroepithelium,[16,17] bladder smooth muscle cells,[18] osteocyte,[9] neural stem cells,[19] chondrocyte,[10] retinal pigment epithelium,[20,21] corneal endothelial cells,[21] salivary epithelial cells,[22] liver cells,[23] and adipose cells.[24] However, there have been no reports of airway epithelial cells being successfully cultured on a PLGA membrane.

The PLGA membrane used in this study has many pores, and the pore size was approximately 3 μm in diameter (Figure 1C). An attempt was made to determine the most suitable PLGA conditions in terms of the pore size and the speed of degradation. There are two factors that need to be considered when deciding the pore size. One is that the size of the pore should be sufficiently large to supply a sufficient quantity of nutrients from the culture medium into the basal surface of the cells. The other factor is that the size of the pore should be smaller than the basal epithelial cell, so that the cells can grow over the PLGA membrane and not fall into the pores. The degradation rate depends on the PLGA concentration and the membrane thickness. After carrying out various experiments, it was decided to use an 8%, 50 μm thickness PLGA membrane because severe degradation occurred 4 weeks later.

This study then examined whether or not HNBE cells can differentiate into the mucociliary epithelium on a PLGA membrane in this culture system. SEM showed that HNBE cells differentiated into the mucociliary epithelium on the PLGA membrane without any morphological damage.

However, the mucociliary differentiation of the HNBE cells on the PLGA membrane occurred slightly later than when the PLGA membrane was not used.[25] The reason for this is believed to be that the PLGA membrane may slightly hinder the supply of nutrients to the cells. This study investigated the MUC5AC and MUC8 gene expression level as a function of differentiation. It was found that the MUC5AC gene expression level increased from 7 days after ALI creation and did not show significant increment thereafter, and the MUC8 gene expression level increased as a function of differentiation. These results concur with our previous report.[25] This suggests that HNBE cells can differentiate into mucociliary epithelium on a PLGA membrane.

V. CONCLUSION

The mucociliary epithelium was successfully induced from HNBE cells on a PLGA membrane in this culture system. This suggests that a PLGA membrane can be used as a scaffold for mucociliary epithelium transfer for airway luminal reconstruction.

REFERENCES

1. Piantanida R, Roselli R, Pellini R, Ferrario F, Boschini P, Spriano G. Reconstruction of major orbital-maxillary defects with free latissimus dorsi myocutaneous flap. Facial Plastic Surg 1999;15:297-302.
2. Pribaz JJ, Morris DJ, Mulliken JB. Three-dimensional folded free-flap reconstruction of complex facial defects using intraoperative modeling. Plast Reconstr Surg 199493:285-293.
3. Duff BE, Wenig BL, Applebaum EL, Yeates DB, Wenig BM, Holinger LD. Tracheal reconstruction using an epithelial equivalent. Laryngoscope 1994;104:409-414.
4. Andhoga MA, Wilson GR, McLaughlin W, McLean NR. Split-thickness skin grafted stent for upper airway patency after medial maxillectomy. Br J Oral Maxillofac Surg 1993;31:385-387.
5. Eliachar I, Sebek BA, Levine S, Tucker HM. Histologic changes in skin implanted into the larynx and trachea by myocutaneous flap reconstruction. Otolaryngol Head Neck Surg 1985;93:754-758.
6. Conley JJ. Regional skin flaps in partial laryngectomy. Laryngoscope 1975;85:942-949.
7. Toohill RJ. Autologous graft reconstruction of the larynx and upper trachea. Otolaryngol Clin North Am 1979;12:909-917.
8. Yoon JH, Kim KS, Kim SS, Lee JG, Park IY. Secretory differentiation of serially passaged normal human nasal epithelial cells by retinoic acid: Expression of mucin and lysozyme. Ann Otol Rhinol Laryngol 2000;109:594-601.

9. Abukawa H, Terai H, Hannouche D, Vacanti JP, Kaban LB, Troulis MJ. Formation of a mandibular condyle in vitro by tissue engineering. J Oral Maxillofac Surg 2003;61:94-100.
10. Rahman MS, Tsuchiya T. Enhancement of chondrogenic differentiation of human articular chondrocytes by biodegradable polymers. Tissue Eng 2001;7:781-790.
11. Kim CH, Kim HJ, Song KS, Seong JK, Kim KS, Lee JG, et al. MUC8 as a ciliated cell marker in human nasal epithelium. Acta Otolaryngol 2005;125:76-81.
12. Benicewicz BC, Hopper PK. Polymers for absorbable surgical sutures. J Bioact Compat Polym1991;6:64-94.
13. Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices. Biomaterials 2000;21:2475-2490.
14. Hansbrough JF, Cooper ML, Cohen R, Cohen R, Bartel RL, Naughton G. Evaluation of biodegradable matrix containing cultured human fibroblasts as a dermal replacement beneath meshed skin grafts on athymic mice. Surgery 1992;111:438-446.
15. Yang J, Shi G, Bei J, Wang S, Cao Y, Shang Q, et al. Fabrication and surface modification of macroporous poly(L-lactic acid) and poly(L-lactic-co-glycolic acid) (70/30) cell scaffolds for human skin fibroblast cell culture. J Biomed Mater Res 2002;62:438-446.
16. Merguerian P, Chavez DR, Hakim S. Grafting of cultured uroepithelium and bladder mucosa into deepithelized segments of colon in rabbits. J Urol 1994;152:671-674.

17. Pariente JL, Kim BS, Atala A. In vitro biocompatibility assessment of naturally derived and synthetic biomaterials using normal human urothelial cells. J Biomed Mater Res 2001;55:33-39.
18. Korkmaz M, Guvenc BH, Bilir A, Karal-Yilmaz O, Kumbasar A, Caferler J, et al. Isolation and culture of adult and fetal rabbit bladder smooth muscle cells and their interaction with biopolymers. J Pediatr Surg 2003;38:21-24.
19. Lavik E, Teng YD, Snyder E, Langer R. Seeding neural stem cells on scaffolds of PGA, PLA, and their copolymers. Methods Mol Biol 2002;198:89-97.
20. Lu L, Yaszemski MJ, Mikos AG. Retinal pigment epithelium engineering using synthetic biodegradable polymers. Biomaterials 2001;22:3345-3355.
21. Hadlock T, Singh S, Vacanti JP, McLaughlin BJ. Ocular cell monolayers cultured on biodegradable substrates. Tissue Eng 19995:187-196.
22. Aframian DJ, Cukierman E, Nikolovski J, Mooney DJ, Yamada KM, Baum BJ. The growth and morphological behavior of salivary epithelial cells on matrix protein-coated biodegradable substrata. Tissue Eng 20006:209-216.
23. Karamuk E, Mayer J, Wintermantel E, Akaike T. Partially degradable film/fabric composites: Textile scaffolds for liver cell culture. Artif Organs 1999;23:881-884.
24. Patrick CW Jr, Chauvin PB, Hobley J, Reece GP. Preadipocyte seeded PLGA scaffolds for adipose tissue engineering. Tissue Eng

1999;5:139-151.

25. Yoon JH, Moon HJ, Seong JK, Kim CH, Lee JJ, Choi JY, et al. Mucociliary differentiation according to time in human nasal epithelial cell culture. Differentiation 2002;70:77-83.

ABSTRACT(IN KOREAN)

기도결손 복원을 위한 기도 상피세포배양에서 생분해성 PLGA 막의 이용

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배 정 호

목적. 기도결손 복원에는 주로 피부이식이 사용된다. 이번 연구의 목적은 피부이식의 대체물인 기도점액섬모상피의 배양에 사용할 생분해성 지지체로 PLGA 막의 유용성을 알아보고자 하였다. *방법.* Immersion precipitation method를 이용하여 PLGA 막을 합성하고 주사전자현미경을 이용하여 촬영하였다. 생분해 실험은 합성된 막을 배양액에 넣은 후 시간 경과에 따른 분해정도를 주사전자현미경으로 관찰하였다. 새로 합성한 PLGA 막 위에서 사람 정상 코점막 상피세포를 배양하면서 주사전자현미경을 이용하여 형태학적인 분화과정을 분석하였고 MUC5AC와 MUC8 mRNA의 발현정도를 RT-PCR로 측정하였다. *결과.* 점액섬모상피를 배양하기 위한 PLGA 막은 성공적으로 합성이 가능하였다. 막은 지름 24mm, 두께 50μm, 그리고 pore 사이즈 약 3μm 정도로, 이

막은 배양액 속에서 7일째 분해되기 시작하여 약 3주가 경과하자 분해가 빠르게 진행되어 4-6주 이후에는 다공성 구조가 많이 파괴된 것을 관찰할 수 있었다. PLGA 막 위에서의 배양 실험에서 사람 정상 코점막 상피세포는 형태학적으로, 그리고 유전자 수준에서 점액섬모 상피로 분화가 가능하였다. *결론*. PLGA 생분해성 막은 기도점액섬모상피 배양을 위한 지지체로 사용될 수 있었다.

핵심되는 말 : 사람 정상 코점막 상피세포, 이식, PLGA, 분화