A Study on Proliferation and Behavior of Retinal Pigment Epithelial Cells on Purified Alginate Films

Su Mi Jeong¹, Eun Young Kim¹, Ji Hye Hwang¹, Ga Young Lee¹, Su Jin Cho¹, Ji Yeon Bae¹, Jeong Eun Song¹, Kun Ho Yoon², Choun-Ki Joo³, Dongwon Lee¹, Gilson Khang¹

¹Department of BIN Fusion Technology, Polymer Fusion Research Center, Department of Polymer Nano Science Technology, Chonbuk National University, Jeonju, ²Division of Endocrinology and Metabolism, Department of Internal Medicine, ³Department of Ophthalmology and Visual Science, College of Medicine, The Catholic University of Korea, Seoul, Korea

Background and Objectives: Alginate, an anionic polysaccharide distributed widely in the cell walls of brown algae, is used in biomedical applications. However, alginate's performance as a biomaterial, has limited by its several contamination such as endotoxins, proteins and polyphenols.

Methods and Results: To overcome this problem, we have developed using modified *Korbitt* method for alginate purification. After purification, we made alginate films and used for retinal pigment epithelial cell (RPEs) regeneration. ARPE-19 cells were seeded in non-purified and purified alginate films, and then cell viability and proliferation were estimated by MTT assay and RT-PCR was performed to assess specific cell expression. ARPE-19 cell-loaded alginate films were evaluated specific protein expression by through AEC staining and we examined the cell adhesion by scanning electron micro scopy (SEM).

Conclusions: In this result, ARPE-19 cells in purified alginate films had higher cell proliferative rate and phenotypic expression than those on non-purified alginate films. The results suggest that purified alginate is useful for RPEs regeneration.

Keywords: Alginic acid, Film, Purify, RPE

Introduction

The pigment layer of retina of retinal pigment epi-

thelium (RPE) is the pigmented cell layer just outside the neurosensory retina that nourishes retinal visual cells, and is firmly attached to the underlying choroid and overlying retinal visual cells. The RPE basement membrane contains collagen type, fibronectin, laminin, heparin sulfate, chondroitin sulfate and glycoamino acid, such as hylauric acid. These ingredients accelerate the reattachment and adhesion of the RPEs (1-5). Also, hexagonal-celled monolayered RPE, which placed on to the inner surface of the Bruch's membrane, is important to performed the visual function that is the surrounding tissue, by controlling the interaction with the photo-receptor and the choriocapillaris, that is the surrounding tissue (6). The funtional indisposition can be caused in the macular part photo-receptor, atrophy can be caused to the choriocapillaris positioned in the lower part, and those can act on the recovery

Accepted for publication October 6, 2011

Correspondence to **Gilson Khang**

Department of BIN Fusion Technology, Polymer Fusion Research Center, Department of Polymer Nano Science Technology, Chonbuk National University, 567 Baekje-daero, Jeonju 561-756, Korea
Tel: +82-63-270-2355, Fax: +82-63-270-2341
E-mail: gskhang@jbnu.ac.kr

Co-correspondence to **Dongwon Lee**

Department of BIN Fusion Technology, Polymer Fusion Research Center, Department of Polymer Nano Science Technology, Chonbuk National University, 567 Baekje-daero, Jeonju 561-756, Korea
Tel: +82-63-270-2344, Fax: +82-63-270-2341
E-mail: dlee@jbnu.ac.kr

of eyesight as the obstacle (7-14). Because of this fact, RPE had been being presented in one method of the treatment about many macular flava diseases.

Age-related macular degeneration (AMD) is the leading cause of visual impairment and blindness in the elderly population. About 20% of AMD patients suffer from the exudative form of macular degeneration. And main complication of macular degeneration is the development of subretinal choroidal neovascularization (CNV). Following disruption of Bruch's membrane by the invading choroidal vessels and the degeneration of RPE cells (15), photoreceptor cells degenerate resulting in visual impairment (16). Surgical treatment of CNV consists of the removal of CNV membranes often resulting in the removal and damage of additional RPE cells as well as disruption of the RPE-photoreceptor interface (17).

A number of investigators have transplanted RPE cells. There exist the methods of transplantation of RPE cells, one is suspension, 10~15 cells, form a monolayer when transplanted to the subretinal space and the other one is cultivating the RPE cells on the scaffold (18-21). Above them, using the former method to transplanted RPE cells causes the fibrosis at the subretinal space along with the formation of the RPE of the multilayer (22). In this research, RPE cells were cultured on the manufactured scaffolds for transplantation and results are going to applying the tissue engineering.

Originally tissue engineering is a term, the produced tissue from cultured cells on a variety of porous absorbent substrate, the cells in tissue engineering are fundamental components and are widely being used in our study. Goal of tissue engineering is improvement and reconstruction of damaged tissues (23). Biological signals, such as cells, scaffolds and cytokines, are very important factors. Since cells secrete extracellular matrix (ECM) and grow through interaction with scaffolds, choice of scaffolds is very important. On treatment tissue engineering, scaffolds must induce cell adhesion and proliferation and differentiation effectively, and have high biocompatibility, degradation and absorption *in vivo* (24). There are lots of synthetic polymers and natural polymers for transplantation of RPE cells. Among them, Alginate is quickly gelatinized with divalent cations, such as calcium chloride and barium chloride and has possibility for using on diverse parts due to including cells and drugs (25). However, because of contaminants, such as protein and endotoxin in the Alginate, some recent studies have shown the results that proliferation and adhesion of cells on alginate films were reduced (26-28).

In previous studies, we successfully manufactured the

high purified-alginate removed the contaminants and reduced mannuronic acid which is reported as inducer of immune rejection. The experiment was performed based on the ingredient of the alginate containing the various growth factors promoting the cell activating. To confirm the compatibility as a scaffolds of RPE cells, alginate films were manufactured and then RPE cells were seeded on films to study the influence on growth and adhesion of cells.

Materials and Methods

Material

The alginate used that average molecular weight is 75,000~200,000 g/mol. And it used the alginate (Pronova UP-LVG, Novamatrix, Oslo, Norway), the content of the guluronic acid is the ~60%. All chemicals of the methylene chloride and in addition to and organic solvent used HPLC class.

Culture of ARPE-19 cells

ARPE-19 cells, a human RPE cell line obtained from the American Type Tissue Culture Collection, were cultured in a 1 : 1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 (DMEM/Ham's F12) supplemented with 10% fetal bovine serum (FBS), 1% 100 U/ml penicillin, 100 μ g/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Alginate purification

This method changed and manufactured the *korbutt* method and as follows; Before purification, 4% w/v activated charcoal (Sigma) was bleached in 0.1 mol/L sodium perchlorate (Sigma, St. Louis, Mo, USA), followed with two washes in distilled water and two washes in 85% ethanol. The charcoal was allowed to air-dry overnight. Alginate powder was dissolved in distilled deionized water (10 g/L). Bleached activated charcoal was added to the solution (20 g/L) and stirred for 3 h. The charcoal/alginate solution was then passed through a succession of filters: first filter paper, and then 0.22 μ m filter (Millipore, Bedford, MA, USA). Magnesium chloride (20 g/L) (Sigma) was added and the solution was stirred for 30 mins. A solution water was added next and stirred for another 30 mins. The precipitated alginate was then recovered and re-dissolved in 0.1 mol/L EDTA 10 mmol/L HEPES buffer (Sigma) at a concentration 75 ml/g of original alginate dissolved. Once dissolved, 0.125 g NaCl per gram of alginate was added and stirred for 30 mins. This solution was then centrifuged at 5,000 g using 1,000 Da ultra-filtration

tubes (Millipore, Bedford, MA), discarding the distillate. Next, 60% ethanol was added to the final solution, refrigerated at 4°C, and allowed to settle for 3 h. The mixture was centrifuged at 2,000 rpm at 4°C for 5 mins to pellet the alginate. The supernatant was removed, and the precipitate was washed and centrifuged five times more with increasing concentrations of ethanol (60%, 80% ×2 and 100% ×3) as before and allowing the alginate to dry overnight. Alginate pellet was then dissolved into the distilled water with 1% concentration gradient and then passed through of 0.22 μm filter and then freeze-dried for two days. Final alginate dissolved in KRH buffer and filtration using 0.22 μm filter (29).

Cell viability test

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used as an index of cell viability and was performed as described follow. The cells were seeded on the alginate films with 1×10^5 concentration and it measured in 1, 3 and 5 days after cultivating. If the violet crystal was generated, add the dimethylsulfoxide (DMSO) solution (1ml/well), until the crystallization melted completely and dissolved with the ultrasonic cleaner for 30 mins. ARPE-19 cells were incubated in medium containing 100 μl MTT (50 mg/ml) at 37°C for 4 hours. After 0.5 h, the MTT solution was aspirated and optical densities of the supernatant were read at 570 nm using a Microplate Reader (Thermomax, Molecular Device Co. USA).

Reverse transcription-polymers chain reaction (RT-PCR)

In terms of genes, RT-PCR was performed to confirm the specific expression gene markers of ARPE-19 cell, such as RPE-65 and GAPDH. The cells were seeded and cultured on the alginate films with a density of 1×10^5 cells/cm². Total RNA was extracted from the RPE cells centrifuged at 12,000 g at 4°C for 15 mins using 1ml of Trizol (Invitrogen, Life Technologies Co, Groningen,

Netherlands) and 0.2 ml of chloroform. The supernatants precipitated with 0.5 ml of isopropanol (Sigma) and 5 μl of Polyacryl Carrier (Molecules Research Center, Inc., Cincinnati, OH, USA). Complementary DNA templated from each sample were prepared from separated RNA with Oligo (dT) primer (Invitrogen), 5× first strand buffer (Invitrogen), dNTP (dGTP, dATP, dTTP, dCTP, Gibco), Rnase inhibitor (Invitrogen), SuperScript II (Invitrogen), RNase H reverse transcriptase (Invitrogen) and DNase/RNase free water (Gibco) by Authorized thermal cycler (TP 600, Takara Bio Inc, Japan) reversed cDNA amplified the specific gene of DNA. Each of primers (GAPDH, RPE 65) were extended using PCR Master kit (Roche, Germany) including 2 unit Taq DNA polymerase. We observed that all PCR products were separated via electrophoresis on 1.5% (w/v) agarose gel containing SYBR Green (Nucleic Acid Gel Stain, Cambrex, UK) and visualized under UV light (Vilber Lourmat ETX-20.M, France) at 300 nm. The primers used in this study had been purchased from Genotec (Daejeon, Korea) and the gene sequence of RT-PCR primers are listed in Table 1.

Immunocytochemical evaluation

Immunocytochemistry was performed to certify the distribution and growth of RPE cells. The RPE cells seeded on the alginate films with a density of 1×10^5 cells/cm². The cells are incubated in primary antibodies including diluents of mouse anti-cytokeratin (Sigma) in 1 : 800 and rabbit anti-RPE65 (Santa Cruz Biotechnology, California, USA) in 1 : 100 for 1 hour. Then anti-rabbit immunoglobulin antibody (Immunotech, France) was added and reacted for 30 mins at room temperature. In addition, the reaction products were mixed with avidin-biotin-peroxidase (Immunotech) and reacted for another 30 mins at room temperature. We evaluated the color change with chromogen (Immunotech) and observed through a microscopy with object 100 times.

Table 1. PCR primers and protocol for thermal cycling

Species	Gene	Primer sequence	Protocol	Cycles
Human	GAPDH	F: 5'-acc aca gtc cat gcc atc a-3'	D=95°C; 1 min A=56°C; 1 min	24
Rabbit	RPE 65	R: 5'-tcc acc acc ctg ttg ctg ta-3' F: 5'-gcc ctc ctg cac aag ttt gac ttt-3'	E=72°C; 1 min D=94°C; 30 sec A=60°C; 30 sec	35
		R: 5'-agt tgg tct ctg tgc aag cgt agt-3'	E=72°C; 1 min	

D: denature; A: annealing; E: extension.

Scanning electronic microscopy (SEM)

To observe the cell attachment and morphology of RPE cells on the alginate films, we cut into the films with diameter of 1.5 cm of manufactured alginate film and fixed the films with silicon tube in 24 well plates and than sterilized by using 70% ethanol. Cell suspension of RPE was seeded with a density of 1×10^5 cells/cm² and cultured for 1, 3 and 5 days. After remove the culture medium first and then washed out with PBS and fixed with 2.5% glutaraldehyde (Sigma) at room temperature for 24 hours before dehydrated using a graded series of ethanol concentrate each for every 30 minutes. The films of cross section were prepared by cutting dried films and fixed on folder. The cell-attached surface was gold deposited with plasma sputter (Emscope SC 500K, London, UK) under argon gas and the morphology and behavior were examined using Bio-LV SEM (SN-3000 Hithchi, Japan).

Statistics

Statistical analysis was carried out using the student's t-test.

Results

Alginate purification and culture of ARPE-19 cells

Used ARPE-19 cells in this study are shown in Fig. 1. The alginate film was manufactured after purifying the alginate using *Korbutt* method. In the previous research, the result of manufacturing the high purity alginate has shown that mannuronic acid and proteins effecting on the cell division were reduced and also has shown increasing stability of cells with invreased gluronic acid. ARPE-19 cells were seeded on the alginate film with a density of 1×10^5 cell/cm².

Cell viability test

We compared proliferation rate of RPE on high purified alginate films by MTT assay using the capability of the mitochondria reducing MTT tetrazolium which is the watersoluble medium of the yellow by the dehydrogenase reaction to MTT formazan of the non-aqueous having the blue violet. The cell growth rate at 1, 3 and 5 days was shown on Fig. 2. If refined materials without many contaminants declining proliferation of cells have increasing solubility and can be interacting between cells directly. As a result, the higher growth rate was observed in the purified alginate film than non-purified alginate film. We thought that purified alginate films as designed scaffold for RPE cells becomes the important factor.

Reverse transcription-polymers chain reaction (RT-PCR)

To determine the level of expression of a specific mRNA, RT-PCR was performed to cerfity that the effect

Fig. 2. ARPE-19 cell proliferation in purified and non-purified alginate after 1, 3 and 5 days *in vitro* (* $p < 0.001$).

Fig. 1. Photographs of ARPE-19 cells morphology (A) $\times 40$ and (B) $\times 100$.

Fig. 3. SEM microphotographs of the proliferation on alginate films surface for 1, 3 and 5 days respectively (mafnification; ×500).

Fig. 4. Effect of alginate film on the gene expression of retinal pigment epithelial cell-specific markers.

Fig. 5. Gene expression profiles of GAPDH, RPE65 as analyzed by RT-PCR on day 1, 3 and 5. The result of agarose gel electrophoresis Normalization of by RPE65 by GAPDH (* $p < 0.001$).

of a phenotype of RPE cell on the purified alginate film at 1, 3 and 5 days each. Fig. 4 shows the band indicating the level of expression of RPE65 as a specific gene marker for RPE cells *in vitro*. The expression of RPE65 was observed differently between non-purified alginate and purified alginate film at first day, it means primary adhesion was high in the purified alginate film. And band expression of RPE65 showed up higher in the purified alginate film as the time passed. Fig. 5 shown the level of expression of mRNA after standardization into GAPDH as a housekeeping gene using each primer. The cell culture on the purified alginate film is helpful to maintain cell expression. And alginate film is positively effects on proliferation and maintenance of morphology of cells.

Immunocytochemical evaluation

The immunocytochemical staining was performed by AEC staining proving the distribution of cell by the macroscopy easily by staining the protein of RPE65, that is the specific gene of the RPE cell by the antigen-antibody reaction in order to evaluate the morphology of the cell and distribution aspect. The Fig. 6 shown the level of RPE65, a specific gene marker of RPE cell became darked as time passed and we confirmed that expression of the RPE65 and cell proliferation was increased on purified alginate film. The mucopolysaccharide, proteoglycan and the substrates of the protein from the purified alginate are helpful to the cell adhesion of the RPE cell. Alginate is suitable scaffolds also influence on proliferation, survival and maintaining morphology of cells. In the future, it is considered that alginate plays a important role as the scaffold to design for regeneration of the RPE cell.

Scanning electronic microscopy (SEM)

As shown in Fig. 3, SEM analysis was performed in order to observe the morphology, adhesion and proliferation of ARPE-19 cell by macroscopy. We observed that cell proliferation was enhanced and confirmed that cell spread out rapidly on the alginate film surface as time passed. It was considered that high purity alginate has the positive effect on the proliferation of the cell in comparison with the original alginate. In the original alginate film, it was unable to be helpful to the cell adhesion by the hydrophobicity, however in the purified alginate film, it is considered to supplement the hydrophobicity and enhance the attachment of the cell as the material stabilize the cell rapidly.

Discussion

In this research, we manufactured the high purity alginate which had an effect on the cell division and causes the immune rejection. After manufacturing the alginate film, in order to confirm the conformance by the scaffold of the RPE cell and the cells were seeded on the alginate films to study the influence that it reaches to the attachment of the cells and growth *in vitro*. The result of confirming the cytotoxicity evaluation of the cells reached to the RPE cell was confirmed by the MTT assay and immunohistochemical stain through the expression of the specific marker of the RPE cells indicated that it could observe to influence the positive effect on the primary cell attachment and cell survival than the original alginate film in the high purity alginate film and it confirmed that it was useful to the alive of the RPE cell as the time passed. It confirmed to reach the positive effect to the RPE cell growth and maintenance of phenotype to the high mRNA expression of RPE65. The positive effect is given to the RPE cell growth and proliferation from the protein and proteoglycan generated in the purified alginate and it is suitable for the application to tissue engineering with the scaffold which is suitable for the transplantation.

Acknowledgements

This research was supported by WCU (R31-20029) and XRC of Ministry of Health and Welfare (A040004).

Potential conflict of interest

The authors have no conflicting financial interest.

Fig. 6. Microphotographs of AEC method staining of retinal pigment epithelial cells with specific rabbit anti-cytokeratin protein and specific rabbit anti-RPE65 protein (magnification $\times 100$, scale bar: 250 μ m). (A) 1 day. (B) 3 days. (C) 5 days.

References

1. Naumann GOH, Apple DJ. Pathology of the Eye. New York: Springer-Verlag; 1986.245
2. Gouras P, Cao H, Sheng Y, Tanabe T, Efremova Y, Kjeldbye H. Patch culturing and transfer of human fetal retinal epithelium. Graefes Arch Clin Exp Ophthalmol 1994;232: 599-607
3. Aisenbrey S, Bartz-Schmidt KU, Walter P, Hilgers RD, Ayerterey H, Szurman P, Thumann G. Long-term follow-up of macular translocation with 360 degrees retinotomy for exudative age-related macular degeneration. Arch Ophthalmol 2007;125:1367-1372
4. Ho TC, Del Priore LV. Reattachment of cultured human retinal pigment epithelium to extracellular matrix and human Bruch's membrane. Invest Ophthalmol Vis Sci 1997;38: 1110-1118
5. Song MK, Lui GM. Propagation of fetal human RPE cells: preservation of original culture morphology after serial passage. J Cell Physiol 1990;143:196-203
6. Pollack JS, Del Priore LV, Smith ME, Feiner MA, Kaplan HJ. Postoperative abnormalities of the choriocapillaris in exudative age-related macular degeneration. Br J Ophthalmol 1996;80:314-318
7. Korte GE, Reppucci V, Henkind P. RPE destruction causes choriocapillary atrophy. Invest Ophthalmol Vis Sci 1984;25:

- 1135-1145
8. Valentino TL, Kaplan HJ, Del Priore LV, Fang SR, Berger A, Silverman MS. Retinal pigment epithelial repopulation in monkeys after submacular surgery. *Arch Ophthalmol* 1995;113:932-938
9. Del Priore LV, Hornbeck R, Kaplan HJ, Jones Z, Valentino TL, Mosinger-Ogilvie J, Swinn M. Débridement of the pig retinal pigment epithelium in vivo. *Arch Ophthalmol* 1995;113:939-944
10. Del Priore LV, Kaplan HJ, Hornbeck R, Jones Z, Swinn M. Retinal pigment epithelial debridement as a model for the pathogenesis and treatment of macular degeneration. *Am J Ophthalmol* 1996;122:629-643
11. Desai VN, Del Priore LV, Kaplan HJ. Choriocapillaris atrophy after submacular surgery in presumed ocular histoplasmosis syndrome. *Arch Ophthalmol* 1995;113:408-409
12. Nasir MA, Sugino I, Zarbin MA. Decreased choriocapillaris perfusion following surgical excision of choroidal neovascular membranes in age-related macular degeneration. *Br J Ophthalmol* 1997;81:481-489
13. Castellarin AA, Sugino IK, Nasir M, Zarbin MA. Clinicopathological correlation of an excised choroidal neovascular membrane in pseudotumour cerebri. *Br J Ophthalmol* 1997;81:994-1000
14. Smidsrød O, Skjåk-Braek G. Alginate as immobilization matrix for cells. *Trends Biotechnol* 1990;8:71-78
15. Bressler NM, Bressler SB, Fine SL. Age-related macular degeneration. *Surv Ophthalmol* 1988;32:375-413
16. Vingerling JR, Hofman A, Grobbee DE, de Jong PT. Age-related macular degeneration and smoking. The Rotterdam Study. *Arch Ophthalmol* 1996;114:1193-1196
17. Ishibashi T, Kohno T, Sorgente N, Patterson R, Ryan SJ. Fibronectin of the chorioretinal interface in the monkey: immunohistochemical and immunoelectron microscopic studies. *Graefes Arch Clin Exp Ophthalmol* 1985;223:158-163
18. Tezel TH, Del Priore LV, Berger AS, Kaplan HJ. Adult retinal pigment epithelial transplantation in exudative age-related macular degeneration. *Am J Ophthalmol* 2007;143:584-595
19. Aisenbrey S, Lafaut BA, Szurman P, Grisanti S, Lücke C, Krott R, Thumann G, Fricke J, Neugebauer A, Hilgers RD, Esser P, Walter P, Bartz-Schmidt KU. Macular translocation with 360 degrees retinotomy for exudative age-related macular degeneration. *Arch Ophthalmol* 2002;120:451-459
20. Lopez R, Gouras P, Brittis M, Kjeldbye H. Transplantation of cultured rabbit retinal epithelium to rabbit retina using a closed-eye method. *Invest Ophthalmol Vis Sci* 1987;28:1131-1137
21. Li LX, Sheedlo HJ, Turner JE. Long-term rescue of photoreceptor cells in the retinas of RCS dystrophic rats by RPE transplants. *Prog Brain Res* 1990;82:179-185
22. Lopez R, Gouras P, Kjeldbye H, Sullivan B, Reppucci V, Brittis M, Wapner F, Goluboff E. Transplanted retinal pigment epithelium modifies the retinal degeneration in the RCS rat. *Invest Ophthalmol Vis Sci* 1989;30:586-588
23. Kim SJ, Jo EH, Kim OY, Lee EY, Song JE, Lee DW, Khang G. Porcine small intestinal submucosa sheets as a scaffold for retinal pigment epithelial cells. *International Journal of Tissue Regeneration* 2010;1:94-100
24. Thumann G, Viethen A, Gaebler A, Walter P, Kaempf S, Johnen S, Salz AK. The in vitro and in vivo behaviour of retinal pigment epithelial cells cultured on ultrathin collagen membranes. *Biomaterials* 2009;30:287-294
25. Tam SK, Dusseault J, Polizu S, Ménard M, Hallé JP, Yahia L. Impact of residual contamination on the biofunctional properties of purified alginates used for cell encapsulation. *Biomaterials* 2006;27:1296-1305
26. Dusseault J, Tam SK, Ménard M, Polizu S, Jourdan G, Yahia L, Hallé JP. Evaluation of alginate purification methods: effect on polyphenol, endotoxin, and protein contamination. *J Biomed Mater Res A* 2006;76:243-251
27. Klöck G, Frank H, Houben R, Zekorn T, Horcher A, Siebers U, Wöhrle M, Federlin K, Zimmermann U. Production of purified alginates suitable for use in immunisolated transplantation. *Appl Microbiol Biotechnol* 1994;40:638-643
28. Wandrey C, Vidal DS. Purification of polymeric biomaterials. *Ann N Y Acad Sci* 2001;944:187-198
29. Mallett AG, Korbitt GS. Alginate modification improves long-term survival and function of transplanted encapsulated islets. *Tissue Eng Part A* 2009;15:1301-1309