 DOI: 10.1002/masy.200750321

The Effects of Co-Immobilization of Epidermal Growth Factor and Adhesion Factor on the Proliferation of Ligament Cell

Yong-Soon Park,[*1] *Young-Kwon Seo,*[2] *Hwa-Sung Lee,*[3] *Soon-Yong Kwon,*[3] *Jung-Keug Park*[2]

Summary: The epidermal growth factor and cell adhesion factor were co-immobilized on a tissue culture plate to allow the proliferation of human ligament cells. The co-immobilized surfaces were prepared by irradiating a photo-reactive polypeptide containing azidophenyl-derivatized side-chains with UV. The immobilized cell adhesion factor induced initial cell adhesion on the surfaces, and the immobilized cell growth factor appeared to induce cell proliferation. The ligament cells proliferated on the EGF-immobilized plate until 10 days post-culture. The co-immobilization of the epidermal growth factor appears to be an effective method for healing an anterior cruciate ligament through the rapid proliferation of ligament cells.

Keywords: adhesion factor; co-immobilization; epidermal growth factor; ligament; photo-immobilization; proliferation

Introduction

Compared with an injured medial collateral ligament (MCL), an injured anterior cruciate ligament (ACL) has a very poor healing potential, which can result in knee instability, meniscal damage, and osteoarthritis. Although the fibrinous hematome plays important role in the healing process it cannot act satisfactorily due to its dilution by the joint fluid. Other etiological factors associated with the poor healing potential of the ACL include intrinsic biochemical and biomedical differences, the synovial effect and inadequate blood supply.[1–3] For these reasons, the prognosis for a simple cast or primary suture treatment of a ruptured ACL is poor.

On the other hand, reconstructive surgery using a patellar tendon (autografts or allografts) or semitendinous tendon is the preferred treatment.[4] However, problems associated with an autograft include the lengthy rehabilitation as well as the persistent patellar pain. Allografts also carry the risk of disease transmission, and their procurement is often difficult and costly. Furthermore, additional surgery on a reconstructed ACL are often needed because differences in the surgical results according to the technique or the rehabilitaion methods can make the ligament unstable.[6–8] Permanent synthetic ACL prosthesis including Gore-Tex prosthesis, Stryker-Dacron ligament, and Kennedy ligament augmentation device can be used, and may show satisfactory performance in the short term but they tend to deteriorate and fail in the long term.[9–11] In addition, a prosthesis-induced disturbance in the release of cytokine by lymphocytes can cause defects in the host defense against a bacterial infection.[12,13]

Many studies aimed at enhancing the healing potential of an injured ACL using

[1] Dep. Medical Eng., Kwangju Health College, Kwangju, 706-701 Korea
Fax: (+82) 62 958 4946
E-mail: peter@kjhc.ac.kr
[2] Dep. Chemical & Biochemical Eng., Dongguk University, Seoul, 100-715 Korea
[3] Dep. Orthopedics, Catholic University, Seoul, 150-713 Korea

various growth factors have been reported. Recent studies have shown that various growth factors play important roles in stimulating fibroblast proliferation and may influence ligament healing.[14–17] However, if native cytokines or growth factors are infused directly into an injured site, then continuous supplementation will be needed for healing because a part of them can bind to the receptor of the target cells. The remainder of these cytokines is released into the circulation.

Typically, a cell can grow in the presence of serum. However, proliferation without serum, which was achieved by immobilizing growth factor onto polymer substrate, has been reported.[18] The authors reported that cell growth could be enhanced by immobilizing the epidermal growth factor (EGF) under serum-free conditions. Therefore, in this study, EGF and two types of adhesion factors were co-immobilized onto a tissue culture plate using the photo-reactive polypeptide. The effects of the co-immobilized EGF and adhesion factor on the viability and proliferation of ligament cells of the ACL under serum-free conditions were investigated.

Experimental Part

Synthesis of Poly(aspartic acid)

Poly(aspartic acid) (PAsp) was synthesized according to the method described earlier.[19] Poly succinimde (PSI) was synthesized by the polycondensation of L-aspartic acid in the presence of an acid catalyst as *o*-phosphoric acid. A light-brown colored PSI was obtained (Yield: 18.94 g, 94%). PSI (14.0 g, 0.144 mol) was added to 100 mL of an aqueous sodium hydroxide solution (0.144 mol), and the mixture was stirred with cooling. After the mixture had been stirred for 3 h at room temperature, 35% aqueous hydrochloric acid was added to the solution until a neutral solution was obtained. The solution was then poured into methanol (300 mL), and the precipitate was filtered and dried overnight at 50 °C under a reduced pressure.

The synthesized polymers were characterized using Fourier Transform ^{1}H NMR and ^{13}C NMR (FT-NMR 11.75T, Oxford superconducting magnet 500MHz, Oxford Instrument, UK). The average molecular weight (M_w) of the PAsp was analyzed using a HPLC system (Dynamax SD-200, Rainin, USA) with a gel permeation chromatography column (Ultrahydrogel 250 and 2000, Waters, USA) and with a refractive index detector (Dynamax RI-1, Rainin, USA). A calibration curve was obtained using commercial poly(ethylene oxide) of a known molecular weight.

Preparation of Photo-Reactive PAsp (PAsp-Az)

A PBS solution (10 ml) containing PAsp (1.0 g) and 4-azidoaniline (70 mg) was stirred until it was completely dissolved. The water-soluble carbodiimide (200 mg) was added to this solution in ice-bath. The solution was stirred continuously under 4 °C for 24 h, and then at room temperature for 24 h. The dark brown solution obtained was filtered using molecular a weight cut-off filter (Ultra filter unit, cut-off below 10,000, Advantec Co.) to eliminate the unreacted reagents. The azidophenyl groups from 4-azidoanilin were reacted with the carboxyl groups on the PAsp side chains. The content of the bound azidophenyl group in PAsp was determined from the absorbance at 260 nm.

Photo Immobilization

Figure 1 shows a schematic diagram of the of the immobilization method used in this study. Various aqueous solutions (100 μl) containing PAsp-Az, EGF (Sigma Chemical CO., MO) and fibronectin (FN)/collagen (COL) (Sigma Chemical CO., MO) were cast on a 24-well tissue culture plate. The cast plates were air-dried under light-tight conditions for 24 hours. After drying, the plates were irradiated for 30 s with a UV lamp (200 W) from a distance of 5 cm. The plates were then rinsed several times with distilled water until the absorbance of azidophenyl group in the washing liquid at 280 nm became undetectable.

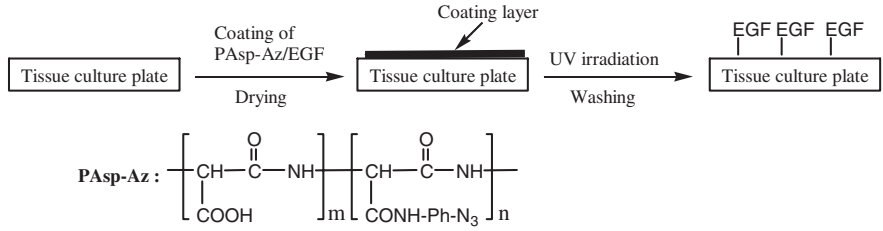

Figure 1.
Photo-immobilization of azidophenyl-derivatized PAsp (PAsp-Az) containing EGF and the adhesion factors.

Human ACL Cell Culture

The normal ACL explants were obtained from a patient, who underwent thigh amputation surgery and provided informed consent. The human ACL cells were isolated using Collagenase IA (C-9891, Collagenase IA 500 mg, Sigma-Aldrich, St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRL, Gaithersburg, MD) containing 10% FBS, nonessential amino acids (0.1 mM), L-glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 g/ml) and fungi zone (0.25 g/ml) was used to culture the cells at 37 °C in an atmosphere containing 5% CO_2, and produce a cell suspension (1×10^5 cells/ml). The subcultured ligament cells were harvested by incubation with PBS containing EDTA and trypsin at 37 °C for 5 min. The ligament cells were washed once with DMEM and then suspended in DMEM for 1×10^5 cells/mL before use.

Cell Culture and Assay Methods

The prepared cellular suspension (100 μl) was added to each immobilized culture plate containing 900 μl of the prepared media. The ligament cells were incubated in the immobilized 24-well tissue culture plate under a 5% CO_2 atmosphere at 37 °C for 72 h. All the cell cultures on the immobilized plates were examined under serum-free conditions to observe the the immobilized EGF and/or adhesion factor effect only. The serum-free cultured cells were observed daily for any behavioral changes and the proliferation rates in each group were calculated at the certain times post-culture using phase contrast microscopy (CK40, Olympus, Tokyo, Japan).

The cell viability was determined using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay. For the MTT assay, serum-free DMEM was added to each cell cultured well, supplemented with MTT (0.33 mg/ml) and then incubated in the dark at 37 °C in an atmosphere containing 5% CO_2 for 2 h. Subsequently, the supernatant was aspirated. Isopropyl alcohol (1.0 ml) was added and the well-plate was shaken slowly for 15 min. The absorption was measured at 504 nm.

Statistical Analysis

The proliferation rates in each group were analyzed using a paired t-test to obtain the statistical significance of the time-dependent cellular growth rates. The results from each group were compared using ANOVA Scheffe's test. A p value <0.05 was considered significant.

Results and Discussion

PAsp was synthesized by the hydrolysis of PSI, which that had been synthesized by the polycondensation of aspartic acid in the presence of o-phosphoric acid as the acid catalyst, with mesitylene and sulfolane as the co-solvent. The ^{1}H-NMR spectrum of PAsp in D_2O indicated methyne protons at 4.5 and 4.7 ppm. The methylene protons were observed as methylene at 2.7 and 2.8 ppm. The area ratio between the α-form

and β-form of the methylene protons was 1:3. The ^{13}C NMR spectrum shows that the carbonyl carbons of the amide groups and carboxyl groups in the 170–185 ppm region. The methyne and methylene carbons were also observed at 50–55 ppm and 35–40 ppm, respectively (data not shown). The weight-average molecular weight (M_w) of the obtained PAsp was approximately 52,000 (DP = about 520), and the molecular weight distribution was 1.7.

The UV spectrum of the PAsp-Az aqueous solution showed a peak derived from the azidophenyl groups at 280 nm. After UV irradiation, the PAsp-Az formed a hydrogel that was insoluble in water. This means that the azidophenyl groups were attached to the PAsp side-chains and could react after UV irradiation. Indeed, approximately 5% of the carboxyl groups in PAsp were bound with azidophenyl groups.

The coating layer containing PAsp-Az, EGF or the adhesion factors on the plate surface formed a hydrated layer through the irradiation of UV-light. Once formed, the hydrated layer did not detach from the plate again. The immobilized EGF, which was bound with the FITC-conjugated anti-human IgG antibody, is expressed as a fluorescence in the green-colored background by the immunostain. The luminescence was observed on the surface of the culture plate after the immobilization of EGF (data not shown).

The ACL cells were cultured on a PAsp-immoblized plate. Figure 2 shows the phase-contrast micrographs of the ACL cells after 6 h and 4 days culture. The ACL cells were attached and spread well over the PAsp-immobilized plate. In addition, the ACL cells cultured on the PAsp-immobilized surface showed the typical morphology of fibroblasts. This indicates that PAsp do not affect the viability of ACL cells during culture.

Table 1 shows the cell densities of the ACL cells after 6 h and 10 days cultured without serum on the FN- or COL-immobilized plate. The ACL cells adhered well to the plate in the short term. However, no ACL cell growth was observed at any loaded concentration. An immobilized cell adhesion factor such as FN and COL accelerated the initial cell adhesion on the surfaces but did not induce cell proliferation.

On the other hand, ACL cell growth was observed on the EGF-immobilized plate containing either FN or COL. Table 2 shows the cell densities on the EGF/FN- and EGF/COL-immobilized plate after 6 h and 10 days incubation. In the case of the control and PAsp-immobilized plate, there were no significant changes compared with the previous extent 10 days post-culture. However, the cell densities on the EGF co-immobilized plate increased remarkably. The cell densities on the EGF/adhesion factor-immobilized plates were higher (approximately 40% higher) than the non-immobilized or PAsp-immobilized plates. The paired t-test and ANOVA Scheffe's test showed that the differences were significant.

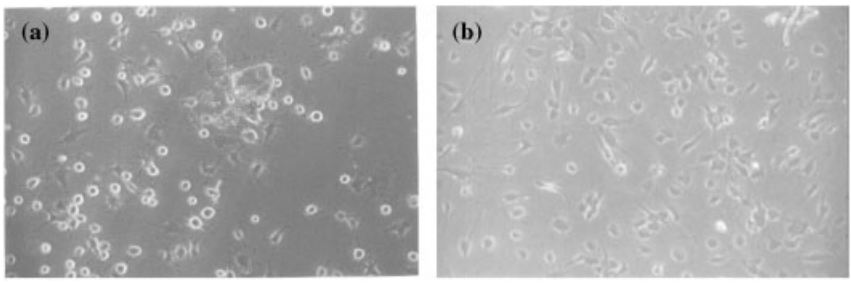

Figure 2.
Photographs of the ACL cells cultured on a PAsp-immobilized plate after 6 h (a), and 4 days (b). The cells were cultured without serum.

Table 1.
The cell densities of the ACL cells on the FN-immobilized plate, and COL-immobilized plate after culturing for 6 h and 10 days.

Sample of immobilized plate (loading amount; μg)	Cell density (10^{-4} cells/ml)*	
	6 h post-culture	10 days post-culture
Control	1.42 ± 0.2	1.40 ± 0.3
PAsp-Az	1.52 ± 0.2	1.50 ± 0.2
FN (1)	1.53 ± 0.3	1.35 ± 0.4
FN (5)	1.55 ± 0.2	1.45 ± 0.3
FN (10)	1.48 ± 0.1	1.46 ± 0.3
FN (25)	1.49 ± 0.1	1.34 ± 0.4
FN (50)	1.49 ± 0.3	1.30 ± 0.4
COL (1)	1.59 ± 0.3	1.35 ± 0.4
COL (5)	1.57 ± 0.5	1.36 ± 0.5
COL (10)	1.56 ± 0.4	1.40 ± 0.4
COL (25)	1.55 ± 0.3	1.39 ± 0.5
COL (50)	1.50 ± 0.4	1.45 ± 0.4

* The p value was <0.05.

However, although cell proliferation was observed, there were no obvious effects of the loading amount on proliferation. Chen et al.[18] reported that 130 ng/cm^2 of the immobilized EGF had produced a single layer of cell proliferation on the surface and the cells grew continuously grown until they reached that concentration. At higher concentrations, the cells did not proliferate to any large extent. In our case, the loading amount did not influence cell proliferation because it would be much higher than necessary.

Table 2.
The cell densities of the ACL cells on the EGF/FN- and EGF/COL-immobilized plate after incubation for 6 h and 10 days.

Sample of Immobilized plate (loading amount; μg)	Cell density (10^{-4} cells/ml)*	
	6 h post-culture	10 days post-culture
Control	1.42 ± 0.2	1.40 ± 0.3
PAsp-Az	1.52 ± 0.2	1.50 ± 0.2
EGF(20)	1.49 ± 0.3	2.08 ± 0.4
EGF (1)/FN (2)	1.52 ± 0.4	2.05 ± 0.4
EGF (5)/FN (2)	1.48 ± 0.2	2.10 ± 0.2
EGF (10)/FN (2)	1.50 ± 0.3	2.17 ± 0.4
EGF (20)/FN (2)	1.52 ± 0.2	2.13 ± 0.5
EGF (1)/COL (2)	1.58 ± 0.3	2.09 ± 0.3
EGF (5)/COL (2)	1.56 ± 0.2	2.17 ± 0.3
EGF (10)/COL (2)	1.57 ± 0.2	2.19 ± 0.2
EGF (20)/COL (2)	1.58 ± 0.2	2.15 ± 0.4

* The p value was <0.05.

Figure 3 shows images of ACL cells cultured on EGF/COL-immobilized plate (EGF10-COL2) after 3 h (Figure 3a) and 10 days cultured (Figure 3b). At 3 h post-culture, the cells formed a round-shape. However, after 10 days incubation, the cells had proliferated on the co-immobilized plate.

Generally, a cytokine such as EGF binds the specific cell-surface receptor of the target cells through endocrine, paracrine or autocrine signaling. Subsequent intracellular signaling cascades control the various cellular functions.[20] This may include the downregulation of several genes and their transcription factors, which in turn causes an decrease in the number of surface receptors.

It was reported that membrane-anchored EGF, unlike other cytokines, functioned by juxtacrine stimulation, which maintained the combined status on the cellular membrane of the target cell.[21,22] An artificial model also showed that once the cytokines had been immobilized on the surface of the polymer substrate and the cell cultures were performed, the immobilized cytokines would continue to function without exhausting the cytokines, compared with the native cytokines.[18,23–25] This is because they did not internalize into the cell as a result of immobilization.

In this study, the ACL cells were cultured under the serum-free condition to exclude the effect of other cytokines that were present in serum. Inoue et al reported that it was possible to carry out a serum-free culture for up to 10 days, and cell growth would decrease in 2-4 days post-culture.[26] However, in this case, the primary cultured ACL cells underwent necrosis on the typical culture plate after few days under serum-free condition. On the other hand, the ACL cells proliferated on the EGF-immobilized plate until 10 days post-culture even if the ACL cells had proliferated slowly. This suggests that the immobilized EGF can enhance cell proliferation in the knee joint, which is a poor environment

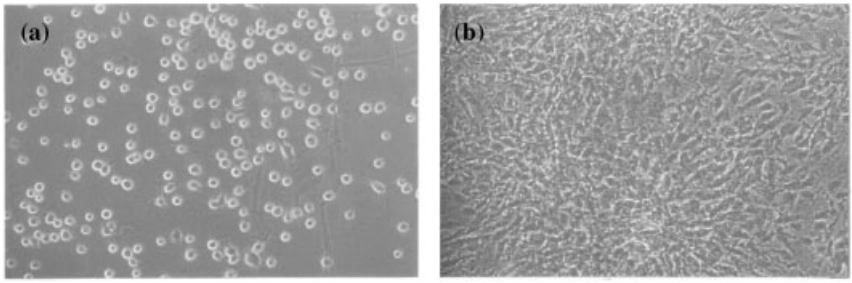

Figure 3.
Phase-contrast micrographs of ACL cells on the EGF/FN-immobilized plate (loading amount; EGF = 10 μg and COL = 2 μg) after 3 h (a) and 10 days cultured (b).

for cell proliferation. The application of an immobilized EGF/adhesion factor enhances the healing potential of the ACL and it has possible clinical applications in the treatment of a ruptured ligament.

Acknowledgements: This work was supported by a grant from the Korean Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (0405-BO01-0204-0006).

[1] D. Amiel, K. K. Ishizue, F. L. Harwood, L. Kitabayashi, W. H. Akeson, *J. Orthop. Res.* **1989**, *7*, 486.
[2] J. Andrish, R. Holmes, *Clin. Orthop.* **1979**, *138*, 279.
[3] S. P. Arnoczky, R. M. Rubin, J. L. Marshall, *J. Bone Joint Surg.* **1979**, *61-A*, 1221.
[4] J. A. Feagin, W. W. Curl, *Am. J. Sports Med.* **1976**, *4*, 95.
[5] P. P. Weitzel, J. C. Richmond, G. A. Altman, T. Calabro, D. L. Kaplan, *Orthop. Clin. North. Am.* **2002**, *33*, 653.
[6] C. H. Jr, Brown, E. W. Carson, *Clin. Sports Med.* **1999**, *18*, 109.
[7] J. W. Jaureguito, L. E. Paulos, *Clin. Orthop.* **1996**, *325*, 25.
[8] F. R. Noyes, S. D. Barber-Westin, *J. Bone Joint Surg.* **2001**, *83-A*, 1131.
[9] G. K. McPherson, H. V. Mendenhall, J. H. Roth, *Clin. Orthop. and Related Res.* **1985**, *196*, 186.
[10] W. Roolker, T. W. Patt, C. N. van Dijk, M. Vegter, R. K. Marti, *Knee Surg. Sports Traumatol. Arthrosc.* **2000**, *8*, 20.
[11] C. E. Keen, A. A. Amis, *Histopathology* **1999**, *35*, 74.
[12] E. S. Grood, F. R. Noyes, *J. Bone. Joint. Surg. Am.* **1976**, *58*, 1083.
[13] G. K. McPherson, H. V. Mendenhall, J. H. Roth, *Clin. Orthop. and Related Res.* **1985**, *196*, 186.
[14] E. A. DesRosiers, L. Yahia, C. H. Rivard, *J. Orthop. Res.* **1996**, *14*, 200.
[15] J. A. Hannafin, E. T. Attia, R. F. Warren, M. M. Bhargava, *J. Orthop. Res.* **1999**, *17*, 398.
[16] D. Kobayashi, M. Kurosaka, S. Yoshiya, K. Mizuno, *Knee. Surg. Sports Traumatol. Arthrosc.* **1997**, *5*, 189.
[17] C. C. Schmidt, H. I. Georgescu, C. K. Kwoh et al., *J. Orthop. Res.* **1995**, *13*, 184.
[18] G. Chen, Y. Ito, Y. Imanishi, *Biochimica et Biophysica Acta* **1997**, *1358*, 200.
[19] T. Nakato, K. Atsushi, K. Toyoji, *J. Polm. Sci.: Part A: Polym. Chem.* **2000**, *38*, 117.
[20] R. A. Goldsby, T. J. Kindt, B. A. Osborne, "*Cytokines. In Kuby Immunology*", WH Freeman and Company, New York **1999**.
[21] S. Higashiyama, R. Iwamoto, K. Goishi, N. Raab, M. Taniguchi, M. Klagsbrun, E. Mekada, *J. Cell Biol.* **1995**, *128*, 929.
[22] J. Massague, A. Pandiella, *Annu. Rev. Biochem.* **1993**, *62*, 515.
[23] Y. Ito, J. Zheng, K. Yonezawa, M. Kasuga, Y. Imanish, *Cell Biology* **1996**, *93*, 3598.
[24] Y. Ito, G. Chen, Y. Imanish, *Biotechnol. Prog.* **1996**, *12*, 700.
[25] Y. Ito, G. Chen, Y. Imanish, *Bioconjugate Chem.* **1998**, *9*, 277.
[26] H. Inoue, H. Osada, K. Kojima, M. Tomioka, H. Ishida, *J. Cardiovasc. Surg.* **1990**, *40*, 435.

