표피성장인자가 치주인대 섬유 모세포의 증식과 부착에 미치는 영향

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국문초록

손상 받은 치주 조직의 치유과정은 치주인대 섬유 모세포의 세포 활성도에 영향을 받는다. 또한 치유과정 중 치주인대 섬유 모세포간의 재부착이 이루어져야 한다. 본 연구의 목적은 표피성장인자가 치주인대 세포의 증식과 부착에 미치는 영향을 알아보는 것으로 이를 바탕으로 향후 완전 탈구된 치아의 보관용액이나 재식전 처리제로서의 표피성장인자의 효용성을 고찰해 보기 위함이다. 발치된 사람의 제 1소구치에서 치주인대 섬유모세포를 채취 및 배양한 후, 세포독성을 및 세포의 최고 활성도를 평가 하기 위해 MTT assay를 시행하였다. 표피성장인자가 첨가된 실험군과 대조군의 세포증식의 차이를 비교하였고, western blot을 통해서 세포 부착에 관여하는 섬유결합소의 발현을 실험군과 대조군을 통해 비교하여 다음의 결과를 얻었다. 표피성장인자는 치주인대 섬유모세포에 대하여 세포독성을 보이지 않으며, 최고의 활성도를 보이는 농도는 10ng/ml였다. 또한 치주인대 섬유모세포의 배지내 증식정도는 10ng/ml의 실험군에서 대조군에 비해 유의하게 높았다. 세포간 부착에 관여하는 섬유결합소의 발현율이 실험군에서 대조군에 비해 유의하게 증가하였다. 본 연구를 통해 표피성장인자는 치주인대 섬유모세포의 재생을 촉진하며 따라서 완전 탈구된 치아의 보관용액이나 재식전 처리제로 사용될 수 있음을 시사한다.

주요어 : 표피성장인자, 섬유결합소, 치주인대 섬유모세표, 치아 완전탈구

Ⅰ. INTRODUCTION

The tooth avulsion constitutes 0.5%~16% of all traumatic injuries to permanent anterior teeth[1]. The successful replantation of avulsed tooth depends on the existence of viable periodontal ligament fibroblasts(PDLFs) that are capable of proliferation over the denuded damaged root surfaces[2,3].

Therefore, in the case of periodontal injury from tooth avulsion, the maintenance of viable PDLFs would be the most important factor considering the prognosis. PDLFs play an important role in periodontal regeneration. Thus, the early recruitment and restoration of fibroblasts in periodontal injury, and the accelerated repair of periodontal ligament are regarded as critical events for successful periodontal regeneration[4-7]. Previous studies also have suggested that successful periodontal regeneration requires the intimate reattachment of periodontal ligament cells[5,8,9]. Recently, with the development of microbiology, growth factors that act on tissue healing have been applied to the field of tissue regeneration[10].

Polypeptide growth factors(PGFs) represent a group of biologicals that have a high potential to be used as the chemotactic and mitogenic stimuli to specific cells with measurable numbers of cell-surface receptors to these factors[11-13]. PGFs resemble hor-

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mone in structure and function. However, recent studies have indicated that their sites of synthesis and means of transport to specific target cells are more variable than their true hormonal analogues. Most of these factors are released in a continuous manner to diffuse to the target cells[14].

There are various PGFs: epidermal growth factor (EGF), platelet-derived growth factor(PDGF), transforming growth factors(α, β TGFs), insulin-like growth factors(IGF Ⅰ & Ⅱ).

EGF has been studied in many aspects since had been found at the process of nerve growth factor (NGF) purification. EGF is known to be mainly secreted from major salivary glands and stimulates the growth of most tissues. One of earliest findings with respect to the *in vivo* effects of EGF was the precocious eyelid opening and early eruption of incisors in the neonatal rodents[15]. As indicated earlier, a variety of mesodermal cells have been shown to respond to EGF, including corneal endothelial cells, vascular smooth muscle cells, chondrocytes, and fibroblsts. Fibroblasts and perhaps endothelial cells respond to EGF with increased production of cell surface protein or fibronectin which is the ground substance required for normal growth and differentiation[16]. Several recent reports have described some of the possible *in vitro* effects of polypeptide growth factors on periodontal ligament cells[4,17,18].

The purpose of this study was to evaluate the effects of EGF on the proliferation and attachment of PDLFs and to verify the efficacy of EGF as a storage media or pre-replantation conditioner of traumatically avulsed tooth.

Ⅱ. MATERIALS AND METHODS

1. Experimental materials

1) EGF

Human recombinant epidermal growth factor(easyf®, Daewoong Pharm Co. Ltd. Korea) is the biochemical synthetic material which has same structure and equivalent activity of human EGF. For *in vitro* study, EGF was dissolved and diluted with 0.1 M phosphate buffered saline(PBS) and 100ng/ml EGF stock solution was made. It was then diluted to different other concentration before experiment.

2) Periodontal ligament fibroblasts(PDLFs)

Human PDLFs were obtained and cultured from the explant tissue of human healthy periodontal ligament taken from several first premolars that had been extracted for orthodontic reasons.

2. Experimental methods

1) Cell preparation

After removing calculus and plaque out of premolar in mouth, it was extracted and rinsed 3 times with Hanks' balanced salt solution(HBSS) to remove the blood clot of root surface. The periodontal ligament tissues from the middle third of the roots were minced, put in culture dishes and incubated in α-MEM with 10% fetal bovine serum(FBS), 100mg/ml streptomycin, 0.5mg/ml amphotericin-B, and 100 unit/ml penicillin at 37℃ in a humidified atmosphere of 5% CO_2 - 95% air. The media was changed every 3 days until dense single cell layer was gained. After outgrowth cells reached confluency, they were trypsinized with 0.25% trypsin-EDTA in PBS for secondary culture. Cultures were maintained until confluency and passed at 1:4 split ratios. All the experiments were done in cells within five and seven passages.

2) Cell toxicity test

3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide(MTT) assay, usually used as cytotoxicity test, is the method to evaluate viable cell proliferation. MTT test uses the principle which tetrazolium salt be reduced by mitochondrial reducing enzyme(succinate dehydrogenase) so that the toxicity of viable cells and cellular differentiations can be measured. The reduced tetrazolium salt is converted into colored water-insoluble formazan salt, and it can be evaluated spectrophotometically once the MTT-formazan has been dissolved in an organic solvent.

In this study, the PDLFs were cultured in media containing 0, 0.1, 1, 10, 100ng/ml of EGF. About 10^4 PDLFs were grown on a 96-well plate in 200 ㎕ α-MEM/10% FBS for 3 days. When PDLFs were fully filled in assay plate, the culture media was replaced with 200㎕ α-MEM/2% FBS containing 0, 0.1, 1, 10, 100ng/ml of EGF. Cell proliferation was measured by

MTT assay every 24 hrs for 3 days. Every 24 hrs the culture media was replaced with 200㎕ of fresh medium and 50㎕ MTT and incubated at 37℃ in culture incubator for 4 hrs. The purple formazan product was dissolved in 200㎕ of organic solvent for 5-10 min at room temperature. The plate was read on a spectrophotometer(ELISA reader) at 570nm and 630nm. The optimum EGF concentration that showed no toxic effect and maximum proliferation of PDLFs was determined from this assay.

3) Experimental grouping

Control group was incubated in α-MEM/2% FBS without EGF. And experimental group was incubated in the same media containing 10ng/ml EGF, which was determined from MTT assay.

4) Proliferation rate of PDLFs

About 2×10^4 cells were plated on culture dishes containing α-MEM/10% FBS and were incubated overnight to allow attachment prior to the addition of EGF. After 24 hrs of incubation period, fresh α-MEM/2% FBS containing 10ng/ml EGF was replaced in experimental group and same media without EGF was replaced in control group. Not to alter the concentration of EGF, this assay was performed without change of culture media for 5 days. At 1, 3 and 5 days of incubation, cells of both groups were harvested by trypsinization and counted in a hemocytometer (Superior Co., Germany).

5) Fibronectin expression (western blot)

After incubating period of 3 days in control and experimental group, cultured soup was used for western blotting experiment. The protein concentration was determined using BCA protein assay. It was then mixed with sample buffer containing SDS and β-mercaptoethanol and was boiled at 95℃ for 5 min. For western blot analysis, protein extracts were resolved by 6% SDS-polyacrylamide gel (Bio-Rad Co., Hercules, CA, U.S.A) at 80 volt for 12 hrs and transferred onto nitrocellulose membrane(Bio-Rad Co., Hercules, CA, U.S.A). The blot was incubated in a blocking solution containing 5% dry milk on phosphate buffered saline plus 0.1% Tween-20 (Sigma Chemical Co., St Louis, U.S.A.) at RT for 2 hrs. The blot was then probed with the primary antibody (rabbit polyclonal antibody of fibronectin) overnight at 4℃, followed by detection with horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotech Inc., U.S.A.) and ECL Western Blotting Detection System (Amersham Co., Arlington Heights, IL, U.S.A). The blots then were exposed to a film in the dark room. Degree of fibronectin expression was evaluated with band intensity on the film using MetaMorph Analyzing System (Universal Imaging Co., U.S.A.).

6) Data analysis

Statistical significances of differences between groups of observations were tested using the Kruskal-Wallis and Mann-Whitney test at 95% significance level.

Ⅲ. RESULTS

1. Cell toxicity test

From the MTT assay, there was no cellular toxic effect of EGF on PDLFs(Table 1, Fig. 1). And maximum cellular proliferation was reached at 10ng/ml EGF on 2nd and 3rd day of MTT assay, but there was not statistically significant. From this test, the concentration of EGF for the following cell culture study was determined at 10ng/ml, which showed maximum PDLFs activity.

Table 1. Result of MTT assay with EGF on the proliferation of hPDLFs.

Concentration of EGF (ng/ml)	Absorbance (Mean ± SD)	
	after 2 days	after 3 days
0	0.779 ± 0.035	0.771 ± 0.039
0.1	0.709 ± 0.019	0.986 ± 0.040
1	0.721 ± 0.097	1.078 ± 0.099
10	0.803 ± 0.082	1.144 ± 0.074
100	0.662 ± 0.050	0.686 ± 0.026

2. Proliferation test

PDLFs proliferation of control and study group was compared on 1st, 3rd and 5th day of incubation. The proliferation of experimental group, cultured in the media containing 10ng/ml EGF, significantly increased from baseline at 1st, 3rd and 5th day but not significant in control group. The degree of proliferation of experimental groups was significantly enhanced on 1st, 3rd and 5th days compared to that of control group(Table 2, Fig. 2).

3. Fibronectin expression

After incubating control and experimental group for 3 days, cultured soup was used for protein analysis(western blotting experiment). The expression rates of fibronectin acting on PDLFs attachment were compared to evaluate the effect of EGF on attachment. Fibronectin expression was significantly increased in the media containing 10ng/ml EGF compared to control group(Fig. 3, Table 3).

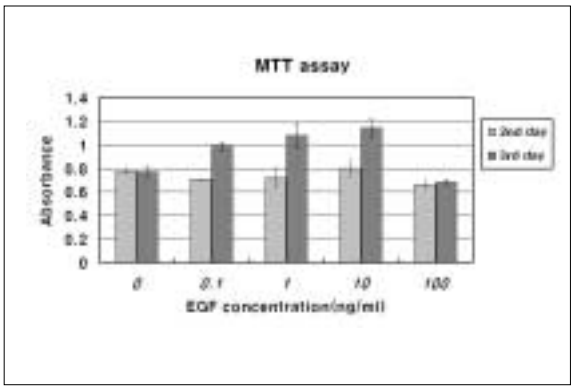

Fig. 1. Result of MTT assay with EGF on the proliferation of hPDLFs.

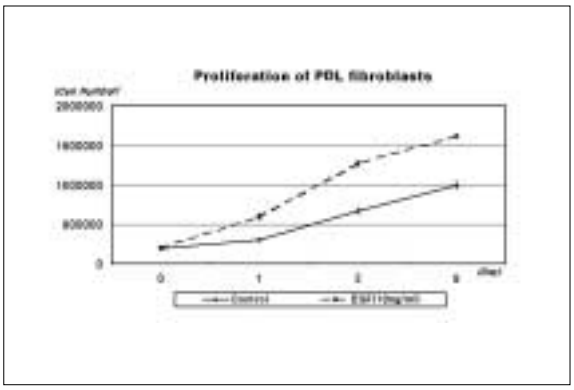

Fig. 2. Comparison of proliferation between control and experimental groups.

Table 2. Comparison of proliferation between control and experimental groups *(unit:10^5)*.

Concentration of EGF (ng/ml)	number of cells (Mean ± SD)			
	Baseline	after 1 day	after 3 days	after 5 days
0	1.94 ± 0.10	3.07 ± 0.21	6.73 ± 0.36	10.00 ± 0.51
10	2.07 ± 0.06	6.00 ± 0.39*#	12.7 ± 0.30*#	16.20 ± 0.24*#

* Statistically significant from baseline at $p<0.05$
\# Statistically significant from 0ng/ml at $p<0.05$

Table 3. Effect of EGF on fibronectin expression with the method of western blot.

	Densitometric analysis (Mean ± SD)	
	EGF(-)	EGF(+)
soup(μm^2)	11.506 ± 1.55	18.848 ± 0.468*

* Statistically significant at $p<0.05$

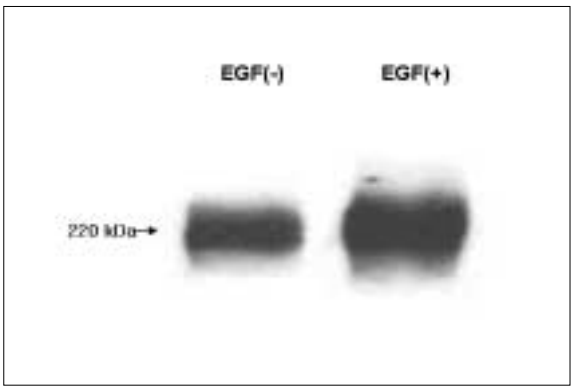

Fig. 3. Effect of EGF on fibronectin expression of PDLFs.

Ⅳ. DISCUSSION

The periodontal ligament is a specialized connective tissue having functions to attach the tooth to the alveolar bone[19]. Fibroblasts, the major cell type of periodontal ligament, form a heterogeneous population that can differentiate into either cementoblasts or osteoblasts, depending on needs and conditions[20]. Periodontal ligament is a dense connective tissue between the root cementum and the alveolar bone that holds the tooth and maintains the structural integrity of this mineralized tissue and has a role in accelerating injured tissue repairments[21].

Proper periodontal tissue regeneration could be achieved by replantation within 30 min after traumatic avulsion of tooth or by placing the tooth in a suitable storage medium until the patient could be seen by a dentist for replantation[22]. The optimal storage medium should be able to preserve the viability, mitogenicity and clogenic capacity of the injured PDLF and their progenitors. The quick repopulation of denuded root surface with PDLF and the prevention of osteoclasts attaching the cementum would be essential for good prognosis[23].

The frequent complications following replantation of a tooth are inflammatory and replacement root resorption, which often leads to the loss of tooth[24]. Replacement resorption is an irreversible process that is related to the death of periodontal ligament cells and cementum. Replacement resorption occurs when the damage to the periodontal tissues has caused an area of resorption that is not healed by secondary cementum but by the bone. Osteoblasts directly form bone tissue on the dentine at the root surface and the tooth becomes involved in the remodeling process of the alveolar bone which is progressively resorbed.

The important factors that may affect replacement resorption are extra-alveolar time and storage condition of avulsed tooth, especially dry storage time[25]. Studies have shown that storage conditions and type of storage media affect the viability of periodontal ligament cells[26-28]. Exposure of PDL cells to saliva lead to reduce a clogenic capacity of only 1.5% and cold milk or cold HBSS to 3% by 60 minutes, while these media maintains the vitality of PDL cells for several hours[8].

In order to obtain successful regeneration of injured PDL tissues, clinical approaches have been attempted to accelerate the proliferation of healthy PDLFs with PGFs which locally help tissue repair as the conventional storage media has limitations[29].

Extracellular matrix(ECM) is important in growth, maintenance of morphology and function of cells. It also may regulate the interaction of different cell types[30]. Fibronectin and Laminin are types of ECM. Fibronectin modulate intercellular attachment of PDLFs and it was reported to have cellular specificity and bind to the specific surface receptor of cell. In other words, fibronectin promotes attachment, chemotaxis and growth of mesenchymal cells and inhibits epithelial cell adhesion and growth. Laminin promotes adhesion, chemotaxis and growth of epithelial and endothelial cell and inhibits fibroblasts adhesion and growth[31]. This shows that laminin and fibronectin act oppositely with cellular specificity. *In vivo* study, fibronectin has been shown to stimulate wound repair probably by preferentially stimulating the attachment, growth and matrix production of fibroblasts, suggesting that fibronectin may induce regeneration of PDLFs[11,21].

In this study, the effects of EGF on the proliferation and attachment of PDLFs were evaluated to verify the efficacy of EGF as a storage media or pre-replantation conditioner of avulsed tooth. From this study, EGF has no toxic effect on PDLFs, and the optimal cellular concentration of EGF on PDLFs was determined at 10ng/ml. Matsuda et al. reported that mitogenic effect of PDLFs was increased at 10ng/ml EGF[22,32]. Carpenter and Cohen reported that the concentration of hEGF in human saliva ranged from 6-17ng/ml[33]. Proliferation rate of experimental group, cultured in the media containing 10ng/ml EGF, was significantly increased from baseline at 1st, 3rd and 5th days in the proliferation test but not significantly increased that in control group. The degree of proliferation of experimental groups on 1st, 3rd and 5th days was significantly enhanced compared to that of control group. The expression rate of fibronectin functioning on PDLFs attachment was compared to evaluate the effect of EGF on attachment. The expression of fibronectin was significantly increased in the media containing 10ng/ml EGF compared to that of control group. We can be suggested that EGF in-

creased the expression of fibronectin and may affect the regeneration and reattachment of injured PDLFs. In conclusion, EGF had no toxic effect on PDLFs. And EGF effectively accelerated the cellular proliferation and the intercellular attachment of PDLFs.

Additional research is needed to verify the effects of EGF on the micro-structure of PDLFs and on the regeneration and attachment of PDLFs to the traumatic tooth injury *in vivo*.

From this study we could conclude that EGF had no toxic effect on PDLFs. The maximum cellular proliferation of PDLFs was reached with 10ng/ml EGF. This concentration of EGF enhanced the proliferation of PDLFs with statistical significance($p<0.05$). From the protein analysis, 10ng/ml EGF increased the expression of fibronectin out of PDLFs participating in intercellular attachment with statistical significance($p<0.05$).

Ⅴ. CONCLUSIONS

The present study demonstrated the effect of EGF on proliferation and attachment of PDLFs. EGF was proved to have no toxic effect on PDLFs through MTT assay. To study the effect of EGF on proliferation and intercellular attachment of PDLFs, proliferation rate and fibronectin expression of the control and study group were compared.

1. EGF showed no toxic effect on PDL fibroblasts. The highest proliferation was reached at 10ng/ml.
2. Degree of proliferation of PDL fibroblasts was significantly high for EGF study group at 10ng/ml.
3. No morphological difference was noted at 10ng/ml between EGF study group and control group.
4. Expression of fibronectin participating in intercellular attachment was significantly high for EGF study group at 10ng/ml.

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Abstract

THE EFFECTS OF EGF ON PROLIFERATION AND ATTACHMENT OF HUMAN PERIODONTAL FIBROBLASTS

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In considering the healing process of injured periodontal tissue, healing rate would be influenced by the cellular activity of periodontal fibroblasts(PDLFs). In addition, the reattachment among PDLFs should be induced for healing process. The purpose of this study was to evaluate the effects of epidermal growth factor(EGF) on the proliferation and attachment of PDLFs and to verify the efficacy of EGF as a storage media or a pre-replantation conditioner of traumatically avulsed tooth.

Human recombinant epidermal growth factor(hrEGF) and human periodontal fibroblasts from first premolar were prepared. At first, MTT assay was done to evaluate the toxic effect on human periodontal fibroblast and the maximum cellular growth of EGF. Cellular proliferation rate was then compared between control group and 10ng/ml EGF added group. Also, western blot was done to evaluate the expression of fibronectin in both groups. The results were as follows:

1. From MTT assay, EGF showed no toxic effect on PDL fibroblasts. The highest proliferation was shown at 10ng/ml EGF.
2. In 10ng/ml EGF added group, the degree of proliferation of PDLFs was significantly higher than that in control group.
3. Fibronectin expression of EGF added group was also significantly higher than that of control group.

From this study we could conclude that EGF enhanced the regeneration rate of periodontal fibroblast, which could be used as a pretreatment agent or a storage media for traumatically avulsed teeth.

Key words : EGF, Fibronectin, PDL fibroblasts, Tooth avulsion