

EPIDERMAL GROWTH FACTOR PROMOTES THE PROLIFERATION AND DIFFERENTIATION OF PROGENITOR CELLS DURING WOUND HEALING OF RAT SUBMANDIBULAR GLANDS

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ABSTRACT

Background and Aim: The objective of this study was to develop an animal model of surgically wounded submandibular glands (SMGs) and to investigate the effects of a collagen gel containing epidermal growth factor (EGF) on the tissue regeneration of surgically wounded SMGs in vivo.

Material and Method: The animal model was produced by making a surgical wound in rat SMGs using a 3 mm diameter biopsy punch. Each wound was then filled with a collagen gel without EGF (Control group) or with EGF (EGF group).

Results: In surgically wounded SMGs, smaller collagen gels were observed in salivary gland tissue without scar tissue around the wound area. At after operation day (AOD) 5, small round cells and spindle-shaped cells had invaded the collagen gels in both groups and their numbers were dramatically increased at AOD 7. At AOD 14, the collagen gels were almost completely replaced by the host tissue. At AOD 7, invading cells in the EGF group were positive for α SMA, CD49f, c-kit and AQP5. Similarly, mRNA expression levels of α SMA, CD49f, keratin19 and AQP5 were increased.

Conclusion: This study suggests that using collagen gels containing EGF improves the potential for salivary gland regeneration.

Clin Dent Res 2016; 40(3): 87-94

Keywords: EGF, Progenitor Cell, Regeneration, Tissue Engineering, Wound Healing

Submitted for Publication: 03.25.2016

Accepted for Publication : 07.29.2016

EPİDERMAL BÜYÜME FAKTÖRÜ SIÇAN SUBMANDİBULAR TÜKRÜK BEZLERİNDEKİ YARA İYİLEŞMESİ SÜRECİNDE PROGENİTÖR HÜCRELERİN ÇOĞALMA VE FARKLILAŞMASINI UYARIR

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ÖZ

Amaç: Bu çalışmanın amacı cerrahi olarak yara bölgesi oluşturulmuş submandibular tükrük bezleri bulunan hayvan modelleri oluşturmak ve epidermal büyüme faktörü içeren kollajen jelin submandibular bezlerdeki cerrahi yara bölgeleri üzerindeki doku rejenerasyonuna olan etkilerini in vivo olarak araştırmaktır.

Gereç ve Yöntem: Hayvan modeli, sıçan submandibular tükrük bezinde 3 mm çapında punch biopsisi ile yara bölgesi oluşturularak elde edildi. Oluşturulan yara bölgeleri Epidermal Büyüme Faktörü içeren (EGF grubu) ve içermeyen (kontrol grubu) kollajen jel ile dolduruldu.

Bulgular: Cerrahi olarak yara bölgesi oluşturulan submandibular tükrük bezlerinde, yara bölgesinde skar dokusu oluşmaksızın tükrük bezi dokularında daha küçük kollajen jeller gözlemlendi. Operasyondan sonraki 5. günde her iki grupta da küçük yuvarlak hücrelerin ve iğne biçimli hücrelerin kollajen jel içerisine invaze olduğu ve operasyondan sonraki 7. günde sayılarının önemli ölçüde arttığı görüldü. Operasyondan sonraki 14. günde kollajen jelin neredeyse tamamı konakçı hücreleriyle yer değiştirdi. Operasyondan sonraki 7. günde EGF grubuna invaze olan hücrelerde α SMA, CD49f, c-kit ve AQP5 pozitifliği. Benzer şekilde α SMA, Cd49f, keratin 19 ve AQP5'in mRNA ekspresyonu da arttı.

Sonuç: Bu çalışma Epidermal Büyüme Faktörü içeren kollajen jel kullanmanın tükrük bezi rejenerasyon potansiyelini arttıracak olduğunu önermektedir.

Clin Dent Res 2016; 40(3): 87-94

Anahtar Kelimeler: EGF, Progenitör hücre, Rejenerasyon, Doku Mühendisliği, Yara İyileşmesi

Yayın Başvuru Tarihi : 25.03.2016

Yayına Kabul Tarihi : 29.07.2016

INTRODUCTION

Glandular tissues, including salivary glands, have weak regenerative capacities at the site of tissue damage since they are composed of well-differentiated epithelial cells.¹⁻³ When salivary glands are damaged, the resulting hyposalivation disturbs pronunciation and the ability to swallow, thus hindering its function as a lubricant and its role in enzymatic digestion.⁴⁻⁶ Moreover, saliva contains antibacterial agents and antibodies that not only prevent microbial proliferation in the oral cavity but also play a key role in maintaining homeostasis in the oral cavity, such as pH maintenance, due to its buffering action.⁴⁻⁸ The loss of those functions significantly affects a patient's quality of life (QOL),¹⁻³ and therefore, finding strategies to regenerate damaged salivary gland tissues is an important challenge.

There are some wound models which represent the pathological condition of decreased saliva secretion in submandibular glands (SMGs), such as duct ligation, radiation and surgical wounding. In the surgical wound model, the wound is made by cutting parenchymal tissue, so changes in the cellular composition in the wound area are attributed to the surrounding connective tissues rather than to the SMG cells.^{9, 10} In the case of primary healing, such as the surgical wound model, the wound healing starts with a vascular response followed by cell migration into the wounded area. This is followed by the infiltration of inflammatory cells and degenerative changes of the acinar cells.^{9, 10} After that, intercalated ducts, which are usually positive for stem cell markers and myoepithelial cell markers, start to proliferate and differentiate into acinar cells.^{9, 10} However, it has been reported that when secondary healing occurs in salivary gland tissues, it usually heals with scar tissue without regeneration of the salivary gland tissue¹¹ so it is necessary to use a tissue engineering technique to obtain cells which are in the salivary gland in an appropriate three dimensional configuration. Surgical treatment of salivary glands sometimes results in secondary healing and functional disorders. We hypothesized that in order to get regenerative tissue rather than scar tissue, a method to increase the infiltration of fibroblasts from the surrounding connective tissue into the wound area, and to accelerate the differentiation of progenitor cells, is needed. Therefore tissue engineering techniques, including cells, scaffolds and growth factors, should be employed.

A variety of growth factors are involved in the regeneration and wound healing of organs. Epidermal growth factor

(EGF) was originally detected and isolated from mouse salivary glands by Cohen.¹² EGF is a single-chain polypeptide of 53 amino acids. The EGF ligand/receptor system plays a crucial role during organ development, morphogenesis, repair and epithelial regeneration.¹³⁻¹⁸ It has been reported EGF is activated when SMGs are developing and it promoted branching in an in vitro study.^{19,20}

We have been reported the surgical wound model is useful for observation of cells during wound healing.²¹ Furthermore, it has been reported cells, which were expressed in the wound healing, have an ability for the differentiation to the acinar cell.²¹

The aim of this study was to investigate the tissue regeneration of surgical wounded salivary glands using EGF in a collagen gel in vivo.

MATERIALS AND METHODS

1. Animals

This study was conducted in compliance with the Guidelines for the Treatment of Experimental Animals at the Tokyo Dental College (Approval Number 243208). Thirty adult male Sprague-Dawley rats, each weighing about 200 g (Sankyo Lab Service, Tokyo, Japan), were used in this study. During the experimental periods, none of the animals were infected or died.

2. Wound model in SMGs

According to the protocol of Kobayashi et al.²¹, we developed an in vivo wound model in rat SMGs (Figure 1. A, B). Rats were anesthetized by intraperitoneal injection of thiopental (0.2 mL/100 g; Ravonal; Mitsubishi Tanabe, Osaka, Japan). A skin incision, approximately 2 cm in length, was made along the center of the anterior neck using a surgical knife. Both sides of the SMGs were exposed, and a wound that passed through the SMGs was created using a 3mm diameter biopsy punch (Kai Industries, Gifu, Japan), without injuring the principal artery and main duct. Bleeding was arrested with gauze, and collagen gels with EGF (EGF group) or without EGF (control group) by inserting them into the wounds. Wounds with collagen gels were covered with a Gore-Tex membrane (GORE, Tokyo, Japan) to prevent the migration of surrounding fibroblasts into the collagen gel.

3. Preparation of EGF in the collagen gel

Twenty µg recombinant EGF powder (20 µg) (Pepro Tech Inc, Rocky Hill, NJ, USA) was dissolved in 1 ml phosphate-buffered saline (PBS) to make the EGF stock solution (20 µg/

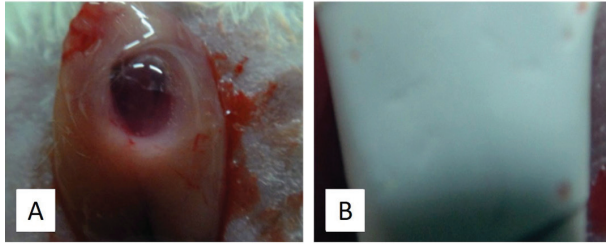


Figure 1. A cylindrical defect that passed through the SMG was made using a 3 mm diameter biopsy punch (A). After arrest of the bleeding, collagen gels were placed in the wounds (A). Finally, salivary glands were over-molded with a Gore-Tex membrane (B).

ml). This EGF solution was diluted 1000-fold in the collagen gel solution described above to a final concentration of 20 ng/ml EGF in the collagen gel.

4. Histological observations

Two rats in each group were euthanized at specified times after the operation days (AOD) 5, 7, 10 and 14 (n=16). Animals were anesthetized by an intraperitoneal injection of thiopental (Ravonal; Mitsubishi Tanabe, Osaka, Japan) and perfusion fixation was performed by transcardial injection of 10% neutral buffered formalin for 1 hr. After the perfusion fixation, SMGs were removed by mechanical means and were immersed in the same fixative solution for 24 hr at room temperature. The specimens were dehydrated in ethanol before being embedded in paraffin. Paraffin sections, 3 μ m in thickness, were cut horizontally using a sliding microtome. For light microscopy observations, paraffin sections were stained with hematoxylin and eosin (HE).

5. Immunohistochemical observations

Paraffin sections of specimens at AOD 5 and 7 were used for immunohistochemical observation. After being deparaffinized with xylol, they were microwaved with a 0.01 M citric acid buffer solution (pH 6.0) for 15 min at 65°C for antigen retrieval. Sections were incubated in 3% hydrogen peroxide with methanol for 30 min at room temperature to block endogenous peroxidase activity. To block non-specific binding, the sections were treated with 3% bovine serum albumin for 10 min at room temperature. The monoclonal antibody supplied in the kit was used as the primary antibody. The sections were incubated at 4°C overnight and were then incubated with a biotinylated secondary antibody, NICHIREI-Histofine simple-stain MAX-PO® (Nichirei, Tokyo, Japan), for 30 min at room temperature. Thereafter, the sections were rinsed with PBS and stained

with NICHIREI-Histofine simple-stain Diaminobenzidine® (Nichirei) and counterstained with hematoxylin. Specimens were observed by light microscopy (Axio-photo 2; Carl Zeiss, Oberkochen, Germany). Antibodies to vimentin (Dako, Glostrup, Denmark; diluted 1:100) as a fibroblast marker, α -smooth muscle actin (α SMA) (Santa Cruz Biotechnology, Dallas, US; diluted 1:50) as a muscle marker, Pan-cytokeratin (Pan-CK) (Abcam, Cambridge, UK; diluted 1:40) as an epithelial cell marker, CD49f (Santa Cruz Biotechnology, diluted 1:50) as a progenitor cell marker, c-kit (Santa Cruz Biotechnology; diluted 1:50) as a progenitor cell marker, and aquaporin 5 (AQP5) (Abcam, diluted 1:500) as an acinar cell marker, were used as primary antibodies. Immunopositive cells for those antibodies were counted using an Axio microscope system in the collagen gel of each group.

6. Quantitative RT-PCR

For messenger RNA (mRNA) expression studies, total RNAs were extracted using the acid guanidium thiocyanate/phenol chloroform method as follows. Four rats of each group were sacrificed at AOD 7, and the SMGs were removed mechanically (n=8). The collagen gels were washed with PBS and were then mechanically removed from the salivary glands using a stereoscope. The collagen gels were homogenized in 500 ml TRIzol Reagent (Invitrogen Corp, Carlsbad, CA, USA), according to the manufacturer's instructions, and total RNA was reverse-transcribed to complementary DNA (cDNA) using a QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD, USA). Quantitative RT-PCR was carried out using TaqMan Gene Expression Assays (Applied Biosystems, Life Technologies Corp, Carlsbad, CA, USA) for the target genes: vimentin, α SMA, keratin 13 (expressed in differentiated epithelium such as ductal cells), keratin19 (expressed in undifferentiated epithelium such as ductal cells and basal cells), CD49f (expressed in progenitor cells), AQP5 (expressed in acinar cells) and GAPDH (endogenous control) using the primers shown in Table 1. All PCR reactions were performed using a real time PCR 7500 fast system (Applied Biosystems). Expression levels of mRNAs of interest were normalized against the expression of GAPDH and are designated as an expression coefficient.

7. Statistical analysis

Quantitative data are presented as means $\pm$ standard deviation and were analyzed using Mann-Whitney's U test by the MS Excel 2008 add-in. Differences where the p value is <0.05 are considered to be statistically significant.

Table 1. Primers used for real-time reverse transcription-polymerase chain reaction

Primer	Gene Name	Assay ID
Cytokeratin 13	keratin 13	Rn01464229_ml
Cytokeratin 19	Keratin 19	Rn01496867_ml
CD49f	integrin alpha6	Rn01512708_ml
α SMA	smooth muscle alpha-actin	Rn01759928_gl
vimentin	vimentin	Rn00579738_ml
Aquaporin5	aquaporin5	Rn00562837_ml
GAPDH (endogenous control)	glyceraldehyde-3-phosphate dehydrogenase	Rn01775763_gl

RESULTS

1. Wound healing is accelerated by EGF

In low power fields of both groups at AOD 5 (Figure 2 A, K), the collagen gel was observed in its original state in the wound area. At AOD 5 and AOD 7 (Figure 2 B, L), numerous cells were observed indicating increased cell infiltration. At AOD 10 (Figure 2 C, M), large numbers of cells had infiltrated into the collagen gel, such that the collagen gel and host tissue could not be distinguished clearly. From AOD 14 (Figure 2 D, N) onward, significant absorption of the collagen gel was observed, and it was almost completely replaced by the host tissue. During this period, although the collagen gel was absorbed and replaced by the host tissue, salivary gland tissue was not observed in the collagen gel. Although the volume of the collagen gel in the wound for each group became progressively smaller, collagen gels in the EGF group were significantly smaller compared to the Control group at each of the time periods (Figures 2, 3).

At high power magnification, oval-shaped cells and spindle-shaped cells were observed near the edges of the wounded salivary glands in the collagen gel at AOD 5 in the Control group (Figure 2 A, F). At AOD 7, the number of spindle-shaped cells had increased in the collagen gel, especially in the inner area of the collagen gel (Figure 2 B, G). At AOD 10, the numbers of oval-shaped cells and spindle-shaped cells in the collagen gel had increased. There was a high density of these cells in the collagen gel (Figure 2 C, H). A decrease in the size of the collagen gel could be observed clearly. At AOD 14, the remaining collagen gel was quite small (Figure 2 D, I).

At high power magnification, many cells were seen in the collagen gel of the EGF group compared to the Control group at AOD 5 (Figure 2 K, P). Many cells that were observed in

the collagen gel were spindle-shaped cells. At AOD 7, cells had infiltrated into the collagen gel uniformly (Figure 2 L, Q). At AOD 10, there were aggregated cells in the collagen gel and the area between the collagen gel and salivary gland tissue could not be seen clearly (Figure 2 M, R). At AOD 14, the collagen gel had almost disappeared and was replaced with host tissue (Figure 2 N, S).

2. Characterization of cell types in the wound area

Myoepithelial cells

At AOD 5, spindle-shaped cells located in the peripheral area of the collagen gel were positive for vimentin (Figure 3 A) and α SMA (Figure 3 B).

At AOD 7, numerous spindle-shaped cells were positive for vimentin (Figure 3 M) and α SMA (Figure 3 N) positive cells were observed in the peripheral area and the entire area of the collagen gel at AOD 7. In addition, spreading of those cells was observed.

Progenitor cells

Although cells positive for CD49f (Figure 3 C) and c-kit (Figure 3 G) were not observed at AOD 5, at AOD 7, oval-shaped cells and a few spindle-shaped cells stained positive for CD49f (Figure 3 O) and c-kit (Figure 3 S). At AOD 5 in the EGF group, cells positive for CD49f (Figure 3 F) and c-kit (Figure 3 J) were seen. These cells decreased compared with the Control group at AOD 7 (Figure 3 R, V).

Ductal epithelial cells

At AOD 5 in the Control group (Figure 3 H), cells positive for Pan-CK were not observed, however oval-shaped Pan-CK positive cells were observed in the EGF group (Figure 3 K). At AOD 7, the number of Pan-CK positive cells was slightly

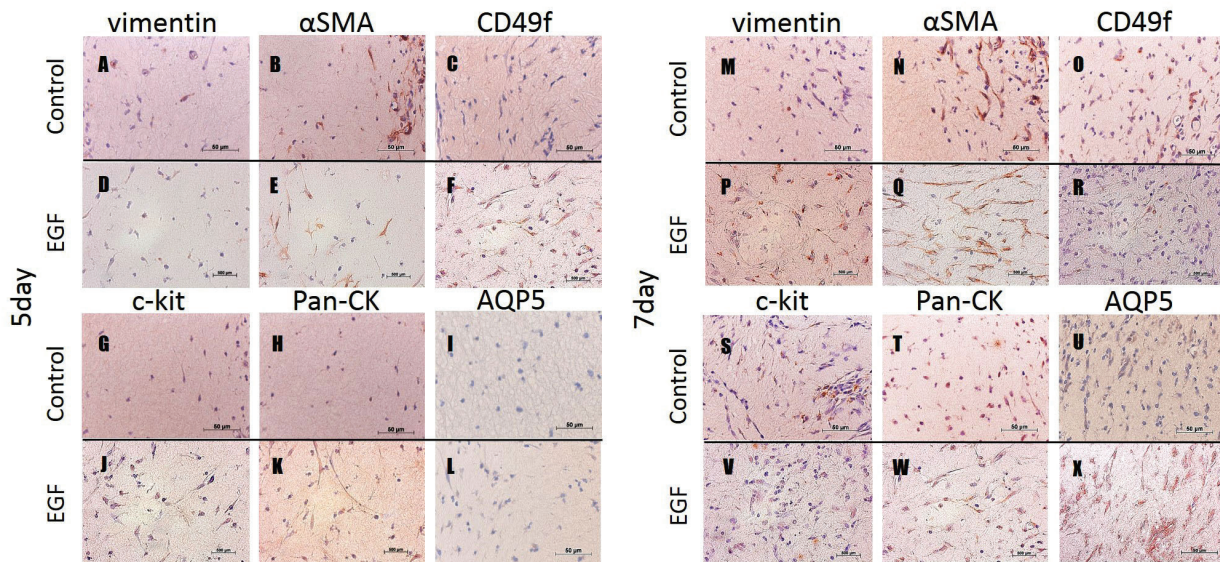


Figure 2. Immunohistochemical staining (original magnification $\times 400$). Control group (A-C, G-I, M-O, S-U) and EGF group (D-F, J-L, P-R, V-X). Collagen gel stained with antibodies to vimentin, α SMA, Pan-CK, CD49f, c-kit and AQP5 as noted at AOD 5 (A-L) and AOD 7 (M-X). In the Control group at AOD 5, vimentin and α SMA positive cells were observed in the peripheral areas of the collagen gel and Pan-CK, CD49f, c-kit and AQP5 positive cells were not observed. In the EGF group at AOD 5, CD49f and c-kit positive cells were observed but AQP5 positive cells were not observed. In both groups at AOD 7, α SMA positive cells were observed in the inner area of the collagen gel. Vimentin, Pan-CK, CD49f, c-kit and AQP5 positive cells were observed in peripheral areas of the collagen gel.

increased (Figure 3 T, W).

Acinar cells

At AOD 5, neither group had AQP5 positive cells (Figure 3 I, L). However, at AOD 7, many cells positive for AQP5 were observed in the peripheral area of the collagen gel (Figure 3 U, X).

3. Effect of EGF on cell proliferation and differentiation

Based on the immunohistochemical observations at AOD 7, the ratio of positive cells for α SMA, pan-CK and AQP5 were significantly higher in the EGF group compared to the Control group. Further, the ratio of positive cells for CD49f and c-kit were lower in the EGF group compared to the Control group (Figure 3).

The mRNA expression levels of α SMA, keratin 19, keratin 13 and AQP5 were also significantly higher in the EGF group compared to the Control group at AOD 7, and CD49f was lower in the EGF group compared to the Control group (Figure 4).

DISCUSSION

In the case of salivary gland resection, the resected area is generally filled with a muscular scar tissue that decreases the function of salivary gland. In the present study, it was observed that the surgical wound model is an appropriate model to evaluate wound healing in salivary glands in

agreement with previous studies.²¹ In this method, the healing with scar tissue formation can be prevented.

In this study, we analyzed the expression of genes and proteins that are affected by EGF during wound healing using a surgical wound model in rat SMGs.

Intercalated ducts, striated ducts, excretory ducts, myoepithelial cells and progenitor cells (except acinar cells) should be engaged with the wound healing of salivary glands.²² Salivary gland tissue is composed of myoepithelial cells, fibroblasts, epithelial cells, acinar cells and progenitor cells. There are well-known specific markers for myoepithelial cells, epithelial cells and fibroblasts. CD49f and c-kit are progenitor cell markers of the salivary gland. It has been reported that cells positive for those markers increased when damaged salivary glands were regenerating.^{23,24} Furthermore, these cells are pluripotent.^{25,26} One study revealed that cells expressing those progenitor cell markers are located in the regenerating salivary gland, where it has been suggested that cells positive for these markers are related to the regeneration of salivary glands.

Acinar cells are particularly necessary for the regeneration of salivary glands. The aquaporin expressed in salivary glands is AQP5, which is expressed in acinar cells of those glands.²⁷ Furthermore, AQP5 is expressed during ductal formation, which has the ability to differentiate to acinar

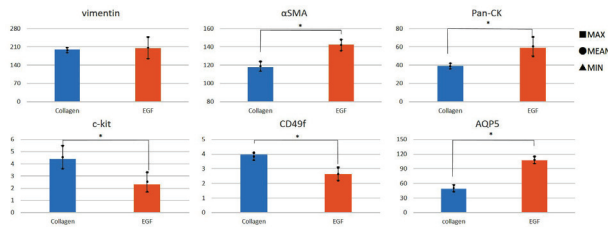


Figure 3. Immunopositive cell ratio. At AOD 7, α SMA, Pan-CK and AQP5 showed a significantly higher ratio in the EGF group compared to the Control group. CD49f and c-kit was significantly lower in the EGF group compared to the Control group. Data represent means $\pm$ SD; * $P < 0.05$.

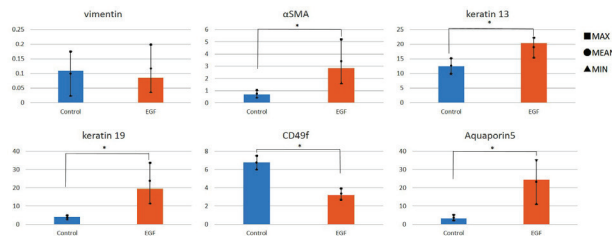


Figure 4. mRNA expression levels At AOD 7, RT-PCR analysis indicated that the mRNA expression levels for α SMA, keratin 13, keratin 19 and AQP5 were significantly higher in the EGF group compared to the Control group. CD49f mRNA was significantly lower in the EGF group compared to the Control group. Data represent means $\pm$ SD; * $P < 0.05$.

cells.²⁷ What is more, the importance of AQP5 in saliva secretion for water movement across the plasma membrane has been well established.²⁸ The reported reduction in AQP5 expression in ligated glands is in agreement with the defective fluid secretion for atrophic glands, but AQP5 expression is recovered with salivary gland regeneration, thus it is thought that AQP5 could be used as an index for salivary gland regeneration after injury.²⁹

It has been reported that EGF promotes the proliferation of myoepithelial cells and ductal cells of salivary glands.³⁰ Furthermore, EGF promoted the differentiation of progenitor cells to acinar cells in an *in vitro* study.³¹ It is known EGF promotes cell proliferation and differentiation, however, over-expression of EGF inhibits those processes.^{32, 33} In this study, the concentration of EGF used (20 ng/ml) is the same as used in an *in vitro* study reporting a three dimensional cell culture model.^{34, 35} It was suggested that cells which express EGF into the collagen gel promote proliferation and differentiation, which is consistent with the results of our study in this wound model. The results of the immunohistochemical study and RT-PCR analysis showed that cells positive for Pan-CK, α -SMA and AQP5 were increased in the EGF group compared with the

control group as their mRNA expression levels. Moreover, cells positive for CD49f and c-kit were decreased in the EGF group compared with the control group. Corentin Cras-Me'neur et al.³¹ have reported that EGF effects progenitor cells to proliferate and to keep pluripotency until 7 days, after 7 day, it effects these cells to differentiate into acinar cells in the *in vitro* study. In this study, we have got similar results. This suggests that EGF in the collagen gel promotes the proliferation of ductal cells and myoepithelial cells and stimulates the differentiation of progenitor cells to acinar cells.

Furthermore, AQP5 positive cells were observed in the collagen gel at AOD 7 and this may suggest that regenerated intercalated duct cells might differentiate into acinar cells.

Thus, we have developed a salivary gland surgical wound model, which is suitable for observation of the wound healing process. Taken together, collagen gel scaffolds induce the proliferation of ductal cells, myoepithelial cells and progenitor cells, but not their differentiation to acinar cells. However, in this study, which included EGF in the collagen gel, many AQP5 positive cells were observed and this may suggest the occurrence of acinar cell regeneration.

CONCLUSION

This study suggests that using collagen gels with EGF for the repair of salivary gland injury improves the potential for salivary gland regeneration.

ACKNOWLEDGEMENTS

The authors would also like to acknowledge all contributors to the field whose work could not be included due to space constraints.

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