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Comparison of EGF and bFGF Expression *In Vivo* and their Effect *In Vitro*

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Abstract

Oral mucosal wound healed faster and with minimal scar formation compared with dermal wounds. The present study tried to found useful information in scarless oral wound healing process. This study firstly evaluated the effect of EGF and bFGF on the proliferation and migration of isolated fibroblasts from oral mucosa and skin. Then a liner wound was made in SD rats on transplanted oral mucosa and control skin. *In vitro* study found that both bFGF and EGF could promote the migration of dermal fibroblasts but not for oral fibroblasts. The oral fibroblasts proliferation was more sensitive to different concentrations of EGF, while bFGF had no significant effect on cell proliferation in this experiment. Our in vivo results demonstrated that oral mucosal wound healed with minimal scar after transplanting to skin and expressed higher EGF. No regular bFGF expression was found either on the transplanted oral mucosal or dermal wound. The results suggested that oral mucosa healed with minimal scar mainly due to its inherent cell phenotypes. The results also suggested the important role of EGF played in scar formation. The ultimate goal of this study is to apply this knowledge to find new ways to reduce scar formation in dermal wound.

Keywords: Inflammation; Healing; Wounds; Remodelling

Introduction

Scar formation in skin is a common result of the wound healing process. The clinicians have a common impression that human oral mucosal wounds heal faster with minimal scar formation compared with skin wounds [1]. The formal animal studies have indicated that wounds in oral mucosa heal quickly with little inflammation compared with skin [2,3]. So, oral mucosa provide an ideal adult study subject to explore the scarless wound healing. Some studies indicated that the minimal scarring in the oral cavity was due to the presence of bacteria that stimulate wound healing and the moist environment and growth factors present in saliva [1,4]. They have proven that saliva application could enhance skin wound healing and reduce inflammation. While other studies suggested that different cell type especially distinct fibroblast phenotype could contribute to the minimal scarring in oral wound healing process [5].

Wound healing is a dynamic process which is including the interaction between growth factors and the cells in the wound beds. Fibroblasts play an important role in wound healing process. They proliferate and migrate into the wound area and help form a vascular and resistant granulation tissue which protects the wound bed by synthesizing new extracellular matrix, aiding keratinocyte migration and remodelling [6]. Another important element in wound healing process is growth factor. A variety of cytokines and polypeptide growth factors have been shown to play key roles in the dynamic process of wound healing, which include the regulation of inflammation, cell migration, proliferation, and synthesis of matrix proteins [7-9]. Fibroblast growth factor (FGF) family which composed of 23 members was one of the mediators in the dynamic process. FGFs can be produced by keratinocytes, fibroblasts, endothelial cells, smooth muscle cells, chondrocytes, and mast cells [10]. The most important member involved in cutaneous wound healing in FGF family is FGF-2 or basic FGF (bFGF). *In vitro* and *vivo* studies have demonstrated that bFGF can help to significantly decrease inflammation [11,12], as well as regulates the synthesis and deposition of various ECM components and promotes the migration of fibroblasts.

Another best-characterized growth factor in wound healing is EGF family. EGF is secreted by platelets, macrophages, and fibroblasts [13]. Recently, growing studies have demonstrated that EGF/EGFR-signalling plays an important role in the inflammation stage of wound healing [14-20]. For example, Yurtçu M et al. reported that ectogenic EGF can significantly decrease inflammation *in vitro* [15-20]. At the same times, *in vitro* studies have shown that EGF could significantly accelerate re-epithelialization and increase tensile strength in wounds [21]. Clinical trials for chronic wound therapeutics showed that the addition of topical EGF increased epithelialization and shortened healing time in skin wounds [22,23].

Several *in vitro* wound healing models are available [24]. Such models include the *in vitro* scratch assay and transwell test, which have been shown to provide the insights into the process of fibroblast migration, and CCK-8 to test the cell proliferation [25]. A great advantage of these methods is that the concentrations of growth factors can be easily altered *in vitro*. Another important model to study wound healing is animal experiment. In this study, in order to gain a better understanding of the molecular and cellular processes involved in wound healing in oral mucosa and skin, both cell culture and animal wound healing models are created. Firstly, the effect of EGF and bFGF on the proliferation and migration of isolated

fibroblasts obtained from the oral cavity and skin was determined. The hypothesis was that the cell responses to growth factors of mucosal fibroblast were different from those of dermal fibroblasts. Then an animal wound healing model was created to reduce the influence of growth factors in saliva and the wet environment in oral cavity. The oral mucosa was firstly transplanted into the right side of the abdomen of wistar male rate and the incision wounds were made at both sides. The present study tried to found useful information in scarless oral wound healing process.

Materials and Methods

Fibroblast culture, cell migration and proliferation

Human dermal fibroblasts were obtained from the dermis of juvenile human foreskin at the time of circumcision, with consent from the patients, as previously described [26]. Oral fibroblasts were isolated and cultured from gingival biopsies of patients undergoing third-molar extractions [27,28]. All tissue samples were obtained from healthy individuals in compliance with a protocol approved by Medical Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology for the Use of Human Subjects in Research.

The effect of bFGF and EGF on cell migration was determined by transwell assay and scrape-wound healing assay as described before [26]. In transwell assay, 1×10^5 dermal or oral fibroblasts were seeded into the upper chamber of the insert (transwell plates are 6.5 mm in diameter with 8 μm pore filters; Corning Costar, Cambridge, MA), with 300 μL of culture medium in upper chamber and 600 μL medium in lower chamber. After the cells adhered, the medium in the upper chamber was changed with serum-free DMEM/F-12. To determine the respective effects of EGF and bFGF, the culture medium in the lower chamber was changed with 10 ng/mL EGF or 20 ng/mL bFGF which was indicated in our former experiment [29]. According to our preliminary studies results [16,29], we chose to evaluate fibroblasts migration at 24 h (the migration of fibroblasts was most active at this time point.) The migrated fibroblasts were digested by trypsin-EDTA and counted under microscopy. All experiments were donefive times.

In scrape-wound healing assay, fibroblasts were seeded into 12-well plates with culture medium until they reached 80% confluence. These monolayers were then scored with a sterile pipette tip to leave a plus shape scratch of approximately 0.4 mm-0.5 mm in width. Culture medium was then immediately removed and changed with optimal concentration of EGF or bFGF.

Proliferation assays: The effects of the EGF or bFGF on fibroblastsproliferation were determined utilizing CCK-8 assay. Briefly, 2×10^3 fibroblasts were seeded into the 96-well plates for 24 h to adhere. Then the medium was changed with different concentrations of EGF or bFGF at 0 ng/mL, 1 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, and 100 ng/mL respectively. We chose to evaluate the cell proliferation in its logarithmic growth phase (day 3). All experiments were performed five times.

Animal experimentation

24 male SD rats, weighting approximately 180 g, were obtained from Animal Experimental Center and Animal Biosafety Level 3 Laboratory of Wuhan University (Wuhan, Hubei, China). The rats were housed at Animal Experimental Center of Union Hospital, Tongji Medical College of Huazhong University of Science and Technology (Wuhan, Hubei, China) with 12 h light/dark cycles and fed antibiotic-free food and water ad libitum. Firstly, oral mucosa grafting surgery was done to the animals. Briefly, the animals were generally anesthetized and a full-thickness excisional wound (d=1 cm) was created on the left abdominal skin, and an identical wound was created on the oral mucosa of the tongue. The full-thickness mucosal tissue was sutured on the wound of the skin. After 21 days, when the wounds were healed, a new, line-like full-thickness excisional wound (1.5 cm in length) was created on the transplanted mucosal tissue site. As a control, an identical wound was created on the right abdominal skin in the same rat. 24 rats were randomized sacrificedat 6 time points (12 h, 1 d, 2 d, 3 d, 5 d, 7 d, n=4) after wounding and full-thickness tissue biopsies were collected from the wounds including 5 mm of the surrounding tissue. The tissue samples were embedded in 4% paraformaldehyde for histopathology study or in RNA later RNA Stabilization Reagent (QIAGEN; Valencia, CA) for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Tongji Medical College.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

For qRT-PCR analysis, 2 rat/ group was sacrificed at 12 h, 1 d, 2 d, 3 d, 5 d, 7 d post wounding. Following sacrifice, the skin tissue (about 100 mg) of the wound edge were obtained and immediately stained in the RNA later RNA Stabilization Reagent (QIAGEN; Valencia, CA) at 4°C overnight and frozen at 20°C. Total RNA was extracted from the samples using TRIZOL reagent (Invitrogen Life Technologies; Carlsbad, CA) followed by a clean process according to the manufacturer's protocols. The value of OD 260/OD 280 of RNA samples were all greater than 1.8. The synthesis of cDNA was performed by reverse transcription of RNA (1 μg) using Revert Aid First Stranel cDNA Synthesis Kit (Fermentas; US) according to the manufacturer's standard protocol. Real-time PCR analyses were performed in fluorescence thermocycler (Applied Biosystems 7500 Real-time PCR, USA) using specific primers. Reactions were performed in triplicate under the following conditions: an initial denaturation at 95°C for 15 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and elongation at 72°C for 33 s. The cDNA amplification was in a 10ul reaction mixture containing 50 ng cDNA solution, 5μl of SYBR premix EX Taq (TAKARA, Japan), 4 pmol each of the forward and reverse primers, and 2 pmol of Rox Reference Dye II (TAKARA, Japan).

Immunhistochemical analysis

Goat anti-EGF (SC-1343) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit anti-FGF basic (ab16828) antibody was purchased from Abcam (Cambridge, MA, USA).

For immunhistochemical analysis, 2 rats/ group were sacrificed at 12 h, 1 d, 2 d, 3 d, 5 d, 7 d post wounding. Following sacrifice, the wounds of the skin including 3 mm of the surrounding tissue were obtained and routinely processed, and embedded in paraffin by the Department of Pathology of Union Hospital. For immunostaining, EGF (1:50 dilution) and bFGF (1:10 dilution) were performed using the Vectastain ABC Elite Kit and Vector DAB Substrate Kit (Vector Laboratories). To this end, samples were fixed and incubated with the primary antibody as above and with the appropriate biotinylated secondary antibody at room temperature for 1 h. The sections were

then incubated with freshly prepared Vectastain ABC reagent at room temperature for 30 min. DAB substrate was prepared according to manufacturer's instructions and allowed to react with the samples until suitable color development was noted. Control staining was performed by replacing the primary antibody with the phosphate buffer saline (PBS) and gave only a very weak nonspecific background staining.

Statistical analysis

SPSS 16.0 analysis software is used for data analysis. Numeric data are presented as means ± SE (standard error). Two-tailed Student's t tests were used for statistical analyses. Statistically significant difference was set at $p<0.05$.

Results

Migration assay of fibroblasts in the stimulation of EGF and bFGF

To characterize the effects of EGF and bFGF on fibroblasts migration, the responses of fibroblasts to 10 ng/mL EGF and 20ng/mL bFGF were examined by transwell assay. The results showed in Figure 1(A) indicated that both the bFGF and EGF could promote the migration of dermal fibroblasts. However, the migration of oral fibroblasts was not promoted by either of the growth factors.

Simultaneously, the effects on fibroblasts migration were also further confirmed by scrape wound healing assay (Figure 2). The results showed that both EGF and bFGF could promote the cell migration of dermal fibroblasts, but not in oral fibroblasts. The result was in agreement with that of the transwell assay.

Proliferation assay of fibroblasts in the stimulation of different concentrations of EGF and bFGF

To further characterize the effects of the EGF and bFGF on fibroblasts proliferation and to determine the optimal cytokine concentration, we examined the responses of fibroblasts to different concentrations of EGF and bFGF (at 0 ng/mL, 1 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, and 100 ng/mL, respectively.). The results at day 3 were chosen to compare the effects of the cytokines at the respective concentrations, as day 3 was a time point of the logarithmic growth phase during which the cells proliferated significantly.

The results showed in Figure 1(B) indicated that both cells showed a mitogenic response to EGF. Dermal fibroblasts proliferation was promoted by EGF at 10 ng/mL and 100ng/mL. In oral fibroblasts, EGF promoted the cell proliferation when it was above 10 ng/mL and the optimal concentration of EGF was 20 ng/mL ($p<0.01$). The results indicated that oral fibroblast proliferation was more sensitive to different concentrations of EGF. bFGF had no significant effect on dermal fibroblasts proliferation in this experiment and this was beyond our expection. Oral fibroblasts proliferation was increased significantly by bFGF at 10 ng/mL and 50 ng/mL ($p<0.01$).

The EGF and bFGF expressions in the transplanted mucosal and dermal wounds

To determine if EGF and bFGF expression levels differ in transplanted mucosal and dermal wounds, we performed quantitative PCR to measure mRNA levels at different time points after wounds. The results in Figure 1(C) showed that higher EGF mRNA were found at 1 d, 2 d, 3 d, 5 d, 7 d post injury and lower at 12 h post injury. The results suggested higher EGF expression in transplanted mucosal wounds at most of the time points post injury. The results also demonstrated that EGF expression was dramatically elevated at the early time-points and was decreased at later time points.

Immunohistochemistry was used to compare the protein levels of EGF in skin and transplanted mucosal wound tissues. As shown in Figure 3, EGF staining was stronger in transplanted mucosal wounds than in skin wounds at most timepoints after wounding occurred. This was consistent with mRNA expression result.

As shown in Figure 1(C), no regular bFGF expression differences was found in transplantedoralmucosaand skin wounds in this experiment.The immunohistochemistry expression of bFGF was shown in Figure 3. The protein expression was similar with the transcript levels of this gene.

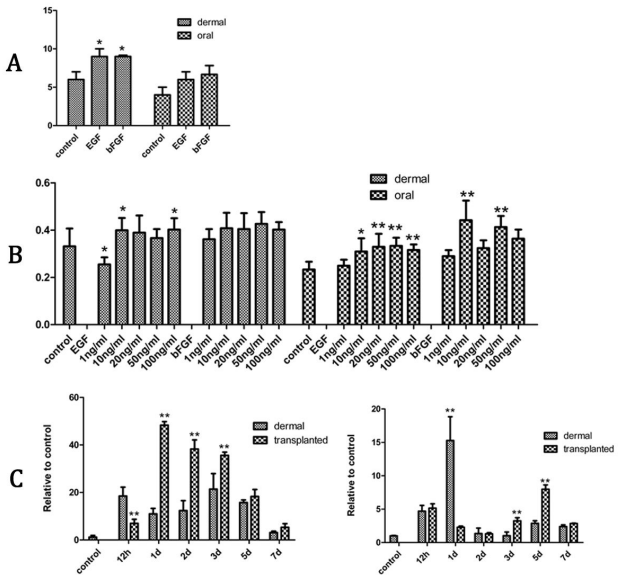

Figure 1: (**A**): The effects of 10 ng/mL EGF or 20 ng/mL bFGF on dermal and oral fibroblasts migration utilizing trans well assay. Migration in dermal fibroblasts was observed being obviously promoted by EGF and bFGF ($p<0.05$). The migration of oral fibroblasts was not promoted by either of the growth factors. (**B**) The effects of EGF or bFGF with different concentrations on proliferation of dermal and oral fibroblasts. Dermal fibroblasts proliferation was promoted by EGF at 10 ng/mL and 100 ng/mL ($p<0.01$). In oral fibroblasts, EGF promoted the cell proliferation when it was above 10 ng/mL and the optimal concentration of EGF was 20 ng/mL ($p<0.01$). bFGF had no significant effect on dermal fibroblasts proliferation. Oral fibroblasts proliferation was increased significantly by bFGF at 10 ng/mL and 50 ng/mL ($p<0.01$). **C:** EGF and bFGF mRNA expression in dermal and transplanted oral mucosal wound. Higher EGF mRNA was found at 1 d, 2 d, 3 d post injury and lower at 12 h post injury ($p<0.01$). No regular bFGF expression differences were found in transplanted oral mucosa and skin wounds in this experiment.

Discussion

Accumulating evidence from human and animal models have indicated that oral mucosal wound healed in general significantly faster and associated with a shorter and milder inflammatory response than skin wounds. In addition, oral wounds often heal without scarring while wounds in other locations do not [3,30]. With this in mind, the present study try to find out the cytokines expression difference in dermal and mucosal wounds and the response difference of dermal and oral fibroblasts to these cytokines.

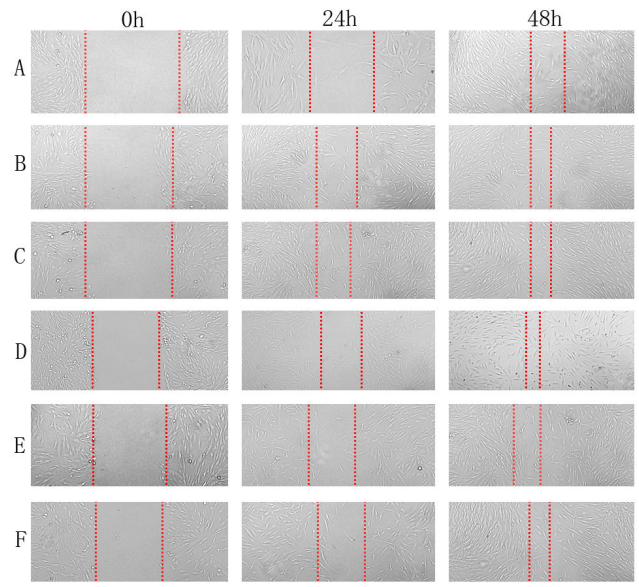

Figure 2: The effect of EGF and bFGF on skin and oral fibroblasts migration utilizing scrape assay. The photomicrographs illustrated the decrease in the size of the cell void area created by a sterile tip (**A**: skin fibroblast control group without growth factors; **B**: skin fibroblast treated with 10 ng/mL EGF; **C**: skin fibroblast treated with 20 ng/mL bFGF at different timepoints; **D**: oral mucosa fibroblast control group without growth factors; **E**: oral mucosa fibroblast treated with 10 ng/mL EGF; **F**: oral mucosa fibroblast treated with 20 ng/mL bFGF at different timepoints). The cell void area has been highlighted by a red line.

Wound healing involves many dynamic cellular processes, such as inflammation, cell proliferation, cell migration, and cell–cell interaction [7]. So, the present study firstly utilized cell culture model to compare the cell migration and proliferation rate of dermal and oral fibroblasts response to EGF and bFGF. Our results found that both bFGF and EGF could promote the migration of dermal fibroblasts but not for oral fibroblasts. Our results also found out that the cell proliferation was more sensitive to different concentrations of EGF in oral fibroblast than dermal fibroblasts, while bFGF had no significant effect on cell proliferation in this experiment. One early study showed that bFGF promotes human neonatal skin fibroblast proliferation, while not having any effect on chemotaxis of the same cell type along a concentration gradient [31]. However, a more recent study has shown that bFGF is both chemotaxic and mitogenic for murine adventitial fibroblasts when present as a concentration gradient [32]. Furthermore, enhanced proliferation and migration of human fibroblasts has been demonstrated on the surface of FGF-2 loaded microthreads [33]. Another study conducted by Barrientos showed that the bFGF appeared to increase proliferation of the limb fibroblasts only, not in oral fibroblast. The different culture condition, different cell type,and different state of the cells may cause the variance of the results.

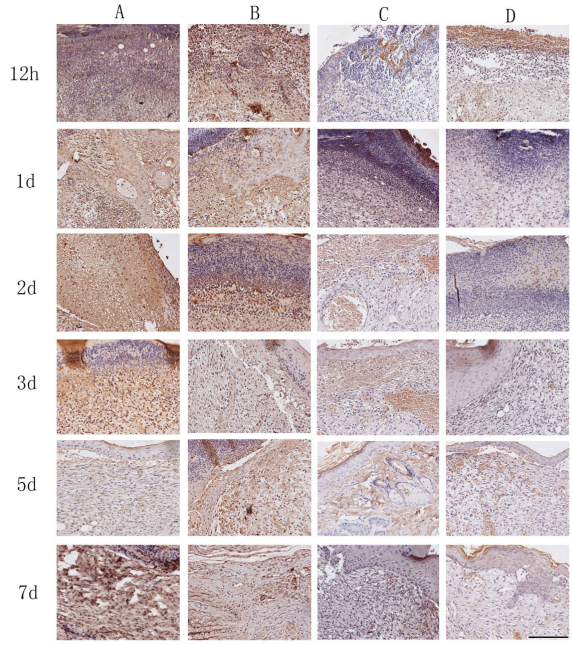

Figure 3: Immunohistochemical analysis of EGF and bFGF expressions in the transplanted mucosal and dermal wounds. Biopsy samples of dermal wound (column A) stained with antibody against EGF or bFGF (column B) were compared with tissues from transplanted oral mucosa wound (column C: EGF staining; column D: bFGF staining). The protein expression was similar with the transcript levels of these genes.

Since wound healing is a complex process involving multiple cells, inflammatory mediators, cytokines, and growth factors, *in vitro* data cannot explain the whole process of wound healing. Thus it is necessary to create in vivo system which represents the complexity of wound healing process. In this study, we firstly transplanted the oral mucosa to skin and a linear wound was secondly made after the transplantation wound healed. The goal of the transplantation was to eliminate the influence of wet environment and growth factors in oral cavity. Our results found that higher EGF mRNA and protein expression were found at 1 d, 2 d, 3 d, 5 d, 7 d post injury and lower at 12 h post injury. The results suggested higher EGF expression in transplanted mucosal wounds at most of the time points post injury. While, no regular bFGF expression differences was found in transplanted oral mucosa and skin wounds in this experiment.

Former studies have shown that topically or subcutaneously applied EGF significantly accelerates reepithelialization and increases tensile strength of the wound [34,35]. Thereby, EGF has been noted for treating impaired dermal wounds and the scope of its application is

now expanded to the treating chronic wounds such as burn wounds and chronic ulcers [34]. However, studies that examined growth factors involved in scar formation used fetus models during murine pregnancy revealed that during the scar formation period on the 19th day; EGF was simultaneously increased [36]. That means higher EGF expression was associated with scar formation. In the present study we found higher EGF expression in transplanted oral mucosal wounds, which was universally accepted that healed with minor scars. In our *in vitro* cell culture model, the oral fibroblasts reacted differently to EGF from dermal cells. The different EGF expression and different cell response may cause the different wound healing result.

Although FGFs have been implicated in a variety of physiological and pathological processes and FGF signalling clearly plays an important role in development, the specific function of FGF in vivo wounding is not yet clear. Some former studies found that topical application of bFGF accelerates healing of skin wounds in animal models, as well as of eye, retina, and corneal wounds [37-39]. While other study had postulated that bFGF might cause a delay in wound maturation by increasing the risk of local infection [40]. The present study did not find regular bFGF expression differences between transplanted oral mucosa and skin wounds.

Although, our in vivo study showed different EGF expression in transplanted oral mucosa wound healing process, one limitation of the experimental design in this study should be addressed regarding the transplantation. To eliminate the influence the wet environment and growth factors in saliva, oral mucosa was transplanted to rat skin and the liner wound was made 3 weeks after transplantation. However, the first transplantation may have influence to the second liner wound. Therefore, to eliminate the influence of the transplantation, the skin should be transplanted to its own wound site as the transplanted oral mucosa. If that, the results of this study would be more distinct.

In conclusion, the present found out that oral fibroblast reacted differently to EGF and bFGF from dermal cells considering the proliferation and migration. In the created rats experiment, the liner wound on the transplanted oral mucosa expressed higher EGF expression indicated the important role that EGF played in the scarless wound healing process.

Acknowledgments

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