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RESEARCH ARTICLE

THE ANTI-SCAR EFFECTS OF BFGF-DERIVEDOLIGOPEPTIDES ON THE WOUND REPAIR *IN VIVO*

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ABSTRACT

Hypertrophic scars (HTS) and keloids are challenging problems. Their pathogenesis results from an overproduction offibroblasts and excessive deposition of collagen. Studies suggest a possible anti-scarring effect of basic fibroblast growth factor (bFGF) during wound healing, but the direct application of bFGF still has many weaknesses. In view of this, we synthesized and investigated the therapeutic effects of bFGF-derived oligopeptides (pFGF) on HTS animal model as well as human scar fibroblasts (HSF) model. We show that pFGF promoted wound healing and reduced the area of flattened non-pathological scars in mouse skin wounds. We provide evidence of a new therapeutic strategy: pFGF administration for the treatment of HTS. The scar elevation index (SEI) and epidermal thickness index (ETI) was also significantly reduced. Histological reveal that pFGF exhibited significant amelioration of the collagen tissue. The levels of fibronectin (FN), tissue inhibitor of metalloproteinase-1 (TIMP-1) collagen I, and collagen III were evidently decreased. These results suggest that pFGF possesses favorable therapeutic effects on hypertrophic scars in vivo, which may be an effective cure for human hypertrophic scars.

INTRODUCTION

Hypertrophic scar was commonly formed after dermalskin injury. The abnormal scar was characterized by red, rigid and raised appearance associated with pain and itchiness. It will lead to cosmetic disturbance and affect the daily activities of a person if the scar is contracted causing joint stiffness and deformities[1].During the repair of a wound proceeds, keloid and hypertrophicscars (HTS) are a common problem. Clinically, they are characterized by excessive deposition of collagen in the dermis and subcutaneous tissues secondary to traumatic [2]. This process is regulated by cytokines and growth factors such as transforming growth factor b (TGF-b), epidermal growth factor (EGF), fibroblast growth factor (FGF) and platelet-derived growth factor(PDGF)[3].

During embryo-genesis, FGFs play key roles inregulating cell proliferation, migration, and differentiation. Inadult tissues, FGFs have various effects, including mediating angiogenesis and neuroprotection, in addition to their stimulatory effects during wound repair [2, 4, 5]. Basic fibroblast growth factor (bFGF) is a potent mitogen and chemo attractant for endothelial cells, fibroblasts and keratinocyte. bFGF stimulates the metabolism, growth of the extracellular matrix (ECM), and the movement of mesodermally derived cells[6]. The goal for wound treatment is the fast and scarless healing, although this is quite difficult for adult tissues. The accelerating wound healing may improve the quality of healing and alleviate the scar. The anti-scarring effects of bFGF have been shown in both animal models and clinical use; postoperative administration of bFGF also inhibits hyperplastic scar without side effects [2, 7, 8]. However, side effects were suggested such as extreme pain, high recurrence rate or the relation with cancer [9-13].

During the wound healing process, the imbalance of collagensyn thesis and degradation resulting in excess accumulation of dermal collagen can lead to the scar complications. Sufficient content of type III collagen may prevent scar tissue formation, while excessive secretion of type I collagen may result in a disorganized fiber structure and hypertrophic scar formation [14, 15].

This study aims to demonstrate whether newly synthesized bFGF-derived 13 amino acids oligopeptide (pFGF) can alleviate or eliminate formed hypertrophic scars in a full-thickness excisional mouse model. To explore the possible mechanism of action, we comparatively evaluated the in vitro effects of pFGF on fibroblasts. Thus, we provide evidence of a new therapeutic strategy: pFGF administration for the treatment of established HTS.

MATERIALS AND METHODS

Synthesis of bFGF-derived oligopeptides

All the peptides used in this study were synthesized by the Fmoc (9-fluorenylmethoxycarbonyl)-based solid-phase method and characterized by Peptron Inc. (DaeJeon, Korea). The purities of all peptides used in this study were greater than 95%, as determined by high-performance liquid chromatography.

Animal Experiments

Male C57BL/6mouse (n = 6), weighing 25 g, were chosen for the experiment. The skin was cleaned with alcoholand two full-thickness wounds (8mm radius) extending through the panniculus carnosus were made on the dorsum on each side of midline under aseptic conditions. the wounds of the experimental group (1 ml for each wound) every other day in concentrations of 1 mg/ml (dissolved in 0.9% w/v saline), while the control group received equal amounts of 0.9% w/v saline treatment for 14 days. Wounds were left uncovered after injury, and wound areas were measured at various time points[7]. The rate of wound closure was calculated using the following formula: Wound closure rate= [(Original wound area-Open area on final day)/Original wound area X 100%.

Histological Examination and Immunohistochemistry Staining

The scar or skin tissues were fixed in 4%paraformaldehyde at 4℃ overnight prior to processing for paraffin embedding, cut in 5 mm sections, and stained with hematoxylin-eosin (HE) or Masson's Trichrome Stain Kit (Sigma–Aldrich, USA). The other half was stored at –80℃forprotein extraction. The scar elevation index (SEI) and epidermal thickness index (ETI) were used for histomorphometric analysis and measured for treated and untreated wounds. The SEI is a ratio of total wound area tissue height to the area of normal tissue below the scar. The height of the normal tissue is determined based on the height of the adjacent unwounded dermis. All measurements were taken within the confines of the wounded area under 40X magnification from the HE stained tissue sections. An SEI of 1indicates that no newly hypertrophied dermis formed, whereas an index >1 indicates HTS formation. The ETI was used to determine the degree of epidermal hypertrophy and was based on measurements taken from H&E-stained tissue sections at 400X magnification. The ETI is a ratio of averaged epidermal height in scar tissue to the averaged epidermal height in normal uninjured skin. ETI >1 indicates hypertrophic epidermis formation.

The immunohistochemical staining of the collagen I and collagen III (Santa Cruz Biotech, USA) was conducted by respective antibody. Sections were dewaxed and hydrated; endogenous peroxidase was blocked with 3% hydrogenperoxide for 10 min; nonspecific binding was blocked with 1%BSA for 30 min. Primaries were applied for overnight at 4℃. Biotinylated secondary antibodies were then applied at 1:200 for 30 min, followed by incubation with horseradish peroxidase (HRP)-streptavid in at 1:400 for 30 min. Color development was performed with DAB for 3 to 5 min for all samples, followed by haematoxylin counterstaining, dehydration and cover slipping. The immunopositive in fields was counted for per sections using Image-Pro Plus software (Nikon, Japan).

Collagen I and Collagen III Quantification

The quantification of collagen I and III used an ELISA Kit (R& D Systems Inc., USA) according to the operation manual. In brief, 100 μL of standards or samples were added to the appropriate well of the antibody pre-coatedmicrotiter plate, followed by 50 μL of conjugate; each well was then covered and incubated for 1 h at 37℃. Next, 50 μL of substrates A and B were added to each well and incubated for10 min at room temperature (to avoid sunlight). Finally, added50 μL of the stop solution was added, and the optical density (O.D.) was read at 450 nm using a microtiter plate reader immediately.

Fibroblasts Culture

NIH3T3 (mouse fibroblast cell line) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) medium containing 10% fetal bovine serum, penicillin/streptomycin (100 U/ml). The medium was replaced every 3 days. The fibroblasts were grown at 37℃ in an atmosphere of 5%CO_2 and were passaged every 2 days by trypsinization.

Statistical Analysis

The data were expressed as the mean ± SEM. Statistical significance was determined with the student's t-test when there were two experimental groups. For more than two groups, statistical evaluation of the data was performed using one-way analysis of variance (ANOVA), followed by Dunnett's post-hoctest, with values of $P<0.05$ considered significant.

RESULTS

Screening and Basic properties modification ofpFGF

The selection of peptide sequence was carefully progressed through the BLAST search protein database (NCBI protein database). From the database, 1/3 part from N-terminus sequence of basic Fibroblast growth factor was selected and its partial fragmentation was tested to identify efficiency. Among many oligopeptides, the partial fragmentation of N-221 to 234 which contains 12 amino acids was noticeable. From this amino acid sequence, 70 amino acid sequences that were close to the FGFreceptor-ligand domain was selected and 12 different shorter sequences that partially overlap each other (pFGF 1.1–1.12) were synthesized (Fig. 1A). Then, each synthesized sequences were tested in its fibroblast ability and pFGF1.6 showed the most prominent cell-activating efficiency in preliminary tests. The final step included addition of Cysteine(C) at C-terminus to facilitate attachment of drug molecules (Fig.1 B). Analysis of pFGF1.6 hydrophobicity index showed that the sequence was slightly hydrophobic with an alpha-helix structure (Fig. 1C). Then, the final pFGF1.6 peptide consisted of 13 amino sequences was lyophilized for convenient use in prospective investigation (Fig. 1D).

pFGF Accelerates Acute Wound Closure in the IncisedInjury Model

Wound healing of the skin incision was determined by the percentage of wound surface covered by regenerating epidermis. The wounds treated by pFGF recovered much more quickly with better skin appearance (Fig. 2A). After day 8, the wounds treated with pFGF were almost scarless, while the wounds in the control had obvious scars. Thus, pFGF significantly contributed to wound healing, compared to the control group ($P<0.05$; Fig. 2B). After day 14, both the pFGF group and the control group showed wound closure mostly, for

natural repair of the mouse skin. These wound closure rates made a good match to the results of HE staining and Masson Staining (Fig. 2C). Longerkeratinocyte migration tongue and collagen expression was observed on day 14 for pFGF-treated wounds; the results demonstrated the accelerating effect of pFGF on the wound reepithelization *in vivo*.

pFGF Alleviates the Scar Formation in the histological sections.

All wounds had adequate scar maturation and showed histologic evidence of scarring. The mean scar thickness (SEI) in the control group was 1.61±0.41 and 1.36±0.37, which was higher than that of the pFGF group with 0.93±0.31, 0.83±0.2446 on days 7 and 14, respectively (Fig.3A). The mean epidermal thickness (ETI) of the control group was 2.08±0.47 and 2.75±0.32 compared with 1.87±0.4232and 2.49±0.3070 for the PFGF group (P<0.05; Fig.3B). This represents a significant epidermal thickness reduction in wounds treated with pFGF.

Histologically, the dermis layer of the control scars thickened significantly, and the boundary between the papillary and reticular layers of dermis was obscure; collagen fibers were dense, with derangements in collagen bundles, which were irregularly arranged in the profound dermis and nodular, circular, or whirled in the superficial dermis. The number of cells also increased, while the basal layer of the epidermis in the scars treated with pFGF for 14 day was flattened. The dermis layer was not significantly thickened, and collagen fibers were well arranged, with few cells (Fig.3C).

Effect of pFGF on Collagen I and Collagen III Synthesis

In order to evaluate the molecular effects of pFGF on matrix production, we measured protein expression of collagen I and III, which constitute the bulk of the scar ECM. As expected, collagen Iexpression was significantly decreased on day 7 (P<0.05), while collagen I expression was insignificantly decreased on day 14 (Fig.4A); collagen III significantly reduced on day 14 (P<0.05) in the pFGF treated group (Fig.4B). The collagen density was significantly reduced in wounds treated with pFGF compared with the untreated wounds by immunohistochemical staining of the collagen III on day 14 (P<0.05; Fig.4C and D).

DISCUSSION

Scarring is a multifactorial process with different clinical presentations that affects more than 40 million people worldwide[16]. Generally, scars can be classified into two categories, pathological scars and non-pathological scars. There are some preventive and therapeutic measures for exuberant scars, such as silicone, pressure therapy, corticosteroids, laser therapy, cryotherapy, radiation, and surgery[17-19]. However, there is noconsensus about the best treatment for complete and permanent improvement of scars with few side effects.

Funato *et al*. [20] reported that bFGF is a possible inducer of apoptosis in myofibroblasts during palatal scar formation. Spyrouand Naylor [21] found that treatment with bFGF inhibited the transient phenotypic change of granulation-tissue fibroblasts into myofibroblasts. In order to identify a means to reduce scar formation of the skin after incision. They found that no patient treated with bFGF had hypertrophic scars, while some patients had hypertrophic or very wide scars in the control group; the ratios of minimum scarring for the bFGF treatment group were statistically significantly higher than those of control group.

However, the direct application of bFGF has many weakness, in case of related with signaling pathway occurred many cancers [11, 22-24], poor protein stability *in vitro* and *vivo*[25-27], also the high cost for manufactured recombinant proteins.

In this study, we newly sysnthesized the effective fragment of bFGF oligopeptides (pFGF) for maintained the pharmaceutical activity of original bFGF and reduced the side-effects and evalutate the therapeutic potential of pFGF for wound healing and HTS animal model as well as fibroblast cell model and analyze the potential mechanisms (Figure 1).

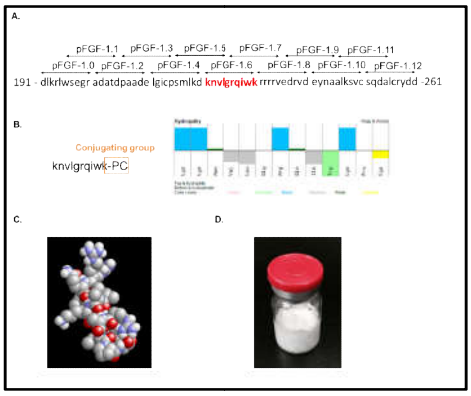

Figure 1 Illustrations of novel anti-scar peptide and its molecular characters (A) 12 different amino acid sequences that partially overlap each other (pFGF 1.1 – 1.12) were initially obtained from 70 amino acid sequence human basic fibroblast growth factor. After CPP test for each sequences, pFGF 1.6 showed the most effective anti-fibroblast ability and subsequently conjugation group consisted of proline and cysteine were attached at (-)end. (B) Molecular structure of pFGF 1.6 was visualized through mobile simulation. (C) pFGF 1.6 was lyophilized for the use of prospective tests.

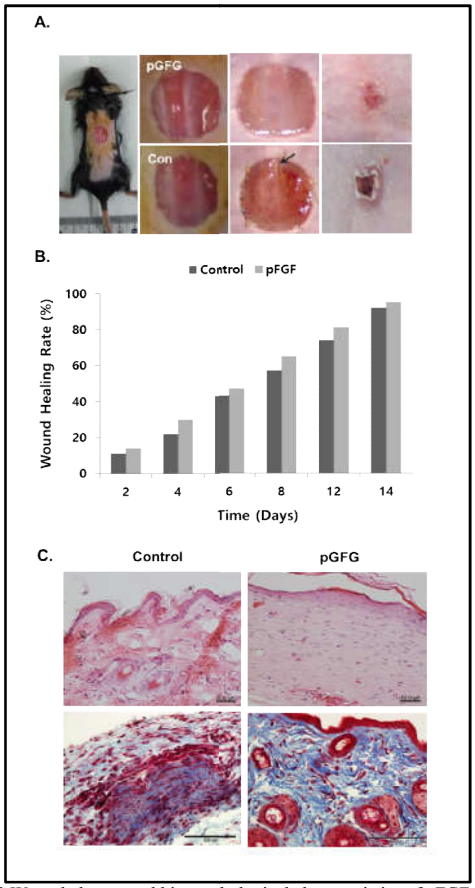

Figure 2.Wound closure and histopathological characteristics of pFGF treated wound healing in mouse. (A) Representative photographs offull-thickness skin wounds at various time points after treatment with or without 1 mg/ml pFGF. (B) The wound healing rates of bFGF. *P<0.05compared to control group, n = 8. (C) Histopathological observation and masson staining of collagen in wound healing at day 14 post-wounding (x 40)

We find that pFGF promoted wound healing and reduced the area offlattened non-pathological scars in rat skin wounds (Figure 2) aswell as the bFGF. Int he early stages of incisional-wound healing, pFGF administration has no adverse effect on the tensile strength of the wound and, atlater stages, can be attributed to the improved the architecture of the neodermis. Type I and III collagens are the central components of ECM products. However, the production of collagen can be a double-edgeds word: on the one hand, it is necessary for wound healing; on the other hand, excess deposition of collagen can result inscarring [28, 29]. Therefore, the appropriate expression of collagen is required for ideal wound healing. Our findings indicate that the treatment of pFGF can accelerate wound healing with increasing collagen production and subsequent collagen deposition (Figure 4). The outcome is an improvement in the quality of wound healing. In the histological analysis, pFGF resulted in a sparse arrangement of the collagen, similar to normal skin collagen distribution, and reduced type I collagen and type III collagen content, which prevented the formation of nodular structures (Figure 3).

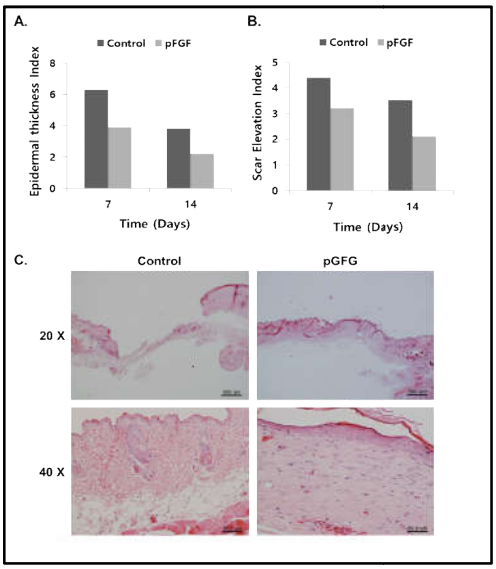

Figure 3 pFGF alleviated the scar formation *in vivo*. (A) The averaged Epidermal Thickness Index (ETI) of the scars. Epidermalhypertrophy was displayed by ETI. ETI >1 depicts a hypertrophic epidermis. (B) The averaged scar elevation index (SEI) of the scars. Dermalhypertrophy is displayed by the SEI, where SEI >1 depicts a hypertrophic scar. *P<0.05, **P<0.01 compared to control group, n = 6. (C) Themicroscopic histology of wounds that control or pFGF at day 14, HE stain

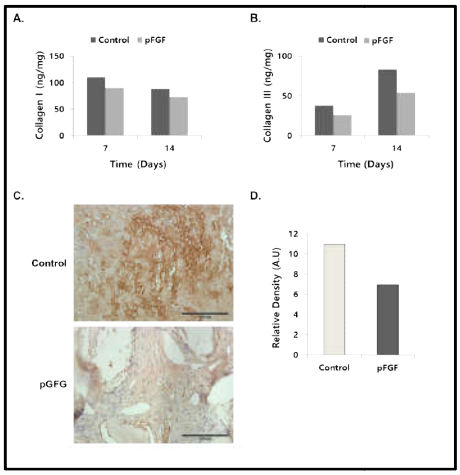

Figure 4 pFGF decreased collagen I and collagen III synthesis. The levels of expression of (A) collagen I and (B) collagen III in scars treated with saline or pFGF, *P<0.05, **P<0.01 compared to control group, n = 6. (C) Immunohistochemistry of the expression of collagen III in scars treated with saline or pFGF (x200). (D) Analysis of relative density collagen III, *P<0.05, **P<0.01 compared to control group, n = 6.

which is beneficial for wound closure and scar diminution. Also, the study by Xie *et al*. [7] supports the potential of bFGF to accelerate wound healing and improve the quality of scars by regulating the balance of collagen synthesis and degradation. Consequently, interfering with one or several components of the ECM metabolism could be a potential therapeutic intervention to alleviate scars. The amount of ECM in the tissue might be controlled through a balance among ECM production, ECM degradation by MMPs, and inhibition of MMPs by tissue inhibitors of metalloproteinases [30]. Fibronectin is one of the most important ingredients of the ECM and plays a particularly important role in wound repair, largely determining the quality of the wound [31]. The deposition and/or polymerization of fibronectin into the ECMcontrols the deposition and stability of other ECM molecules [32]. All of these results indicate that bFG Fregulates ECM metabolism to improve wound healing and hypertrophic scarring. Our future study shows that the stimulation of fibroblast with pFGF in vitro could result in anupregulation of MMP-1 and decreased TIMP-1, FN gene. This was partial explain related with the mechanism of pFGF action.

Thus bFGF might beapplicable as an anti-scarring agent after the surgery of skin or other organs where myofibroblast overgrowth would induce complications for scarring. In conclusion, we provide evidence of a new therapeutic strategy, pFGF administration for the treatment of scar tissue. pFG Fregulate ECM synthesis and degradation via interference in theMMP-1, TIMP-1, lysyl hydroxylase and prolyl hydroxylase gene expression. The efficacy of treatment using pFGF was also demonstrated in animal models and the cell model. However, it no doubt that the limitations of pFGF in scar therapy still need further investigation and improvement. For example, single a dose of pFGF was treated right after injury, a post-injury treatment of optimal dose and extended time would better evaluate the therapeutic value in the future. It is interesting that although we have tried several concentrations of pFGF in our model *in vivo*, there is no obvious enhancement of the protective effect with the increase of pFGF, which may be related to the regulation of absorption and metabolism. Nevertheless, the anti-scarring effect of pFGF in the therapy of HTS is confirmative and feasible, to improve the pharmacodynamic action and demonstrate the mechanism underlying is necessary in the following study.

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