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Fibroblast growth factor 2 dimer with superagonist *in vitro* activity improves granulation tissue formation during wound healing

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ABSTRACT

Site-specific chemical dimerization of fibroblast growth factor 2 (FGF2) with the optimal linker length resulted in a FGF2 homodimer with improved granulation tissue formation and blood vessel formation at exceptionally low concentrations. Homodimers of FGF2 were synthesized through site-specific linkages to both ends of different molecular weight poly(ethylene glycols) (PEGs). The optimal linker length was determined by screening dimer-induced metabolic activity of human dermal fibroblasts and found to be that closest to the inter-cysteine distance, 70 Å, corresponding to 2 kDa PEG. A straightforward analysis of the kinetics of second ligand binding as a function of tether length showed that, as the polymerization index (the number of monomer repeat units in the polymer, N) of the tether decreases, the mean time for second ligand capture decreases as ~$N^{3/2}$, leading to an enhancement of the number of doubly bound ligands in steady-state for a given (tethered) ligand concentration. FGF2-PEG2k-FGF2 induced greater fibroblast metabolic activity than FGF2 alone, all other dimers, and all monoconjugates, at each concentration tested, with the greatest difference observed at low (0.1 ng/mL) concentration. FGF2-PEG2k-FGF2 further exhibited superior activity compared to FGF2 for both metabolic activity and migration in human umbilical vein endothelial cells, as well as improved angiogenesis in a coculture model *in vitro*. Efficacy in an *in vivo* wound healing model was assessed in diabetic mice. FGF2-PEG2k-FGF2 increased granulation tissue and blood vessel density in the wound bed compared to FGF2. The results suggest that this rationally designed construct may be useful for improving the fibroblast matrix formation and angiogenesis in chronic wound healing.

1. Introduction

Healing in chronic wounds including venous, arterial, diabetic [1], and pressure [2] ulcers is impaired due to decreased growth factor production, keratinocyte and fibroblast proliferation and migration, granulation tissue formation, and angiogenesis. Treatment of chronic wounds costs over 9.5 billion U.S. dollars annually, worldwide [3]. Diabetes alone is projected to affect 439 million adults (ages 20–79) by 2030 globally, and 15% of diabetic patients develop chronic foot ulcers [4]. Thus, viable treatment of chronic wounds represents a significant challenge to the medical community.

Fibroblast growth factor 2 (FGF2), a growth factor whose expression is impaired in both diabetic and pressure ulcers [1,2], moderates cell proliferation, differentiation and migration of multiple cell types. FGF2 is critical in wound healing, angiogenesis, bone regeneration, neuroregeneration, and can even result in scarless healing [5,6]. As a result FGF2 released from gels alone or in combination with other proteins and/or heparin has been employed as a strategy to increase angiogenesis, ischemic heart repair, nerve regeneration, etc. *in vivo* [7–10]. While FGF2 alone

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appears to be a good candidate for the treatment of chronic wounds, and is approved in Japan (Fiblast or Trafermin) for skin ulcers, US and European clinical trials (Phase II for treatment of peripheral arterial [11] and coronary [12] disease and Phase III for neuropathic diabetic foot ulcers [13], respectively) have shown minimal effectiveness [14]. Thus, increasing the effectiveness of FGF2 is an important endeavor. This has been undertaken by a variety of approaches, including adding peptides or proteins that bind to both FGF2 and its receptor [15–17], by truncating the FGF2 sequences [18], mutating specific amino acid residues [19,20], covalent modification [21–23], or by utilizing FGF receptor peptide agonists as an alternative [24,25]. In this report, we describe the formation of a FGF2 with superagonist activity *in vitro* that improves granulation tissue formation and blood vessel density during wound healing *in vivo* at low doses through chemical dimerization of FGF2.

FGF2 activity is dependent on the formation of a tetrameric complex, consisting of two FGF2 proteins and two FGF receptors (FGFR1) [26,27]. Many proteins, like FGF2, exist or self-assemble into homodimers or multimers in their native or active state and these structures are often required for protein activity [28,29]. Synthetic routes to protein dimerization are pursued in the scientific community as a means to study protein interactions and to create superagonist growth factor therapeutics [30–33]. Pre-organization of dimeric ligands is known to increase the effective local concentrations, thereby facilitating activation of receptors [34,35]. Heparin or oligoheparins are mimics of membrane-bound heparin-sulfates that are known to facilitate FGF2 receptor binding and dimerization. It has been shown that adding heparin [36,37], or oligoheparins [38–40] is important for FGF2 activity, and it is has been proposed that the heparin molecules promote FGF2 dimerization in heparanoid complexes [41]. Nonspecific chemical crosslinking of FGF2 lysine side-chains through reaction with short (11.4 Å) [21–23] tethers such as bis(sulfosuccinimidyl) suberate has been performed as a means to study the interaction of FGF2 with heparin oligomers. Towards the same end FGF2 has also been oligomerized through biotin–streptavidin binding [42]. In addition, recombinant expression of a dimeric FGF2 has led to enhanced biological activity compared to FGF2 alone [43]. While these prior reports nicely demonstrate the importance of dimerization of FGF2, we hypothesized that much more significant activity could be obtained by 1) conjugating site-selectively to residues spatially separated from both the heparin-binding domain and receptor binding sites and 2) probing the ideal length of the dimerizer.

Site-specific conjugation is imperative in the development of protein homodimers in order to avoid the formation of protein multimers or complex protein-polymer networks. In addition, polymer conjugation at or near an active site, or the addition of multiple polymers to a protein therapeutic can shut down protein activity [44]. Therefore targeting a single reactive site is important to maintain protein activity. Cysteine is an ideal target for site-specific protein modification due to its low abundance and nucleophilicity [45]. FGF2 contains two surface-exposed free cysteines (Cys-78 and Cys-96) [46]. The mutation of either cysteine, as shown by Lappi et al., is not detrimental to protein activity [47], and Kang et al. observed a retention of activity after PEGylation at both surface-exposed cysteines with a 5 kDa PEG [46]. Therefore we chose to install the genetic modification cysteine to serine at amino acid 78 (C78S), resulting in an FGF2 containing a single surface-exposed cysteine, Cys96 (shown in red, Fig. 1).

In addition to site-selective dimerization, the length of the tether is also imperative to protein activity. Linker length is essential to receptor activation for similar growth factors [30,33,34]. Based on the crystal structure of the tetrameric FGF2:FGFR1 complex, we hypothesized that a flexible linker with a length close to the inter-cysteine distance of 70 Å would induce the greatest activity [26,27]. Poly(ethylene glycol) (PEG) was chosen as the linker based on the ease of modification. PEG is also known to improve pharmacokinetics through stabilization and improved circulation time, and many FDA-approved, PEGylated therapeutic agents are on the market [48,49]. In addition, there have been several PEG-FGF2 monoconjugates prepared that have improved circulation life times and other favorable *in vivo* features such as enhanced penetration into the injured spinal cord [46,50–54]. Here, we describe the development of PEG-linked FGF2 dimers as superagonists that could be used to create a more fertile wound healing bed.

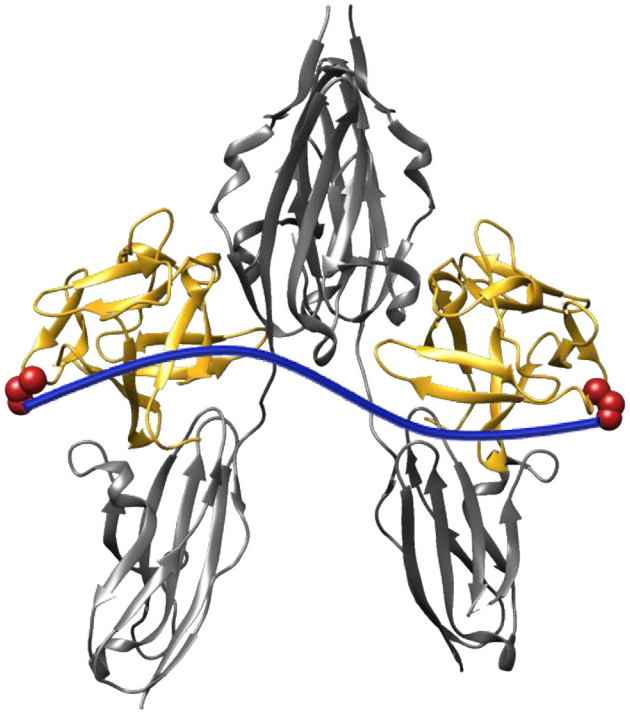

Fig. 1. The active tetrameric complex consisting of two FGF2s (gold) two FGFRs (silver) with Cys96 in red and poly(ethylene glycol) represented in blue. Modified from PDB 1CVS using Chimera software. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

2.1. Materials

All chemicals and reagents were purchased from Sigma–Aldrich and used as received unless otherwise indicated. Enzyme-linked immunosorbent assay (ELISA) Development DuoSet kit was purchased from R&D Systems. Normal Human Dermal Fibroblasts (HDFs), human umbilical vein endothelial cells (HUVECs), and cell media were purchased from ATCC.

2.2. HDF metabolic activity assay

HDF cells (passage 4 – ATCC) were suspended in Ultra-CULTURE™ (Lonza) serum-free medium supplemented with 2 mM L-Glutamine, 100 μg/mL penicillin, and 100 μg/mL streptomycin, and plated at 2000 cells/well in a 96-well plate. The cells were allowed to adhere for 16 h at 37 °C, 5% CO_2. After 16 h the medium was removed by aspiration and replaced with 100 μL of unmodified FGF2, mPEG-FGF2, or FGF2-PEG-FGF2, diluted in UltraCULTURE™