Original Article

Stability and biological activity evaluations of PEGylated human basic fibroblast growth factor

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Abstract

Background: Human basic fibroblast growth factor (hBFGF) is a heparin-binding growth factor and stimulates the proliferation of a wide variety of cells and tissues causing survival properties and its stability and biological activity improvements have received much attention.

Materials and Methods: In the present work, hBFGF produced by engineered *Escherichia coli* and purified by cation exchange and heparin affinity chromatography, was PEGylated under appropriate condition employing 10 kD polyethylene glycol. The PEGylated form was separated by size exclusion chromatography. Structural, biological activity, and stability evaluations were performed using Fourier transform infrared (FITR) spectroscopy, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay and effect denaturing agent, respectively.

Results: FITR spectroscopy revealed that both PEGylated and native forms had the same structures. MTT assay showed that PEGyalated form had a 30% reduced biological activity. Fluorescence spectrophotometry indicated that the PEGylated form denatured at higher concentrations of guanidine HCl (1.2 M) compared with native, which denatured at 0.8 M guanidine HCl.

Conclusions: PEGylation of hBFGF makes it more stable against denaturing agent but reduces its bioactivity up to 30%.

Key Words: 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay, Fourier transform infrared spectroscopy, human basic fibroblast growth factor, polyethylene glycol methyl ether maleimide, stability

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INTRODUCTION

Biotherapeutic agents are successfully employed in the treatments of different pathophysiological disorders ever since the first approval of recombinant insulin in 1982.[1] Hundreds of recombinant drugs have found places in the market after their approval by respective authorities. Such products are

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termed as first generation of recombinant protein/peptides as they show an identical amino acid sequence to a native human protein and replacing or increasing levels of that protein *in vivo*.[1] Though the first generation of biopharmaceuticals have been approved and improved; But they possess limitations such as relatively short *in vivo* circulation time, poor solubility, physicochemical and proteolytic instability and immunogenicity.[2] Furthermore, second generation of biotherapeutic agents was engineered by different approaches like, amino acid manipulation to reduce immunogenicity and proteolytic digestion.[3] Slow release and protection through drug delivery system,[2,4] higher circulation (half-life) and stability[5,6] and efficiency,[5,7-15] tissue permeability[6,16] and lower immunogenicity[17-19] can be achieved as postproduction modifications by conjugating the target protein to some synthetic or natural polymers such as polyethyleneglycol, thereby the patients get benefited as their administration frequency can be reduced to large extent.[2] human basic fibroblast growth factor (hBFGF) is one of the multifunctional biopharmaceutics with growing therapeutic potential in cardiovascular disease, cancer and other disorders.[20] hBFGF is a heparin binding growth factor containing 146 amino acids polypeptide with a high affinity to its trans membrane receptors[21-24] and stimulates the proliferation of a wide variety of cells and tissues causing survival properties.[25] Mitogenic and angiogenic properties,[26-30] effectiveness in burn treatment,[31-34] tissue repair after myocardial infarction[35-38] and improvement in spinal injuries[39-44] are the most important roles of hBFGF.

In this article, with the aim of protection against proteolysis which cause reduced stability, short half-life and immunities;[6,45] our attempts were made to PEGylate recombinant hBFGF and study its biological activity and stability by comparison such properties with that of non-PEGylated form.

MATERIALS AND METHODS

For BFGF production, *Escherichia coli BL21 (DE3*) containing plasmid expressing hBFGF was cultivated in Luria-Bertani (LB) broth (Sigma-Aldrich). The production of target protein was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG: Thermo Scientific: Fermentas) at 30°C for 4 h. The cells were harvested by centrifugation and were slurried in an appropriate buffer and then disrupted by high pressure homogenizer. It was then centrifuged at 4°C and 12000 rpm for 30 min. The supernatant was subjected to cation exchange membrane Sartobind S (Sartorius Co.) and heparin affinity (heparin affinity (HiTrap: GE Health Care Life Sciences), both at a flow rate of 1 ml/min using fast purification liquid chromatography (Bio-Rad). Vivaspin 10 kD was used to exchange the buffer in order to reduce the salt concentration. In the final step, anion exchange membrane chromatography Sartobind Q (Sartorius Co.) at a flow rate of 1 ml/min was used to minimize presence of endotoxin to accepted value. This step was performed to avoid interference of endotoxin in 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Western blot and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) 12% was carried out to confirm the presence of BFGF.

PEGylation of the above mentioned protein was performed in presence of nitrogen gas at dark employing 10 kD maleimide-polyethyleneglycol (Jenken). The PEGylated form was separated from unPEGyalted hBFGF by size exclusion chromatography using a Hiload 16/600 Superde × 75 prep grade column (GE Health care life sciences) at a flow rate of 1 ml/min. Both the forms were treated with different concentrations of guanidine hydrochloride from 0.1 mol/ml up to 4 mol/ml as denaturing agent at 37°C. The effects of guanidine hydrochloride on both PEGylated and unPEGyalted BFGF were studied by fluorescence spectrophotometry (Luminescence spectrometer PERKIN ELMER LS 50 B). This assay use for BFGF as a unique test to separation stable and unstable BFGF molecule from each other.[46]

3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay was used to evaluate the biological activity of the samples. The balb/c 3T3 cells from mouse embryo tissue were cultivated in Dulbecco's modified Eagle's medium (DMEM: Gibco) containing 10% fetal bovine serum (FBS) (Gibco). 10,000 cells/ml per well added to plastic 96 well plates, were cultured at 37°C for 2 h in a humidified 5% CO_2-95% air atmosphere. The cells then were treated with different concentration of hBFGF. The assay was going on by using the MTT exclusion dye. The range of sample concentration was from 15 pg/ml up to 2000 pg/ml. The MTT assay was used for PEGylated form too.

Fourier transform infrared spectroscopy tests of PEGylated and native forms were performed to study the effect of PEGylation on protein secondary structure. Samples were mixed with potassium bromide in 1/200 ratio and then were ground to form a very fine powder. This powder was then compressed into a thin pellet at a pressure of 8 tons. The changes of structure before and after

PEGylation were analyzed at the scan range of 4000–400/cm.

RESULTS

Escherichia coli BL21 (DE3) containing plasmid was grown in LB broth. The expression of BFGF was induced by 1 mM IPTG when the growth reached an OD of 1 at λ 600 nm. The cells were harvested by centrifugation after 4 h of induction and expression of BFGF was confirmed by SDS-PAGE 12% and Western blot. Purification steps were started by passing the cells' slurry through high pressure homogenizer. The cell debris were separated by centrifugation at 20,000 rpm, 4°C for 30 min. Thus the supernatant containing hBFGF was subjected to two steps purification processes including cation exchange and heparin affinity chromatography techniques as mentioned in methods. Figure 1 depicts the purified BFGF.

The purified hBFGF then was PEGylated and purified by size exclusion chromatography. Good separation of PEGylated hBFGF from reaction mixture, was achieved by using sufficient tall column (60 cm), low sample apply volume (2% column packed bed volume) and low flow rate (1 ml/min). Figure 2 shows the SDS-PAGE results of PEGylated recombinant BFGF separated from non-PEGylated form. The PEGylated form of the recombinant protein was separated.

Further, the purified human recombinant BFGF and its PEGylated form were subjected to MTT assay in order to evaluate the biological activity. In this assay 10,000 3T3 clone A31 cell line/well was cultured in RPMI1640 containing 10% FBS at 5% CO_2 atmosphere in the presence of 50–200 ng of PEGylated and native recombinant BFGF and 68 and 98% of proliferation rate were observed respectively [Figure 3].

Free BFGF and BFGF-polyethylene glycol (PEG) were treated with guanidine hydrochloride in order to study their stability by fluorescence spectroscopy. It was observed that BFGF was totally denatured by exposure to guanidine hydrochloride (37°C, 24 h) at 0.8 M concentration whereas BFGF-PEG was denatured by 1.2 M concentration of the same denaturing agent. Figure 4 shows the effect of denaturation on the fluorescence spectrophotometry results.

Figure 5 depicts FTIR of free BFGF-PEG, BFGF and PEG where there is no structural change occurring in BFGF after PEGylation.

DISCUSSION

PEGylation of proteins is a method through which the stability of protein can be improved. Random and nonselective PEGylation occurs at N-termini or lysine residues causing heterogeneous with low biological activity products while selective PEGylation reduces the above disadvantages brought about by nonselective PEGylation. Stability of PEGylated form is measure with different methods like heat stability or exposure to denaturing reagent. In this study, we use exposing to denaturing reagent like guanidine hydrochloride and determine by using fluorescence spectrophotometry as a unique assay for BFGF that recognize the stable, semi stable and un stable form

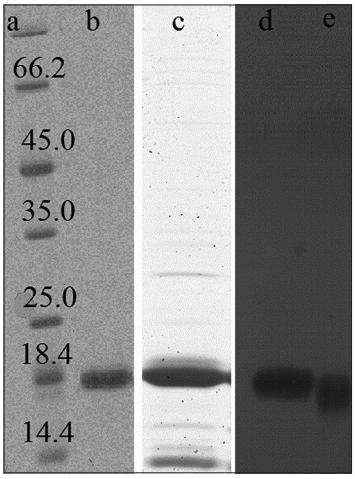

Figure 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results of purified human basic fibroblast growth factor (hBFGF). Lane A: Ladder, Lane B: Heparin affinity eluate, Lane C: Cation exchange eluate, Lane D: Western of standard hBFGF, Lane E: Western of purified hBFGF

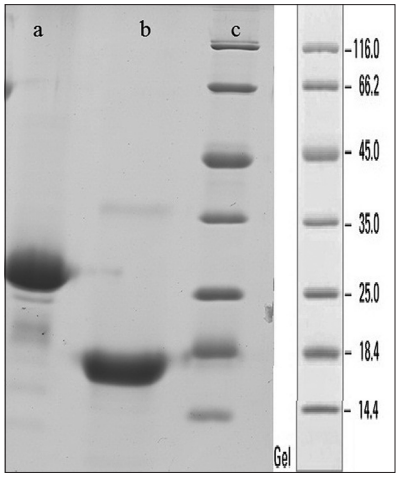

Figure 2: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis results of two fraction separated by size exclusion chromatography. Lane A: PEGylated form in first fraction, Lane B: Non-PEGyalated form in second fraction, Lane C: Ladder

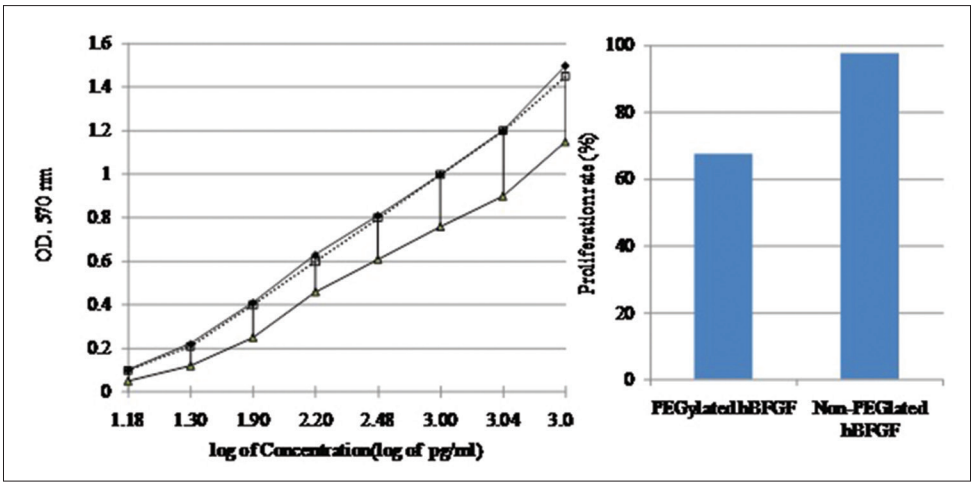

Figure 3: Biological activity analysis. Left: Proliferation assay for standard human basic fibroblast growth factor (hBFGF) (—◆—), PEGylated hBFGF (• • ◘ • •) and non-PEGylated form (—▲—). Right: Comparison of proliferation rate of PEGylated non-PEGylated hBFGF

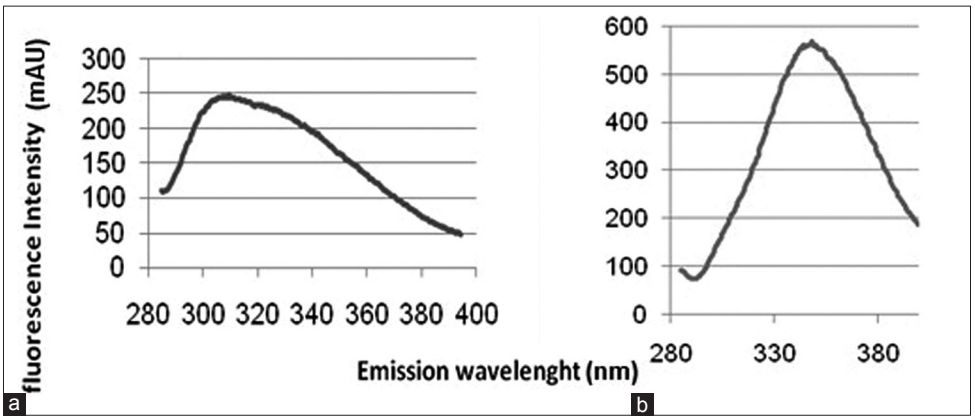

Figure 4: Fluorescence spectrophotometry. (a) intact PEGylated basic fibroblast growth factor (BFGF), (b) denatured form of PEGylated BFGF

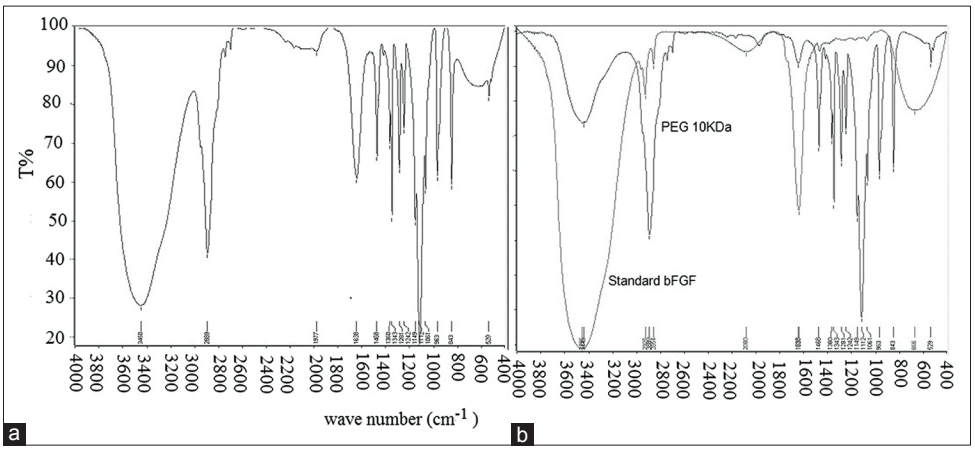

Figure 5: Fourier transform infrared (FITR) spectroscopy of free basic fibroblast growth factor-polyethylene glycol (BFGF-PEG), BFGF and PEG. (a) FTIR of PEGylated form, (b) FTIR of standard BFGF and PEG 10 kDa

of molecule.[46] We showed that the PEGylated form of hBFGF is resistant to denaturing agent like guanidine HCl though its biological activity to some extent is approximately 30% reduced. FTIR assay confirmed that PEGylation did not effect on the protein structure and masking the active site of protein partially is the reason of decreased bioactivity of PEGylated hBFGF.[6]

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