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Site-directed Chemical Modification of Recombinant Human aFGF Mutant with Polyethylene Glycol

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Absract: A new product PEGylated rhaFGF was obtained by site-directed chemical modification. When compared with unmodified rhaFGF, PEGylated rhaFGF showed comparable bioactivity and superior stability at 37°C in mouse serum and the stronger resistant potency to trypsin. This was accompanied by a substantial decreasing immunogenicity. Site-specific PEGylation of rhaFGF may increase its therapeutic potency in humans.

Keywords: rhaFGF, chemical modification, PEGylated rhaFGF.

Human acid fibroblast growth factor (haFGF) is a cytokine which can stimulate all the cells of mesodermal and neuroectodermal origin to divide. As an efficient tissue repaired stimulator and a cardio- and neuroprotector, haFGF has a unique potential for wound healing, bone damage curing and cardio- and neural disease treatment[1-2]. But some defection of haFGF, such as low stability, short half life *in vivo* and strong immunogenic activity, limited its clinical application. It has been proven that modification of proteins with polyethylene glycol (PEG) was an efficient method to enhance the stability as well as to lower the immunogenic activity of proteins[3]. In this paper, a new product PEGylated rhaFGF was obtained by site-directed chemical modification. The results indicated that the mitogenic activity of PEGylated rhaFGF was comparable to that of native rhaFGF *in vitro*, while the resistant potency of PEGylated rhaFGF to temperature and trypsin was much stronger than that of native rhaFGF and the immunogenicity of PEGylated rhaFGF was lower than that of the latter. It seems the PEGylated rhaFGF generating by site-directed PEGylation of recombinant rhaFGF is more suitable for therapeutic application.

Experimental

Before site-directed PEGylation of rhaFGF, a rhaFGF mutant was constructed by replacing the 98 th and the 132 nd cysteine residue with serine residue. The mutant, which retained the bioactivity of rhaFGF, was then conjugated with 5 kD polyethylene

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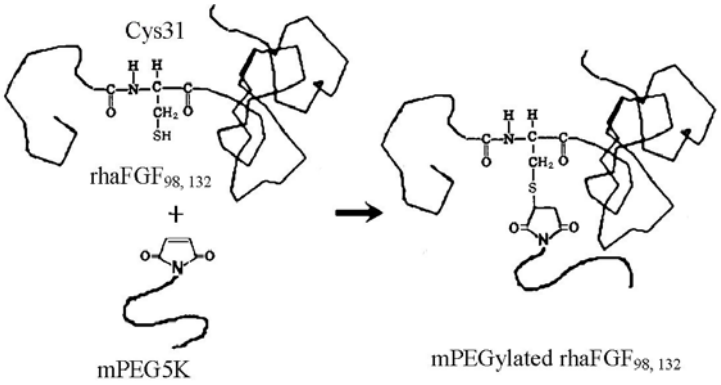
Figure 1 Reaction of rhaFGF$_{98,132}$ and mPEG5K



glycol-maleimide at the 31st cysteine position (**Figure 1**).

A typical procedure for preparation of PEGylated rhaFGF mutant is as follows: rhaFGF mutant with a free thiol group in PBS (pH=7.2) was allowed to react with 30-fold molar excess of 5kD mPEG-maleimide (mPEG5K) at 25°C for 4 h. The reaction mixture was directly loaded on a Sephadex G-25 (Amersham Phamacia) column to eliminate salt and loaded on a Heparin SepharoseTM CL-6B (Amersham Phamacia) column previously equilibrated with 20 mmol/LPB (pH=7.2). Washing the column with buffer A (20 mmol/LPB(pH=7.2)). After the UV absorbance trace return to the basal, the column was eluted with buffer B (0.6 mol/L NaCl in 20 mmol/L PB (pH=7.2)). Collecting the peak fraction to get the PEGylated rhaFGF mutant. The purity of the product was up to 99%, according to the result of SDS-PAGE analysis.

The mitogenic activity of native and PEGylated rhaFGF was assessed by MTT method. The stability of native and PEGylated rhaFGF was determined by incubating them at a final concentration of 1 mg/mL at 37°C in mouse serum. The remaining mitogenic activity of the treated sample was checked by MTT method. The resistant potency of native rhaFGF and PEGylated rhaFGF to trypsin was determined by SDS-PAGE of the equal molar samples, which were incubated with trypsin at 37°C for 0, 5, 10 and 30 min, followed by densitometry scanning. Immugenicity of native and PEGylated rhaFGF was analyzed as follows: Inject the male BAL B/c mice with native and PEGylated rhaFGF at doses of 1.875μmol/mouse in PBS (pH 7.4, 50 mmol/L) containing FCA. The mice were immunized again two weeks after the first immunization. The specific IgG levels in serum were assessed by ELISA.

Result and Discussion

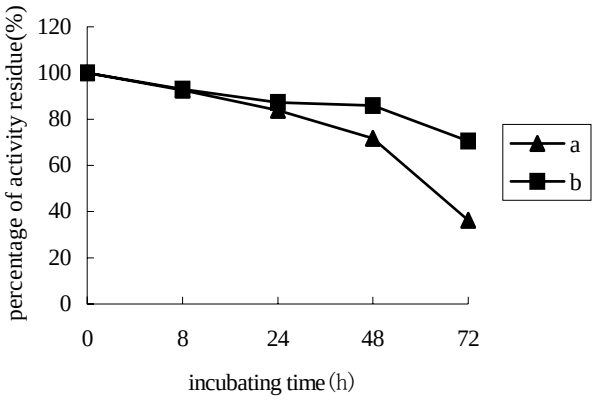
The mitogenic activity of PEGylated rhaFGF is 55.53% of native rhaFGF. Compared with other reported proteins modified by site-directed PEGylation [4], PEGylated rhaFGF showed comparatively high bioactivity. That the chemical modified position at the 31st Cys residue is far from the active site of rhaFGF, which is located in the C-terminal[5], it could be the reason for high bioactivity remaining of PEGylated rhaFGF.

The heat stability of native and PEGylated rhaFGF was assessed by incubating them in mouse serum at 37°C for various periods of time (**Figure 2**). The mitogenic activity of native rhaFGF diminished in a time-dependent manner. In contrast, PEGylated rhaFGF was quite stable. After 48 h, PEGylated rhaFGF retained about 70.51% of their initial activity.

The result indicated that the resistant potency of PEGylated rhaFGF to trypsin was higher than that of the native rhaFGF. Incubated with trypsin for 30 min PEGylated rhaFGF retained about 32% of its amount, however, the native rhaFGF was almost hydrolyzed.

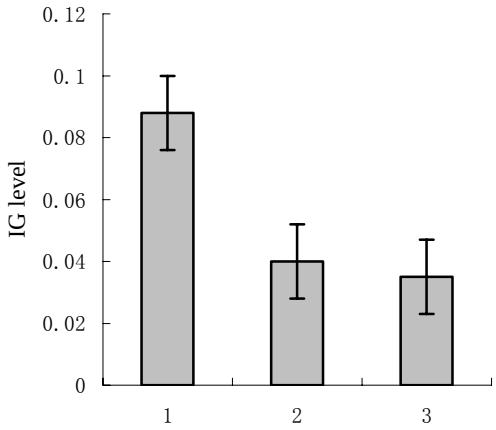
The reason of these results is possible that the PEG sterically hinders rhaFGF from proteolytic attack[6].

Figure 2 Stability of PEGylated rhaFGF in mouse serum.



a-native rhaFGF; b-PEGylated rhaFGF

Figure 3 IgG responses in mice to PEGylated rhaFGF.



1-native rhaFGF; 2-PEGylated rhaFGF; 3-pH7.4, 50 mmol/L PBS

PEGylated rhaFGF was also found to have decreased immunogenicity (**Figure 3**), which could be attributed to reduced degradation by antigen-presenting cells, shielding of some epitopes of peptides after degradation[7], and prevention of binding to receptors on B cells. Additionally, the transportation of PEGylated rhaFGF from blood to immune tissues such as spleen, bone marrow, and lymphoid tissue may be limited[8]. The nonspecific binding and uptake of PEGylated proteins by antigen-presenting cells may also be reduced. Detailed studies on tissue distribution and cell-binding are necessary to clarify these issues.

With higher stability and lower immunogenicity, the new product PEGylated is of great interest for developing therapeutic application. The approach used to modify rhaFGF could be applicable to other recombinant growth factors modification aimed at improving the stability and immunogenicity of them.

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