J. Pharm. Pharmacol. 1994, 46: 168–171
Received June 2, 1993

Aluminium β-Cyclodextrin Sulphate as a Stabilizer and Sustained-release Carrier for Basic Fibroblast Growth Factor

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Abstract—The water-insoluble aluminium salt of β-cyclodextrin sulphate (Al·β-CyD-Sul) was used as a stabilizer and sustained-release carrier for recombinant human basic fibroblast growth factor (bFGF). An adsorbate of bFGF with Al·β-CyD-Sul was prepared by incubating the protein with a suspension of Al·β-CyD-Sul in water. The mitogenic activity of bFGF released from the adsorbate, as indicated by the proliferation of kidney cells of baby hamster (BHK-21), was almost comparable with that of the intact bFGF. Al·β-CyD-Sul significantly protected bFGF from proteolytic degradation by pepsin and α-chymotrypsin, compared with the water-soluble sodium salt. The in-vitro release of bFGF from the adsorbate was sustained in proportion to a rise in the ratio of Al·β-CyD-Sul to the protein in the adsorbate. Of the bFGF preparations evaluated, the adsorbate of bFGF with Al·β-CyD-Sul, when given subcutaneously to the rat, showed the most prominent increase in the formation of granulation tissues, due to the stabilization and slow-release of the mitogen. The limited data presented here suggest that the adsorbate of bFGF with Al·β-CyD-Sul has a potent therapeutic efficacy for wound healing, and may be applicable to oral protein formulations for the treatment of intestinal mucosal erosions.

There has been increasing interest in optimizing the delivery of peptide and protein drugs with the use of rationally-designed carrier materials. Cyclodextrins are useful for eliminating undesirable pharmaceutical properties of this class of drugs, a characteristic which makes them candidates for novel peptide carriers (Brewster et al 1991; Uekama et al 1991). Recently, many kinds of cyclodextrin derivatives have been prepared to extend their inclusion abilities as host molecules. Introduction of sulphate groups onto hydroxyl groups of cyclodextrins confers heparin-mimicking activity on such derivatives (Folkman et al 1989; Shing et al 1990). Sodium salts of cyclodextrin sulphates are highly hydrophilic amorphous mixtures with distributions of the degree of substitution by sulphate groups (Pitha et al 1991) and less toxic than the parent cyclodextrins when given parenterally in the rat (Shiotani et al 1992).

Basic fibroblast growth factor (bFGF) is a potent mitogen that stimulates the proliferation of a wide variety of cells and could play a crucial role in wound healing processes (Gospodarowicz et al 1987). The therapeutic potential of bFGF, however, may have not been fully realized, because of its susceptibility to proteolytic inactivation and short duration of retention at the site of action (Davidson et al 1985; Gospodarowicz & Cheng 1986). Recent studies have demonstrated that sulphated oligosaccharides, including a sodium salt of β-cyclodextrin sulphate (Na·β-CyD-Sul), have high affinity to bFGF and protect it from heat, acid and proteolytic degradation (Kato et al 1990; Kajio et al 1992). Unfortunately, the highly hydrophilic nature of Na·β-CyD-Sul is not suited to the design of bFGF formulations with controlled-release features. In this study, therefore, a water-insoluble aluminium salt of β-cyclodextrin sulphate (Al·β-CyD-Sul) was prepared, and its possible utility in bFGF formulations was investigated.

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Materials and Methods

Recombinant human bFGF was obtained from Scios Nova Inc. (CA, USA). Pepsin from porcine stomach mucosa and α-chymotrypsin from bovine pancreas were obtained from Wako Pure Chemical Ind. (Osaka, Japan) and Sigma Chemical Co. (St Louis, MO, USA), respectively. Na·β-CyD-Sul was prepared according to the method described by Folkman et al (1989). The average degree of substitution of sulphate groups in Na·β-CyD-Sul was confirmed to be 10·7 by fast-atom bombardment-mass spectrometry and elemental analysis (Pitha et al 1991). Al·β-CyD-Sul was prepared by dissolving Na·β-CyD-Sul and aluminium chloride in water, followed by adjustment of the pH to 4·5 with 2 M NaOH. After stirring at room temperature (25°C) for 2 h, the resultant precipitate was washed with water and dried in-vacuo at 60°C for 24 h. An adsorbate of bFGF with Al·β-CyD-Sul was prepared by incubating the protein with a suspension of Al·β-CyD-Sul in water at room temperature. The mitogenic activity of bFGF was assessed by the method described by Neufeld & Gospodarowicz (1985) with slight modification, using baby hamster kidney fibroblast cell line (BHK-21). After three days' cultivation of BHK-21 cells (10^3 cells/well) with bFGF at various concentrations, the increase in protein contents as a measure of cell proliferation was determined by the bicinchoninic acid method (BCA Protein Assay Kit Pierce Co., IL, USA) at 595 nm. The in-vitro release of bFGF from its Al·β-CyD-Sul adsorbate (equivalent to 250 μg as bFGF) into 900 μL 20 mM isotonic phosphate buffer (pH 7·4) was measured at 37°C. After each

sampling, the medium was replaced with 900 μL fresh buffer, and the cumulative amount of bFGF released was recorded.

The degradation of bFGF (250 μg mL^{-1}) in the absence and presence of β-CyD-Sul (25 mg mL^{-1}) was determined at 37°C in pH 1·2 HCl containing pepsin (50 μg mL^{-1}) and in 20 mM phosphate buffer (pH 7·0) containing α-chymotrypsin (50 μg mL^{-1}). The concentration of bFGF was determined by high-performance liquid chromatography under the following conditions: pump, Tosoh CCPM (Tokyo, Japan); detector, Tosoh UV-8011 (Tokyo, Japan) at 277 nm; column, Tosoh TSK-GEL Heparin-5PW (7·5 × 75 mm, Tokyo, Japan). bFGF was eluted with gradient from 0·6 to 3·0 M NaCl in 20 mM Tris-HCl buffer (pH 7·5) containing 1 mM EDTA for 40 min at a flow rate of 1·0 mL min^{-1}.

The effects of bFGF preparations on the formation of granulation tissues were examined by the method of Sakai et al (1988). bFGF preparations consisted of bFGF (500 μg mL^{-1}) in the absence and presence of β-CyD-Suls or $Al(OH)_3$ (50 mg mL^{-1}) in water. Male Wistar rats, 170–190 g, were anaesthetized with ether. A sterile paper disc (0·7 mm in thickness, 8 mm in diameter, Toyo Roshi, Tokyo, Japan) containing 20 μL of each bFGF preparation was implanted under the dorsal skin of the rat. On the seventh day after the implantation, the rats were killed with anaesthetic ether, and the paper discs were carefully removed and the granulation tissues formed around the discs were weighed. Data were analysed statistically by one-way analysis of variance. The significance of difference between two means was evaluated using Fisher's paired *t*-test; $P < 0.05$ was considered to be significant.

Results and Discussion

Al·β-CyD-Sul was obtained as an amorphous fine powder and was slightly soluble in water. The aluminium and sulphur content in Al·β-CyD-Sul were 17·0 and 10·6% by weight, respectively. This molar ratio of aluminium to sulphur at 2:1 in the preparation indicates that the dimer unit, $Al_2(OH)_5$ group, binds primarily to each sulphate group in Al·β-CyD-Sul in a similar manner as reported on a basic aluminium salt of sucrose sulphate (Nagashima & Yoshida 1979). Sulphated oligosaccharides have high affinity to bFGF and regulate the biological activity of the mitogen (Turnbull et al 1992; Gallagher & Turnbull 1992). The adsorbate of bFGF with Al·β-CyD-Sul was readily prepared by incubating the protein with a suspension of Al·β-CyD-Sul in water. For example, when 1 mL 250 μg mL^{-1} bFGF solution was added to 25 mg Al·β-CyD-Sul, 99·8 ± 1·3% of the protein was adsorbed to Al·β-CyD-Sul. With increasing the ionic strength of the medium, the adsorbate was dissociated into each component, a feature which indicates that the association of bFGF with Al·β-CyD-Sul is probably due to electrostatic interaction (Shing et al 1990; Kajio et al 1992). Under these conditions, bFGF was completely eluted from the adsorbate with a buffer composed of 0·1 M citric acid and 0·2 M dibasic sodium phosphate at weak acidic and neutral pH. Fig. 1 shows the typical proliferation profiles of BHK-21 cells treated with bFGF released from its Al·β-CyD-Sul adsorbate as a function of the initial concentrations

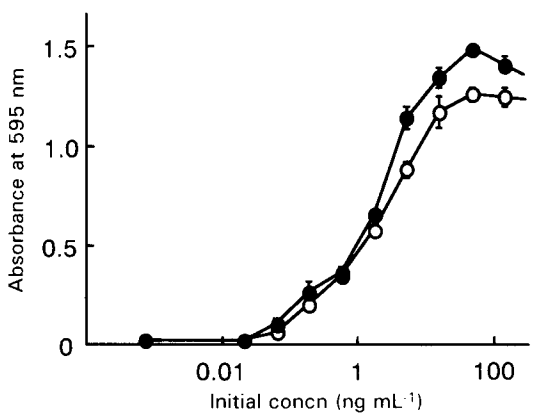

FIG. 1. Proliferation profiles of BHK-21 cells as a function of initial concentrations of bFGF released from its adsorbate with Al·β-CyD-Sul. ○ Intact bFGF, ● bFGF released from the adsorbate. Each value represents the mean ± s.e. of quadruplicate assays.

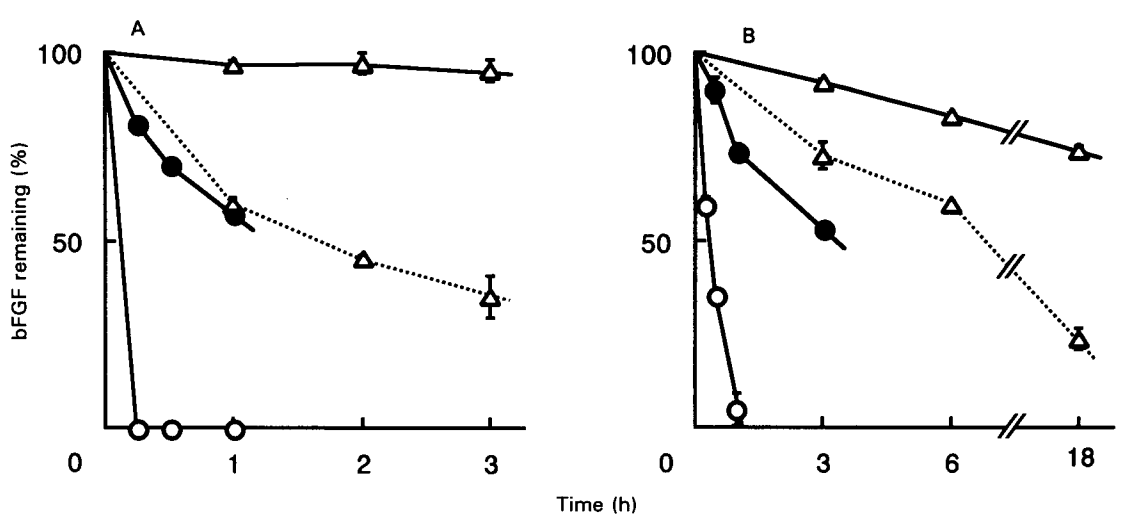

FIG. 2. Effects of β-CyD-Suls (25 mg mL^{-1}) on the stability of bFGF (250 μg mL^{-1}) in the presence of A. pepsin (50 μg mL^{-1}, pH 1·2) and B. α-chymotrypsin (50 μg mL^{-1}, pH 7·0) at 37°C. —○— bFGF alone, —●— bFGF with Na·β-CyD-Sul, —△— bFGF with Al·β-CyD-Sul, ···△··· bFGF with Al·β-CyD-Sul (pH-adjusted). Each value represents the mean ± s.d. of triplicate runs.

of the protein, where the increase in protein content was used as a measure for the proliferation of the cells. It is evident that the mitogenic activity of bFGF was completely recovered by elution from the Al·β-CyD-Sul adsorbate.

Fig. 2 shows the effects of β-CyD-Suls on the stability of bFGF in the presence of pepsin and α-chymotrypsin. When bFGF was incubated with pepsin at 37°C and pH 1·2, the protein was rapidly degraded and almost completely abolished within 15 min. Al·β-CyD-Sul and Na·β-CyD-Sul protected bFGF against peptic digestion, the former being more effective (Fig. 2). It should be noted that in the presence of Al·β-CyD-Sul, the medium pH was shifted to 3·8, at which the catalytic activity of pepsin was minimal. In the pH-adjusted medium, the inhibitory effect of Al·β-CyD-Sul was almost comparable with that of Na·β-CyD-Sul. This indicates that the protection of bFGF by Al·β-CyD-Sul is, in part, ascribable to its intrinsic antacid activity. As shown in Fig. 2, bFGF was degraded within 1 h when incubated with α-chymotrypsin at 37°C and pH 7·0. Both β-CyD-Suls markedly protected bFGF against the enzymatic degradation; in the case of the Al·β-CyD-Sul adsorbate, more than 70% of initial amount of bFGF remained even after 18 h of incubation. Also, the presence of Al·β-CyD-Sul shifted the medium pH to 6·3, at which the specific activity of α-chymotrypsin was reduced by about 50%. Even when the medium pH was adjusted, the stabilizing effect of Al·β-CyD-Sul was greater than that of the soluble sodium salt (Fig. 2).

Fig. 3 shows the release profiles of bFGF from its Al·β-CyD-Sul adsorbate into 20 mM isotonic phosphate buffer (pH 7·4) at 37°C. The degradation of bFGF during the experiment was less than 5% of the initial amount. It is apparent that the release of bFGF was significantly retarded by Al·β-CyD-Sul, the rate depending on the ratio of Al·β-CyD-Sul to the protein in the adsorbate. Davidson et al (1985) have reported that the sustained delivery of growth factors may greatly enhance their effects upon wound repair. These results suggest that water-insoluble Al·β-CyD-Sul, in addition to protecting bFGF from enzymatic inactivation, may also act as a sustained-release carrier for the protein and might potentiate the mitogenic activity of the protein in-vivo.

bFGF is known to increase the formation of granulation tissues in-vivo (Buntrock et al 1982; Yamaoka et al 1991). Table 1 shows the effects of bFGF preparations on the formation of granulation tissues, when they were implanted subcutaneously in the rat. On the seventh day after the implantation, the wet weight of granulation tissues for each bFGF preparation was greater than that of the corresponding protein-free vehicle. In particular, the adsorbate of bFGF with Al·β-CyD-Sul showed the most prominent proliferation-stimulating activity, probably due to the stabilization and sustained-release of the protein at the site of application.

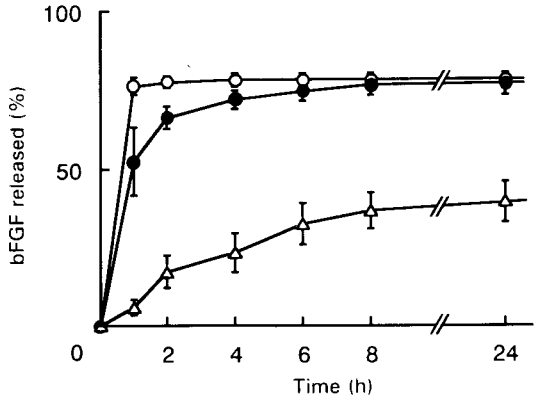

FIG. 3. Release profiles of bFGF from its adsorbate with Al·β-CyD-Sul containing bFGF (250 μg) and Al·β-CyD-Sul at various amounts into isotonic phosphate buffer (pH 7·4) at 37°C. ○ With Al·β-CyD-Sul (10 mg), ● with Al·β-CyD-Sul (20 mg), △ with Al·β-CyD-Sul (40 mg). Each value represents the mean ± s.d. of triplicate runs.

Table 1. The formation of granulation tissues of the rat on the seventh day after subcutaneous implantation of paper discs containing bFGF preparations (10 μg/disc as bFGF).

	Wet weight (mg)	
Treatment	Without bFGF	With bFGF
bFGF alone	98 ± 2	350 ± 46*
with $Al(OH)_3$ (1 mg/disc)	128 ± 14	235 ± 17*
with Na·β-CyD-Sul (1 mg/disc)	91 ± 6	283 ± 19*
with Al·β-CyD-Sul (1 mg/disc)	102 ± 2	1139 ± 334*,**

Each value represents the mean ± s.e. of four rats. *$P < 0.05$ vs each bFGF-free preparation. **$P < 0.05$ vs the bFGF preparation without additives.

Orally administered bFGF appeared to be rapidly degraded by digestive enzymes in the gastrointestinal tract, but the stable adsorbate of bFGF with Al·β-CyD-Sul may solve the problem. Nagashima & Hirano (1980) have demonstrated that an aluminium salt of sucrose sulphate, when administered orally, binds selectively to the ulcer lesion and forms a pepsin-resistant barrier over the surface of the lesion. It is likely that the adsorbate of bFGF with Al·β-CyD-Sul may have a similar affinity to the ulcer locus and may deliver the protein more effectively to the site of action, a situation which may enhance the therapeutic efficacy of the protein intended for treatment of gastrointestinal ulcers.

Acknowledgements

We thank Mr Makoto Okumura, Department of Pharmacology, Central Research Laboratories, Kaken Pharm. Co. Ltd, for his advice on the animal studies.

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