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The interaction of chitosan with fibroblast growth factor-2 and its protection from inactivation

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Abstract

Application of ultraviolet light (UV) irradiation to a photocrosslinkable chitosan (Az-CH-LA) aqueous solution including fibroblast growth factor-2 (FGF-2) results within 30 s in an insoluble, flexible hydrogel. The retained FGF-2 molecules in the chitosan hydrogel remain biologically active, and are released from the chitosan hydrogel upon the in vivo biodegradation of the hydrogel. In view of these findings, we here tested the interaction of chitosan with FGF-2, thereby modifying and stabilizing the FGF-2 activity from inactivations. The photocrosslinkable chitosan hydrogel has a low affinity for FGF-2 ($Kd = 6.12 \times 10^{-7}$ M). Soluble chitosan (CH-LA; Az-CH-LA without photocrosslinkable azide group) substantially prolonged the biological half-life time of FGF-2. Furthermore, CH-LA could protect the FGF-2 activity from inactivation, such as heat, proteolysis, and acid. The effect of chitosan on the FGF-2 activity is of a protective nature, since it had no effect of modifying the FGF-2 activity directly on growth of human umbilical vein endothelial cells (data not shown). Thus, one of the ways by which the chitosan potentiated the FGF-2 activity could be through protecting it from inactivations by the interaction between FGF-2 and chitosan molecules.

Keywords: Chitosan; Fibroblast growth factor-2 (FGF-2); Stabilization; Complex formation

1. Introduction

It has been recognized that growth factors contribute to tissue regeneration at various stages of cell proliferation and differentiation [1,2]. Although many studies using growth factors have been carried out in the field of tissue regeneration, its use has not always been achieved successfully in vivo [3]. Reasons for this difficulty are the high diffusibility and the very short half-life time of growth factors in vivo to retain their biological activity. Thus, it is required to enhance their biological activity in vivo in order to apply growth factors in tissue regeneration.

Among the fibroblast growth factors, FGF-2 was characterized well [2] and currently is available as a pharmaceutical medicine. It is a potent modulator of cell proliferation, motility, differentiation, and survival, and also plays an important role in regeneration processes in vivo, i.e., embryonic development [4], angiogenesis [5], osteogenesis [6], chondrogenesis [7], and wound repair [8]. FGF-2 is known to be stored at various sites in the body,

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interacting with glycosaminoglycans such as heparin and heparan sulfate of the extracellular matrix [9].

FGF-2 binds specifically to heparin and heparan sulfate with a high affinity, and its mitogenic activity and biological stability are modulated by heparin and heparan sulfate [10,11]. Heparin and derived dextrans (mimic heparin) also protect FGF-2 from inactivation by acid and heat, as well as from degradation by proteases [10–12]. Other studies have shown that heparin and heparan sulfate serve as a co-factor to promote binding of FGF-2 to high-affinity receptors, thereby enhancing its activity [10–12]. Based on this in vivo storage mechanism, controlled release of the heparin-binding growth factors has been described from heparin-carrying polystyrene-bound collagen substrata [13], acidic gelatin hydrogels [3], alginate gels containing heparin [14], and FGF-2 containing chitosan/heparinoid hydrogels [15].

Chitin is a linear homopolymer of 1, 4 β-linked *N*-acetyl-D-glucosamine, and chitosan is a partially deacetylated chitin. Chitin and chitosan have been proposed as biomaterials having a range of biomedical and industrial applications attributable to its biocompatibility [16]. Chitosan has been observed to accelerate wound healing [16,17] by inducing infiltration of inflammatory cells into a wound area [18], activation of macrophages [19], production of cytokines [20], as well as possessing anti-infection activity [21]. Several products using chitin and chitosan for wound treatments in a filament, film, membrane, powder, granule, or sponge format have already been in the market [16].

We have previously reported the preparation and characterization of a novel photocrosslinkable chitosan [22]. The material is a viscous solution and is easily crosslinked upon ultraviolet light (UV) irradiation, resulting in an insoluble hydrogel within 30 s. The chitosan hydrogel has been found to induce wound contraction and healing in normal mice [23]. When FGF-2 was added to the photocrosslinkable chitosan hydrogel, 70–80% of the FGF-2 molecules retained in the chitosan hydrogel for several weeks, and was released from the chitosan hydrogel during the in vivo biodegradation of the hydrogel [24]. Furthermore, the application of FGF-2 containing chitosan hydrogels onto a healing-impaired wound in diabetic d*b*/d*b* mice induces a significant wound contraction and accelerates wound closure and healing. However, it has to be investigated whether chitosan molecules interact with FGF-2 and whether the FGF-2 activity remains stable in the chitosan hydrogel for a longer period. The purpose of the present study has been to evaluate the interaction of FGF-2 with chitosan molecules and the stabilization of FGF-2 by chitosan molecules. Here we report that chitosan has a low affinity for FGF-2 molecules and that it is able to protect FGF-2 from heat, acid, and proteolytic inactivation.

2. Materials and methods

2.1. Preparation of soluble chitosan (CH-LA) and photocrosslinkable chitosan molecules (Az-CH-LA)

The chitosan used in this study had a molecular weight of $0.8–1 \times 10^6$ Da with a deacetylation ratio of 0.8 (Yaizu Suisankagaku Industry Co. Ltd., Shizuoka, Japan). For the soluble chitosan (CH-LA), lactose (lactobionic acid) moieties have been introduced through a condensation reaction with amino groups. The introduction of lactose resulted in a water-soluble chitosan at neutral pH-values. It has been estimated that about 2% of the amino groups in the chitosan reacted with lactobionic acid [22]. For the photocrosslinkable chitosan molecules (Az-CH-LA), azide (*p*-azidebenzoic acid) has also been introduced through a condensation reaction with amino groups and it has been estimated that about 2.5% of the amino groups in the chitosan reacted with *p*-azide benzoic acid [22].

A viscous Az-CH-LA aqueous solution (20 mg/ml) has been converted into an insoluble hydrogel within 10 s upon UV-irradiation at a lamp distance of 2 cm (UV-irradiation System, Spot Cure ML-251C/A with a guide fiber unit (SF-101BQ) and 250 W lamp (240–380 nm), Usio Electrics Co. Ltd., Tokyo, Japan) through crosslinking of the azide and amino groups of the Az-CH-LA molecules.

2.2. FGF-2 binding assay to immobilized photocrosslinked chitosan hydrogel

Polystyrene (PS) spherical particles for ELISA (diameter: 2.5 mm, Sumitomo Bakelite Corp., Tokyo, Japan) were rinsed with a 2% Az-CH-LA aqueous solution and UV-irradiated for 1 min on a rotator. It was estimated that about 10 mg of the chitosan hydrogel (containing 0.2 mg of Az-CH-LA) was immobilized to each PS spherical particle. A single binding assay was carried out as follows. The chitosan hydrogel-immobilized PS spherical particle was suspended in phosphate-buffered saline (PBS) and a known concentration of I^{125}-FGF-2 (Specific activity; 70.48 μCi/μg, MP Biomedical, Inc., Irvine, CA, USA) + FGF-2 (Fiblast; Kaken Pharmaceutical Corp., Tokyo, Japan) and binding inhibitor (CH-LA, LMW-chitosan (Chitosan, low molecular weight, average molecular weight (MW): 7000 ± 1500, Seikagaku Corp., Tokyo, Japan), chitosan-hexamer (Seikagaku Corp.), or bovine serum albumin (Globulin free; Wako Pure Chemical Industries Ltd., Osaka, Japan) were added to initiate the binding. Specific binding was determined in the presence of 1 mg/