Control of basic fibroblast growth factor release from fibrin gel with heparin and concentrations of fibrinogen and thrombin

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Abstract

Basic fibroblast growth factor (bFGF) has been known to stimulate the regeneration of a number of tissues including cartilage, nerve, skin, liver, and blood vessel. Delivery of bFGF for a long period in a controlled manner would enhance stimulative effects. The purpose of the present study is to test the hypothesis that the kinetics of bFGF release from fibrin gels could be controlled with heparin and concentrations of fibrinogen and thrombin. The kinetics of bFGF release from fibrin gels with various concentrations of fibrinogen, thrombin, and heparin was determined. The bioactivity of bFGF released from fibrin gels was assessed using dermal fibroblast cell culture. To examine the therapeutic potential of the bFGF delivery system, bFGF-loaded fibrin gels were injected into mouse ischemic limbs. The addition of heparin to fibrin gels decreased the bFGF release rate. As the thrombin content in fibrin gels increased, the bFGF release rate significantly decreased. Similarly, increased concentration of fibrinogen in fibrin gels decreased the bFGF release rate. Basic FGF released from fibrin gels exhibited significantly higher extents of fibroblast growth than bFGF added in a free form daily into the culture medium, suggesting that the fibrin gels may stabilize the bFGF bioactivity. Immunohistological analysis of mouse ischemic limbs indicated that the microvessel density was much higher in the ischemic limbs treated with injection of bFGF-loaded fibrin gels than in the ischemic limbs with no treatment. This study showed that the rate of bFGF release from fibrin gels can be controlled and that the bFGF delivery system has therapeutic potentials for angiogenesis.
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1. Introduction

Basic fibroblast growth factor (bFGF) has shown therapeutic potentials for tissue regeneration. The treatment of ischemia in limb [1] and heart [2–4] with bFGF has been shown to enhance angiogenesis in animal studies and clinical trials. For skin wound

healing, bFGF significantly accelerated soft tissue formation, reepithelialization, and collagen maturation in humans and animals [5–7]. A study showed that bone fracture healing was accelerated by local administration of bFGF in rabbits [8]. However, bFGF undergoes rapid degradation when injected into the body in soluble form [9]. Delivery systems that release bFGF for a long period in a controlled manner could increase the efficacy of bFGF for angiogenesis and other tissue regeneration.

Methods to sustain the release of bFGF have been reported previously. These methods include the encapsulation of heparin–Sepharose-bound bFGF in alginate beads [10], the impregnation of collagen sponges with heparin–bFGF–fibrin mixtures [11], bFGF incorporation into hyaluronate gels [12], and gelatin hydrogels [13,14]. However, the bFGF release from these delivery systems was completed within only 3 days. Basic FGF-loaded poly(D,L-lactide-co-gylcolide) delivery system showed no initial burst and longer release periods [15], but it was difficult to control the release rate and period. The sustained release of bFGF for a longer period in a controlled manner would be desirable so as to be effective as a bFGF delivery system for tissue regeneration.

In recent years, fibrin gel has been used as a matrix to deliver growth factors. Growth factors, such as nerve growth factor [16], bFGF [17], vascular endothelial growth factor [18], glial cell line-derived neurotrophic factor [19], and transforming growth factor-β (TGF-β) [20], were contained within fibrin gels and released for a long period by slowing their diffusion through the fibrin gels. Fibrin gel is formed when fibrinogen is activated by thrombin in the presence of Ca^{2+} ion and factor XIII [21–23]. As a growth factor delivery system, fibrin gel can be easily implanted by injection through a needle without invasive surgery. A study showed that the release rate of TGF-β, a heparin binding growth factor, can be controlled by the presence of heparin in the fibrin gels [20]. However, the control of bFGF release from fibrin gel has not been studied extensively yet.

In this study, we investigated if bFGF release from fibrin gels can be controlled with various parameters and if bFGF released from fibrin gels is bioactive. The rate of bFGF release from fibrin gels was controlled with heparin and concentrations of fibrinogen and thrombin. The bioactivity of bFGF released from

fibrin gels was examined by measuring fibroblast growth in vitro in medium containing bFGF-loaded fibrin gels. In addition, the therapeutic potential of the bFGF delivery system was evaluated by implanting the delivery system in mice's ischemic limbs and by examining angiogenesis.

2. Materials and methods

2.1. Preparation of bFGF delivery system

For synthesis of recombinant human bFGF protein in vitro, a translation system RTS 500 (Roche Applied Sciences, Mannheim, Germany) was used. The feeding solution was prepared by mixing the reconstituted amino acid mix (2.65 ml) without methionine, reconstituted methionine (0.3 ml), and feeding mix (8 ml). The reaction solution was prepared by mixing *Escherichia coli* lysate (0.525 ml), reaction mix (0.225 ml), reconstituted amino acid mix (0.27 ml), methionine solution (0.03 ml), and pET–bFGF DNA (10 μg). The reaction solution and feeding solution were filled into the appropriate compartments of the reaction device separated by a semipermeable membrane according to the instrument manual. The reaction device was loaded into the RTS ProteoMaster instrument (Roche Applied Sciences) and incubated for 24 h at 30 °C and 130 rpm. The reaction mixture was applied to a Ni–NTA agarose resin column (Qiagen, Hilden, Germany) pre-equilibrated in 50 mM sodium phosphate buffer (pH 8.0) at a flow rate of 1 ml/min. The flow-through was discarded and proteins were washed with additional column volumes of buffer A 10 times. The His-tagged bFGF fraction was eluted from the column with 50 mM sodium phosphate buffer (pH 8.0) containing 200 mM imidazole. The volume of protein fractions was reduced to 5 ml by concentration in an Amicon YM10 membrane (Millipore, Billerica, MA, USA), and the proteins were cleaved using thrombin protease (10 U/mg fusion protein). The cleavage mixture of His-tagged bFGF protein was then applied onto heparin–Sepharose CL-6B column (Amersham Biosciences, Uppsala, Sweden) equilibrated in 50 mM sodium phosphate (pH 7.4). The column was washed with the column equilibration buffer, and the bound proteins were eluted using equilibration buffer containing 1.5 M NaCl. The excess salts in preparations