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Local delivery of siRNA using a biodegradable polymer application to enhance BMP-induced bone formation

Tomoya Manaka, Akinobu Suzuki*, Kazushi Takayama, Yuuki Imai, Hiroaki Nakamura, Kunio Takaoka

Department of Orthopaedic Surgery, Osaka City University Graduate School of Medicine, 1-4-3 Abenoku Asahimachi, Osaka 545-8585, Japan

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ABSTRACT

Small interfering RNA (siRNA) is useful tool for specific and efficient knockdown of disease-related genes. However, in vivo applications of siRNA are limited due to difficulty in its efficient delivery to target cells. In this study, we investigated the efficacy of a biodegradable hydrogel, poly-D,L-lactic acid-*p*-dioxanone-polyethylene glycol block co-polymer (PLA-DX-PEG), as a siRNA carrier. PLA-DX-PEG pellets with or without fluorescein-labeled dsRNA were implanted into mouse dosal muscle pouches. The cellular uptake of dsRNA surround the polymer was confirmed by fluorescent microscopy. The fluorescence intensity was dose-dependent of the dsRNA, and exhibited a time-dependent decrease. To investigate its biological efficiency, noggin (antagonoist to BMPs) gene-silencing with siRNA (siRNA/Noggin) was examined by the amount of suppression of BMP-2-induced noggin expression and the level of performance of BMP, indicated by ectopic bone formation. Noggin gene expression induced by BMP-2 was suppressed by addition of siRNA/Noggin to the implant, and the ectopic bone formation induced by implants with both BMP-2 and siRNA/Noggin was significantly greater than those induced by implants with BMP-2 alone. These results indicate the efficacy of local delivery of siRNAs by PLA-DX-PEG polymer, which intensified bone-inducing effects of BMP and promoted new bone formation by suppressing gene expression of Noggin.

1. Introduction

RNA interference (RNAi) is a gene-silencing process whereby double-stranded RNA (dsRNA) can mediate the degradation of sequence-specific mRNA. Short dsRNA fragments called small interfering RNAs (siRNAs) are intermediates in that process, and chemically synthetic siRNA have been shown to be powerful tools not only in functional genomic analyses, but also in therapeutic applications. However, there are few reports about the application of siRNA in skeletal disease or bone regeneration.

Bone morphogenetic proteins (BMPs) are well recognized as efficacious bone-inducing molecules, and BMP-2 and BMP-7 have been used in clinical practice for bone repair and reconstructive surgery [1,2]. However, a current problem in the clinical use of BMPs is the requirement of high doses of BMP in humans and the resulting high cost ($5000–8000/case) required to generate significant bone mass [3]. If BMPs were used in combination with agents that intensified the BMP activity, the required dose of BMP might be reduced. We have previously examined the efficacy of several agents used for the enhancement of BMP activity under in vivo and in vitro conditions [4–12]. One method used to enhance BMP activity is the functional blockade of physiological antagonists of BMP. Previous studies have indicated that noggin is a major antagonist to BMPs [13–15], and noggin gene expression is upregulated by BMP stimulation as a negative regulator in vitro and in vivo [16,17]. Our previous study showed that the silencing of noggin expression by the transfection of siRNA targeting noggin (siRNA/Noggin) suppressed BMP-stimulated noggin expression, resulting in the acceleration of BMP-induced osteoblastic differentiation in vitro [17].

The siRNAs are relatively unstable in blood and serum and do not freely permeate the cell membrane. Thus, a safe and effective intracellular delivery system is essential for in vivo use of siRNAs. To date, several delivery systems for siRNAs have been reported. However, they have some disadvantages with respect to their clinical use [18–20]. Viral vectors are highly efficient delivery systems for nucleic acids, but they have the potential risk of inducing adverse immune responses and inadvertent gene expression changes following random integration into the host genome [21,22]. Cattle-derived atelocollagen has a potential risk of immunologic reactions and the transmission of infectious disease. Cationic lipid has been reported that the delivery of synthetic siRNA

* Corresponding author. Tel.: +81 6 6645 3851; fax: +81 6 6646 6260.
E-mail address: a-suzuki@msic.med.osaka-cu.ac.jp (A. Suzuki).

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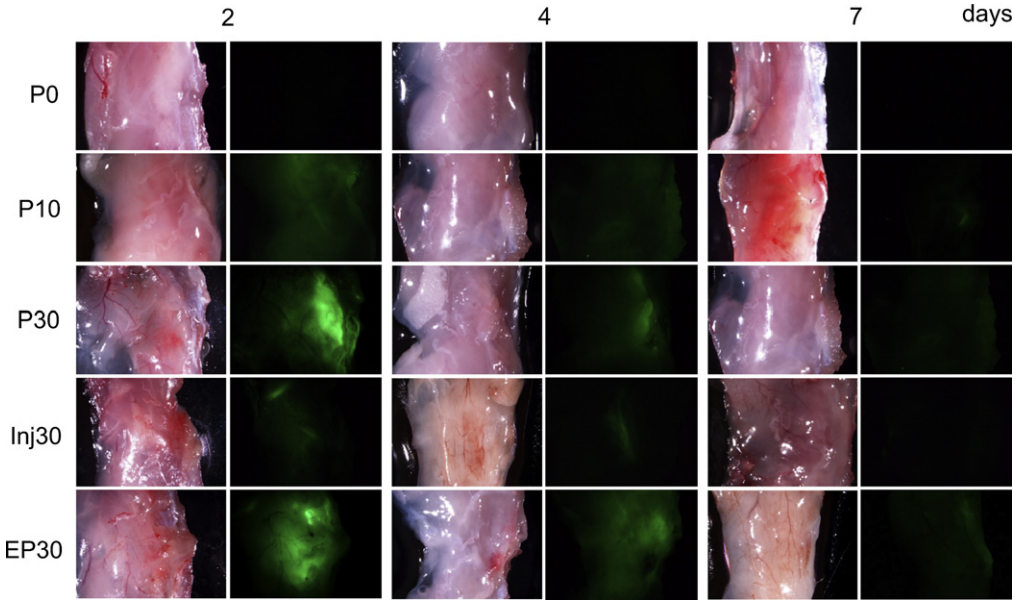

Fig. 1. Stereoscopic images of fluorescence in muscle tissue and their optic images. Note that fluorescent oligonucleotide double-stranded RNA (dsRNA) uptake was observed in muscle tissue adjacent to the PLA-DX-PEG pellets containing fluorescein-labeled dsRNA. The fluorescence increased in a dose-dependent manner, but decreased as time elapsed after implantation of the PLA-DX-PEG pellets containing fluorescein-labeled dsRNA. P0, group implanted with PLA-DX-PEG polymer without fluorescein-labeled dsRNA; P10, group implanted with PLA-DX-PEG polymer containing 10 nmol of fluorescein-labeled dsRNA; P30, group implanted with PLA-DX-PEG polymer containing 30 nmol fluorescein-labeled dsRNA; Inj30, group injected with 30 nmol fluorescein-labeled dsRNA into the back muscle; EP30, group injected with 30 nmol fluorescein-labeled dsRNA and then treated with electroporation.

conjugated with lipids can induce immunological reactions [23]. Electroporation may also be effective for the delivery of non-permeable siRNA into local cells, but there is a potential risk of tissue damage by the electrical charge. In order to avoid the risks associated with these methods and in order to explore a safe method to allow the siRNA to permeate through the cytoplasmic membrane into cells, a biodegradable hydrogel (poly-D,L-lactic acid with randomly inserted dioxanone and polyethylene glycol, PLA-DX-PEG) was tested as a delivery system for the siRNAs in this study. This hydrogel was originally developed as a carrier material for BMP for in vivo local bone induction [24,25]. The efficacy of the PLA-DX-PEG polymer as a carrier of siRNA into cells was estimated using small fluorescent dsRNA. Furthermore, we evaluated the biological efficiency of the siRNA/Noggin transferred into the cells in an in vivo system of ectopic bone induction by BMP in mice.

2. Materials and methods

2.1. Reagents

A biodegradable co-polymer that was composed of poly-D,L-lactic acid with random insertion of p-dioxanone and polyethylene glycol (PLA-DX-PEG polymer, MW 9800, PLA/DX/PEG molar ratio: 3/1/3) was synthesized and donated by Taki Chemical Co., Ltd (Kakogawa, Japan). The chemical and biological characteristics of this polymer have been described previously [24,26,27]. Fluorescein-labeled dsRNA oligomer (Fluorescent Oligo®; 1 mM) was purchased from Invitrogen Corporation (Carlsbad, CA, USA) and used to assay the transfection efficiency in vivo. The siRNA/Noggin was synthesized using Stealth siRNA duplex oligoribonucleotides® (Invitrogen Corporation) with the sequence 5′-AAC ACU UAC ACU CGG AAA UGA UGG G-3′ and 3′-CCC AUC AUU UCC GAG UCG AAC GCG G-5′ [17]. The nonspecific control siRNA duplex was also purchased from Invitrogen Corporation. Recombinant human BMP-2 was provided by Osteopharma Inc. (Osaka, Japan).

2.2. Mice

Male Imprinting Control Region (ICR) mice that were 6 weeks old were purchased from Nippon SLC Co., Ltd. (Hamamatsu, Japan) and housed in cages with free access to food and water. After acclimation for 1 week, experiments were conducted in strict accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals of Osaka City University.

2.3. In vivo assay of transfection efficiency with fluorescence-labeled dsRNA oligomers

In order to prepare a single test implant, 30 mg of PLA-DX-PEG was melted by heating it to 37.5 °C and then mixed and incubated for 20 min with small fluorescein-labeled dsRNA at a concentration of 0, 10, or 30 nmol per implant. The implants were stored at −80 °C overnight. The mice were anesthetized by ketamine and xylazine injections (ketamine, 2.6 mg/kg and xylazine, 0.8 mg/kg), and the PLA-DX-PEG pellets with or without fluorescein-labeled dsRNA were surgically

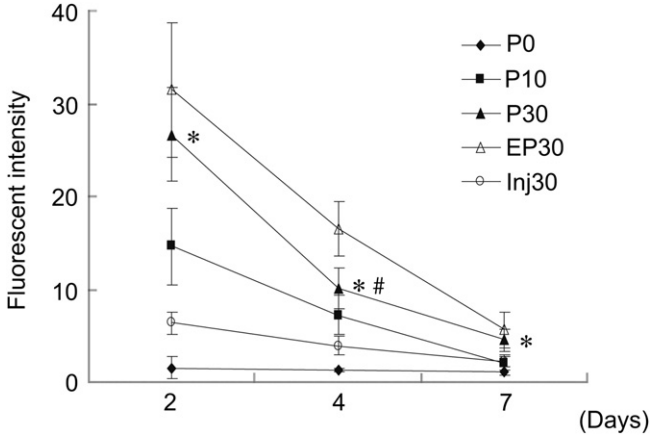

Fig. 2. Quantification of the fluorescence in the stereomicroscopic images. The fluorescence of the muscle treated with the polymer containing 30 nmol dsRNA was significantly stronger than the muscle treated with an injection of 30 nmol dsRNA at each time point. Data represent the mean (SE). P0, group implanted with PLA-DX-PEG polymer without fluorescein-labeled dsRNA; P10, group implanted with PLA-DX-PEG polymer containing 10 nmol of fluorescein-labeled dsRNA; P30, group implanted with PLA-DX-PEG polymer containing 30 nmol fluorescein-labeled dsRNA; Inj30, group injected with 30 nmol fluorescein-labeled dsRNA into the back muscle; EP30, group injected with 30 nmol fluorescein-labeled dsRNA and then treated with electroporation. $^{*}P < 0.05$, compared to injection of 30 nmol dsRNA. $\#P < 0.05$, compared to electroporation with injection of 30 nmol dsRNA.