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Enhancement of bone regeneration by dual release of a macrophage recruitment agent and platelet-rich plasma from gelatin hydrogels

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ABSTRACT

Macrophages play an important role in regulating inflammatory responses and tissue regeneration. In the present study, their effect on bone remodeling is investigated by the simultaneous application of a macrophage recruiting agent, SEW2871 of a sphingosine-1 phosphate agonist, and platelet-rich plasma (PRP). The non-water soluble SEW2871 was solubilized in water through micelles formation with L-lactic acid grafted gelatin, and the resulting micelles with PRP were incorporated into gelatin hydrogels. Mixed SEW2871-micelles and PRP were released from gelatin hydrogels in a controlled fashion both *in vitro* and *in vivo*. *In vitro* migration assay revealed that the presence of PRP synergistically promoted SEW2871-induced macrophages migration. When applied to a bone defect of rats, the hydrogels incorporating mixed SEW2871-micelles and PRP recruited a higher number of macrophages than those hydrogels incorporating either SEW2871-micelles or PRP. The hydrogels incorporating mixed SEW2871-micelles and PRP enhanced the level of tumor necrosis factor (TNF)-α of pro-inflammatory cytokine, 3 days after application, while pro-inflammatory responses coupled with a significant increase in the expression level of osteoprotegerin (OPG) and interleukin (IL)-10 and transforming growth factor (TGF)-β_1 of anti-inflammatory cytokine were observed 10 days postoperatively. The hydrogels incorporating mixed SEW2871-micelles and PRP promoted bone regeneration to a significant great extent compared with those incorporating PBS and either SEW2871-micelles or PRP. It is concluded that macrophages recruitment contributed to PRP-induced bone regeneration.

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1. Introduction

Bone tissue engineering has been noted as an interdisciplinary method to enhance therapeutic efficacy by making use of biomaterials. Cells and bioactive molecules are incorporated into biomaterial scaffolds, which can provide a suitable environment for cell-based bone regeneration. The process of bone healing involves complicated molecular signaling and significant changes in the expression of several genes from various types of cells, which can mediate bone formation and regeneration [1,2]. Among the cells, resident and infiltrated inflammatory cells, such as macrophages, play an immediate and pivotal role in the initiation, maintenance, and resolution of inflammation after bone fracture or injury [3–5]. Macrophages are generally recruited from the blood circulation, function for phagocytosis of apoptotic cells, and play a critical role in the production of growth factors, cytokines, and inflammatory mediators which participate in both modulation of inflammation and regeneration of tissues [6–8]. However, this inflammatory process may fail to resolve the foreign body reactions to implanted materials, resulting in tissue regeneration delay or suppression. Indeed, it has been demonstrated that the inflammation response is closely related to the timing to naturally induce tissue regeneration [9,10].

The modulation of inflammation by macrophages has been increasingly investigated [8,11–13]. It has been demonstrated that the decreased number of macrophages generally implicates their dysfunction of dead cell clearance, the phagocytosis of apoptotic cells, and the up-regulation of pro-inflammatory cytokines, including interleukin (IL)-6, and tumor necrosis factor (TNF)-α. This may induce inflammation and delayed tissue repair. For bone regeneration, macrophages are able to differentiate into multinucleate osteoclasts that induce bone resorption, while osteoblasts differentiated from mesenchymal stem cells stimulate bone formation. The ratio of osteoclasts to osteoblasts affects bone homeostasis. Therefore, the proper number of macrophages may be required not only to modulate inflammation, but also to enhance bone regeneration.

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SEW2871 (5-[4-Phenyl-5-(trifluoromethyl) thiophen-2-yl]-3-[3-(trifluoromethyl) phenyl] 1,2,4-oxadiazole), a sphingosine-1-phosphate $(S1P)_1$-selective agonist, can induce the recruitment of macrophages [14,15]. The S1P binds to a family of G protein-coupled receptors, called S1P receptors 1 to 5. They affect multiple biological functions including cells proliferation, cytoskeletal organization, differentiation, and migration [16]. Among the S1P receptor agonists, SEW2871 is not active for the $S1P_{2-5}$ receptors unlike FTY720 of a non-selective S1P receptor agonist. It has been shown that SEW2871 does not cause coronary artery muscle contraction nor does cause bradycardia, which is mediated via $S1P_2$ and $S1P_3$. Furthermore, it has been found that the receptor binding and signaling of SEW2871 and natural ligand S1P induce $S1P_1$ internalization and recycling. This is in contrast to FTY720, which induces receptor degradation [17]. Consequently, SEW2871 may have advantages over non-selective S1P receptor agonists. However, the water insolubility of SEW2871 may limit the therapeutic application.

Platelet-rich plasma (PRP) has been proposed to enhance tissue regeneration, such as the wound healing of soft and bone tissues [18,19]. It contains several autologous growth factors, such as transforming growth factor (TGF)-β_1, platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), and stromal cell derived factor-1 (SDF-1). Recent studies have demonstrated that bioactive PRP released from gelatin hydrogels enhances angiogenesis and osteogenesis [20,21].

This study is undertaken to investigate the effect of macrophages recruitment on growth factor-induced bone regeneration. It has been experimentally confirmed that PRP release from the gelatin hydrogels promoted bone regeneration [22,23]. No researches on the PRP-induced bone regeneration in terms of macrophages recruitment have been reported.

SEW2871 of a macrophage recruitment agent was water-solubilized through the micelle formation with L-lactic acid oligomer-grafted gelatin micelles. The micelles of SEW2871 water-solubilized and PRP were incorporated into the gelatin hydrogels. Their *in vitro* and *in vivo* release profiles from the gelatin hydrogels were evaluated. The *in vitro* macrophages migration and *in vivo* macrophages recruitment by the hydrogels incorporating SEW2871-micelles and/or PRP were investigated to compare with those of hydrogels incorporating either one. The bone gormation was evaluated by histoloical, radiological, and macro-computed tomographic examinations. We examined the expression levels of inflammatory cytokines and osteoprotegerin (OPG) of osteoclastogenesis inhibitory factor, genes after implantation of the gelatin hydrogels.

2. Materials and methods

2.1. Materials

A gelatin sample prepared by an acidic treatment of porcine skin collagen (isoelectric point (IEP) = 5.0) was kindly supplied by Nitta Gelatin Inc., Osaka, Japan. 5-[4-Phenyl-5-(trifluoromethyl)thiophen-2-yl]-3-[3-(trifluoromethyl)phenyl]1,2,4-oxadiazole (SEW2871) was purchased from Cayman Chemical, Ann Arbor, MI, USA. $Na^{125}I$ (NEZ-033H, >12.95 GBq/ml) and N′-succinimidyl-3-(4-hydroxy-3,5-di[^{125}I] iodophenyl) propionate or [^{125}I] Bolton–Hunter reagent (NEX-120H, 147 MBq/ml) were purchased from Perkine Elmer Life Sciences Inc., Boston, MA.

2.2. Synthesis of L-lactic acid oligomer-grafted gelatin

L-lactic acid oligomer with a number-average molecular weight of 1000 was synthesized from L-lactide monomer by ring-opening polymerization, as reported previously [24]. Briefly, L-lactic acid oligomer (3×10^{-5} mol) was dissolved in 15 ml of Dimethyl sulfoxide (DMSO), while disuccinimidyl carbonate (DSC, 9×10^{-5} mol) and dimethyl amino pyridine (DMAP, 9×10^{-5} mol) were dissolved in 2.5 ml of the DMSO solution. The solution was mixed to activate the hydroxyl groups of L-lactic acid oligomer for 3 h at room temperature. The solution of activated L-lactic acid oligomer was slowly added to the gelatin (IEP = 5) solution in DMSO (33 mg/ml), and the mixture was stirred overnight at room temperature to chemically graft the L-lactic acid oligomer to gelatin. To obtain the L-lactic acid oligomer-grafted gelatin, the resulting solution was dialyzed against double-distilled water (DDW) using a dialysis tube (molecular weight cut off = 12,000 ~ 14,000) at room temperature for 72 h, followed by freeze-drying The ratio of L-lactic acid oligomer grafted to the amino groups of gelatin determined by the fluorescamine assay was 3.1 ± 0.8 mol/mole gelatin, as reported previously [25].

2.3. Preparation of L-lactic acid oligomer-grafted gelatin and SEW2871-micelles

L-lactic acid oligomer-grafted gelatin solution (1 mg/ml) in DMSO and SEW2871 solution (1 mg/ml) in DMSO were prepared. The SEW2871 solution (15 ml) was added to the L-lactic acid oligomer-grafted gelatin solution (30 ml), followed by stirring at room temperature for 3 h. The reaction mixture was dialyzed using a dialysis tube (molecular weight cut off = 1000) for 72 h. The dialysate obtained was centrifuged at 8000 rpm, 4 °C for 10 min to separate water-insoluble SEW2871, and freeze-dried to obtain the SEW2871 water-solubilized by L-lactic acid oligomer-grafted to measure the amount of SEW2871 incorporated into the micelles. The SEW2871-micelles freeze-dried were dissolved in 100 vol% acetonitrile and subjected to high-performance liquid chromatography (HPLC) (LC-8020 model-II, Tosoh, Tokyo, Japan). The concentration of SEW2871 was determined from the calibration curve prepared with the 100 vol% acetonitrile containing various amounts of SEW2871.

2.4. Preparation of PRP

F344 rats (12 weeks old; Shimizu Laboratory Animal Supply Co., Ltd, Kyoto, Japan) were used. All the animal experiments were performed according to the Institutional Guidance of Kyoto University on Animal Experimentation and under permission by animal experiment committee of Institute for frontier Medical Science, Kyoto University. Briefly, rats were anesthetized by the intraperitoneal injection of pentobarbital (Somnopentyl, Kyoritsu Seiyaku Co., Tokyo, Japan) at a dose of 0.65 mg kg^{-1} body weight. PRP was prepared and activated with $CaCl_2$ according to the method reported previously [26]. Briefly, blood (10 ml) was collected from the heart of rats and transferred into tubes containing acid-citrate-dextrose solution formula A (1:4 v/v) anticoagulant. After centrifugation for 7 min at 1000 g and 4 °C, the yellow plasma with the buffy coat was carefully transferred into a BD Vacutainer tube (Becton Dickinson Co., NJ, USA), and then centrifuged for 5 min at 2100 g and 4 °C. The platelet pellet was collected and the thrombolytic pellet in 1.0 ml of plasma was used as PRP, while the supernatant provided platelet-poor plasma (PPP). To allow growth factors to release from PRP, the PRP prepared was mixed with 2 wt% $CaCl_2$ solution at a ratio of 7:1 by volume, and then left for 1 h at 37 °C according to the method reported previously [20].

2.5. Preparation of gelatin hydrogels incorporating mixed SEW2871-micelles and PRP

A gelatin solution (5 wt%, IEP = 5.0) solution was mixed with 0, 7.5 or 15 μg of SEW2871-micelles and cast into a Tissue-Tek® mold (Sakura Finetek Japan Co., Ltd., Tokyo, Japan), followed by freeze-drying. The hydrogels ($2 \times 2 \times 6$ mm^3) were crosslinked dehydrothermal (DHT) treatment at 140 °C for 48 h in a vacuum oven [27] and sterilized by ethylene oxide. Prior to the following experiments, PRP prepared (20 μl) was impregnated into a gelatin hydrogel incorporating SEW2871-micelles, followed by leaving at 4 °C overnight to prepared the hydrogels incorporating mixed SEW2871-micelles and PRP.

2.6. In vitro and in vivo release tests of SEW2871 from gelatin hydrogels incorporating mixed SEW2871-micelles and PRP

To evaluate *in vitro* release of SEW2871-micelles, gelatin hydrogels incorporating mixed SEW2871-micelles and PRP were incubated in 1 ml of 100 mM phosphate-buffered saline solution (PBS, pH 7.4) at 37 °C. At each time point, the PBS supernatant was collected and replaced by fresh PBS. After 24 h incubation, PBS was changed to PBS containing 10 μg ml^{-1} collagenase, and the supernatant was collected at different time intervals. The supernatant containing SEW2871 released was freeze-dried, and then the sample was re-dissolved in 100 vol% acetonitrile. The amount of SEW2871 was measured by the HPLC. The experiment was independently performed for 4 samples per experimental group at each sampling point.

To evaluate the *in vivo* release of SEW2871, gelatin hydrogels incorporating mixed SEW2871-micelles and PRP were implanted into the back subcutis of 6-week-old female ddY mice (Shimizu Laboratory Supply, Kyoto, Japan). At each time point, the hydrogel was collected and incubated with 1 ml collagenase solution (1 mg/ml) at 37 °C until to the complete digestion. The resulting solution was freeze-dried, and then the sample was re-dissolved in 100 vol% acetonitrile, followed by the similar determination of SEW2871 amount. The experiment was performed for 4 samples per experimental group at each sampling point.

2.7. In vitro and in vivo release tests of PRP from gelatin hydrogels incorporating mixed SEW2871-micelles and PRP

TGF-β_1 and SDF-1 were radioiodinated according to the conventional chloramine-T method as previously described [27]. Briefly, 5 μl of $Na^{125}I$ was added

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