TITLE:

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AUTHOR(S):

Kim, Yang-Hee; Furuya, Hiroyuki; Tabata, Yasuhiko

CITATION:

Kim, Yang-Hee ...[et al]. Enhancement of bone regeneration by dual release of a macrophage recruitment agent and platelet-rich plasma from gelatin hydrogels.. Biomaterials 2014, 35(1): 214-224

ISSUE DATE:

2014-01

URL:

<http://hdl.handle.net/2433/180132>

RIGHT:

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

Yang-hee Kim, Hiroyuki Furuya, Yasuhiko Tabata*

*Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences,
Kyoto University, 53 Kawara-cho Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*

Key words: Macrophages, Gelatin hydrogel, Controlled release, S1P₁ agonist (SEW2871),
Platelet rich plasma (PRP), Inflammation, Bone regeneration

* Corresponding author

Yasuhiko Tabata, Ph.D., D.Med.Sci., D. Pharm.

Tel.: +81-75- 751- 4128, Fax: +81-75-751-4646.

E-mail address: yasuhiko@frontier.kyoto-u.ac.jp (Y. Tabata).

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.

Keywords: Macrophages, Gelatin hydrogel, Controlled release, S1P₁ agonist (SEW2871),

Platelet rich plasma (PRP), Inflammation, Bone regeneration

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.

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 S1P₂₋₅ receptors unlike FTY720 of a nonselective S1P receptor agonist. It has been shown that SEW2871 does not cause coronary artery muscle contraction nor does cause bradycardia which is mediated via S1P₂ and S1P₃. Furthermore, it has been found that the receptor binding and signaling of SEW2871 and natural ligand S1P induce S1P₁ internalization and recycling. This is in contrast to FTY 720, which induces receptor degradation [17]. Consequently, SEW2871 may have advantages over non-selective S1P receptor agonists. However, the water insolubility of SEW 2871 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 formation was

evaluated by histological, 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. Na¹²⁵I (NEZ-033H, >12.95 GBq/ml) and N'-succinimidyl-3-(4-hydroxy-3,5-di[¹²⁵I]iodophenyl) propionate or [¹²⁵I] Bolton-Hunter reagent (NEX-120H, 147 MBq/ml) were purchased from Perkin 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 1,000 was synthesized from L-lactide monomer by ring-opening polymerization, as reported previously [24]. Briefly, L-lactic acid oligomer (3×10^{-5} mole) was dissolved in 15 ml of Dimethyl sulfoxide (DMSO), while disuccinimidyl carbonate (DSC, 9×10^{-5} mole) and dimethyl amino pyridine (DMAP, 9×10^{-5} mole) 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 hr 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 hr, 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 mole/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 hr. The reaction mixture was dialyzed using a dialysis tube (molecular weight cut off = 1000) for 72 hr. The dialysate obtained was centrifuged at 8,000 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 CaCl₂ 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 1,000g 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 2,100 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.% CaCl₂ solution at a ratio of 7:1 by volume, and then left for 1 hr 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 x 2 x 6 mm³) were crosslinked dehydrothermal (DHT) treatment at 140 °C for 48 hr 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 hr incubation, PBS was changed to PBS containing 10 $\mu\text{g ml}^{-1}$ 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 μ l of Na¹²⁵I was added into 20 μ l of TGF- β_1 and SDF-1 solution (R&D system, IC., Minneapolis) in 0.5 M potassium phosphate-buffered solution (pH 7.5) containing 0.5 M NaCl. Then, 0.2 mg/ml chloramine-T in the buffer (100 μ l) was added to the solution mixture. After vortex mixing at room temperature for 2 min, 100 μ l of PBS containing 0.4 mg sodium metabisulfate was added to the reacting solution to stop the radioiodination. The solution mixture was passed through a PD-10 desalting column (GE Healthcare Life Sciences, Chalfont St Giles, UK) to remove the uncoupled, free ¹²⁵I molecules from the ¹²⁵I-labeled TGF- β_1 and SDF-1 using PBS as an eluting solution. ¹²⁵I-labeled TGF- β_1 (2 μ l) and ¹²⁵I-labeled SDF-1 (2 μ l) were mixed with PRP (18 μ l), respectively. The mixed solutions of TGF- β_1 (20 μ l) and SDF-1 (20 μ l) were then sorbed into the hydrogels incorporating SEW2871- micelles freeze-dried (2 x 2 x 6 mm³), followed by leaving at 4 °C overnight to allow the solution to incorporate into the hydrogels.

For the *in vitro* release study, the hydrogels incorporating mixed SEW2871-micelles and TGF- β_1 or SDF-1 was incubated in 1 ml of PBS solution (pH 7.4) at 37 °C. At each time-point, the PBS supernatant was collected and replaced by the same volume of fresh PBS. The radioactivity of PBS supernatant was measured by a gamma counter (ARC-380 CL, Aloka Co., Ltd, Tokyo, Japan) to evaluate the time profiles of TGF- β_1 or SDF-1. The experiment was independently performed for 4 samples per experimental group at each sampling point. For the *in vivo* release study, the hydrogel was implanted into the back subcutis of 6-week-old female ddY mouse. At different time intervals, the hydrogels were taken out to measure the radioactivity remaining by the gamma counter. The

experiment was independently performed for 4 samples per experimental group at each sampling point.

2.8. In vivo degradation test of gelatin hydrogels incorporating mixed SEW2871-micelles and PRP

To evaluate the *in vivo* degradation profiles of gelatin hydrogels, the implantation of ^{125}I -labeled hydrogels was performed according to the method previously reported [24]. Briefly, 20 μl of [^{125}I]Boltone-Hunter reagent solution in benzene was evaporated at room temperature. The resultant solid reagent was dissolved in 1 ml of PBS and the resulting solution (20 μl) was sorbed into the freeze-dried gelatin hydrogel, followed by leaving at 4 °C overnight to introduce ^{125}I into the amino groups of gelatin. The radioiodinated hydrogels were washed with double distilled water (DDW) thoroughly for to exclude the uncoupled. Following the implantation into the back subcutis of mice, the hydrogels were taken out at different time intervals to count the radioactivity remaining by the gamma counter. The experiment was independently performed for 4 samples per experimental group at each sampling point.

2.9. In vitro macrophage migration and in vivo recruitment tests

Mouse J774.1 macrophage-like cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10 vol% fetal bovine serum (FBS), and 100 U/ml penicillin and streptomycin at 37 °C in a 5% CO_2 -95% air atmospheric condition. Detached the J774.1 cells (1×10^5 cells/ well) were suspended in the serum-free medium. Lower chambers in transwells (Corning Costar, Lowell, MA) were filled with serum-free medium including various doses of SEW2871 with or without PRP. Cells (1×10^5 cells/ well) were seed into the membrane of upper chamber in transwells and incubated 4 hr at 37 °C. After incubation, the membranes were washed with PBS, and non-migrated cells on the top of the membranes were removed using a cotton swab. The migrated cells were fixed with 4 wt% paraformaldehyde, followed by leaving it for 30 min at room temperature. After washing with PBS, cells were stained with 0.1 vol% crystal violet in 200 mM

2-(n-morpholino)ethanesulfonic acid (MES, pH 6.0) and keep it for 30 min. The membranes were washed with PBS 2 times and observed by a microscope (Carl Zeiss, Jena, Germany). The number of migrated macrophages was counted from the images taken at 20x magnification randomly selected (40 images/experimental group). The experiment was independently performed for 4 samples per experimental group.

For the *in vivo* macrophages recruitment study, gelatin hydrogels incorporating SEW2871-micelles with or without PRP were implanted into a critical-size bone defect of rats and taken out 3 days after implantation. Samples were fixed with 4 wt% paraformaldehyde in PBS at 4 °C for 24 hr, equilibrated in PBS containing 15 wt% sucrose at 4 °C for 24 hr, and then in PBS containing 30 wt% sucrose at 4 °C for further 24 hr. The samples were embedded in Tisse-tek OCT compound (Sakura Finetek Inc., Tokyo, Japan), and frozen in liquid nitrogen. The 6 µm- thickness sections were cut at the center of samples and washed with PBS, blocked with 5 wt% BSA solution for 30 min at room temperature before incubation with a mouse anti-rat CD68 (1:100, mouse igG1, AbD Serotec (ED1) for 1 hr at room temperature. Then, the sections were stained with streptavidin Alexa Flour 488 dye (Molecular Probes, Invitrogen Corporation, Ltd., CA) for 1 hr at room temperature and cell nuclei were finally stained with Hoechst 33258 (Nacalai Tesque Inc., Kyoto, Japan). After washing, the sections were mounted with Vectashield® (Vector Laboratories, Burlingame, CA). The images were taken on a fluorescent microscope (Apotome, Lmager.Z1, Carl Zeiss, Jena, Germany). The number of macrophages recruited to gelatin hydrogels was counted from the images taken at 40x magnification randomly selected (30 images/experimental group). The experiment was independently performed for 4 samples per experimental group.

For histological examination, the samples were fixed with 4 wt% paraformaldehyde in PBS at room temperature for 24 hr, decalcified with PBS containing 9 wt% ethylenediamine tetraacetic acid disodium salt and 10 wt% ethylenediamine tetraacetic acid tetrasodium salt (EDTA) solution at room temperature for 7 days. The EDTA solution was changed very other day. After decalcification, the samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek Inc., Tokyo, Japan), and frozen in liquid nitrogen. The tissue sections (6 µm-thick) were cut at the center of samples, followed

by staining with hematoxyline and eosin (H&E) and masson trichrome to observe the bone tissue newly formed. The images were taken under the microscope (AX80 Provis, Olympus Ltd., Tokyo, Japan) at 10x magnification.

2.10. In vivo bone defect experiment

A bone defect model of rat ulna was prepared to evaluate the bone regeneration after implantation of gelatin hydrogels incorporating SEW2871-micelles containing 15 μ g SEW2871 and/or 20 μ l of PRP. Male F344 (rat 12 weeks old) were used under standard sterile conditions according to the procedure previously reported [28]. Briefly, the rats were anesthetized with an intraperitoneal injection of pentobarbital sodium solution (40 mg/kg body weight). After shaving the hair and disinfection with 70 vol% ethanol, a longitudinal incision was made along the forearm skin of rats. The periosteum was incised circumferentially to approach to the ulna bone. A critical defect of 6 mm length was then created at the middle position of ulna bone by using a side-cutting diamond disk and a high-speed micromotor under an abundant irrigation with the sterile saline solution [29]. The hydrogel was implanted into the defect, while the periosteum and overlying muscle were repositioned with an absorbable polydioxanone suture (Ethicon 5-0, NJ). Then, the wound was closed with a non-absorbable polypropylene suture (Ethicon 5-0, NJ). The experiment was independently performed for 4 samples per experimental group at each sampling point.

2.11. Evaluation of gene expression at bone defects with reverse transcription (qRT-PCR)

The hydrogels incorporating SEW2871-micelles and/or PRP implanted at the defects were taken out 3 and 10 days after implantation. Then, the hydrogels were incubated in 1 ml collagenase solution (1 mg/ml) at 37 °C until to the complete digestion, and then cells were collected. The total RNA was extracted by using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA pellets were then dissolved in 20 μ l of RNase-free water, after which the RNA yields were estimated based on the ratio of A260/A280. cDNA was then synthesized using a

SuperScript VILO cDNA synthesis kit (Invitrogen Corporation, Ltd., CA) according to the manufacturers' instructions. Real-time polymerase chain reaction (PCR) was performed on a Prism 7500 real-time PCR thermal cycler (Applied Biosystems, Foster City, CA) from 10 ng of cDNA in a total volume of 25 μ l containing Power SYBR Green PCR Master Mix (Applied Biosystems) and 10 mM of each primer (Table 1) to analyze the mRNA expression level of TNF- α , IL-10, TGF- β_1 , and OPG genes. The reaction mixture was incubated for the initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles. Each cycle consisted of the following two steps; 95 °C for 15 s and 60 °C for 1 min. Each mRNA expression level was normalized by the expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The expression level of every group was reported as the value normalized to that of the SHAM group (the bone tissue collected from the rats which did not receive the operation and hydrogels implantation). The experiment was independently performed for 4 samples per experimental group.

2.12. Evaluation of bone tissue regeneration at the defects

The bone tissue regenerated at the defect was evaluated 6 weeks later by radiological, micro-computed tomography (μ CT), peripheral quantitative computed tomography (pQCT), and histological examinations. The radiological examination was performed under the softX-ray machine (Hitex-100, Hitachi Ltd., Tokyo, Japan) at 56 kV and 2.5 mA for 20 sec. Three-dimensional images of bone regenerated at the defects were visualized with the CT scans (X-RAY CT System, SMX-100CT-SV3 TYPE, Shimadzu Ltd., Kyoto, Japan). Samples were scanned over a fixed length of bone with a 20 mm sample holder at a resolution of 20 μ m, energy of 30 kV, current of 25 mA, and exposure time of 300 msec. The 2-dimensional images were reconstructed and submitted to the VGStudio MAX 1.2 software (Volume Graphics GmbH, Heidelberg, Germany) for processing to produce the 3-dimensional images of bone regenerated. The bone mineral density (BMD) of whole cortical compartment of bone regenerated was analyzed by using a highly accurate multi-slice pQCT (XCT Research SA β , Stratec Medizintechnik, GmbH, Pforzheim, Germany). The histological examination,

the samples were fixed with 4 wt% paraformaldehyde in PBS at room temperature for 24 h, decalcified and neutralized as described previously. Then, the samples were embedded in Tissue-Tek OCT Compound (Sakura Finetek Inc., Tokyo, Japan), and frozen in liquid nitrogen. The tissue sections (6 μ m-thick) were cut at the center of samples, followed by staining with hematoxyline and eosin (H&E) and masson trichrome to observe the bone tissue newly formed. The images were taken under a microscope (AX80 Provis, Olympus Ltd., Tokyo, Japan) at 10x magnification.

2.13. Statistical analysis

All the statistical data were expressed as the mean $\pm$ standard error of the mean. The data were analyzed by ANOVA to determine the statistical significance between the two mean values. P values less than 0.05 were considered to be significant.

3. Results

3.1. *In vitro* and *in vivo* release profiles of SEW2871 and PRP growth factors from gelatin hydrogels incorporating mixed SEW2871-micelles and PRP and degradation of hydrogels

Figure 1 shows the *in vitro* release profiles of SEW2871, TGF- β_1 , and SDF-1 from gelatin hydrogels. Among various PRP growth factors, TGF- β_1 and SDF-1 of representative factors related to bone regeneration and cells recruitment were chosen to evaluate the PRP release profiles. In PBS, 20 and 30 % of SEW2871 and PRP growth factors were released from gelatin hydrogels for 12 hr, but thereafter no release. After the initial PBS change to collagenase PBS, their release with time was observed. The similar time profile was observed for hydrogels degradation. The time profiles of SEW2871 and PRP release was in a good accordance to those of hydrogels degradation.

Figure 2 shows the *in vivo* profiles from gelatin hydrogels incorporating mixed SEW-micelles and PRP. Within 7 days, about 97 % of SEW2871 was released while the amount of TGF- β_1 and SDF-1 released was about 90 %. SEW2871 was not detected 14 days later, while the radioactivities of TGF- β_1 and SDF-1 still remained. The release profiles of SEW2871 and PRP growth factors were similar to those of gelatin degradation.

3.2. *In vitro* macrophages migration assay

Figure 3 shows the results of *in vitro* macrophages migration by SEW2871 and/or PRP. The number of macrophages migrated in the medium containing 15 μ g of SEW2871 was higher than that of 7.5 μ g and 15 μ l of PRP. Comparing to the control group (without SEW2871 and PRP), the number of migrated macrophages in the medium containing SEW2871 or/and PRP was significantly increased. The number of macrophages migrated in mixed SEW2871 and PRP also increased. Especially, in 15 μ g of SEW2871 and 15 μ l of PRP, the number of macrophages migrated was significantly higher than that of PRP only.

3.3. In vivo macrophage recruitment into gelatin hydrogels incorporating mixed SEW2871-micelles and PRP

Figure 4 shows the *in vivo* recruitment of macrophages into gelatin hydrogels 3 days after implantation. A high number of macrophages recruited and a large amount of collagen formed were around gelatin hydrogels incorporating mixed SEW2871-micelles and PRP implanted. Macrophages were observed around gelatin hydrogels incorporating SEW2871 and/or PRP (Figure 4 C). The number of macrophages recruited to the gelatin hydrogel incorporating 7.5 μ g of SEW2871 or PRP did not significantly increase comparing with that gelatin hydrogel incorporating PBS. The number of macrophages recruited around gelatin hydrogels incorporating mixed SEW2871-micelles containing 15 μ g SEW2871, and 15 μ l of PRP increased to a significantly great extent compared with those incorporating PBS or lower amount (7.5 μ g) of SEW2871.

3.4. Gene expression of tissue around gelatin hydrogels incorporating SEW2871-micelles and/or PRP

Figure 5 shows the gene expression levels of inflammatory cytokines and OPG of cells in the gelatin hydrogels 3 and 10 days after implantation. The expression levels of TNF- α , IL-10, and TGF- β_1 increased for gelatin hydrogels incorporating SEW2871-micelles and/or PRP 3 days later comparing with those of SHAM group. The expression levels of TNF- α and TGF- β_1 were significantly increased by gelatin hydrogels incorporating mixed SEW2871-micelles and PRP. However, significant difference in the levels of IL-10 and OPG expression were not observed. On the other hand, at 10 days, the expression level of TNF- α was decreased, whereas that of IL-10 was increased as compared with the expression levels for gelatin hydrogels alone 3 days after implantation. The mRNA expression levels of TGF- β_1 and OPG of cells in gelatin hydrogels incorporating mixed SEW2871 micelles and PRP significantly increased compared with those of other groups.

3.5. Bone regeneration by gelatin hydrogels incorporating mixed SEW2871-micelles and PRP in bone defect

Figure 6 shows the soft X-ray and micro-CT images of bone tissues regenerated. The bone formation in the defects implanted with gelatin hydrogels incorporating PBS or SEW2871-micelles was scarcely observed. On the contrary, gelatin hydrogels incorporating mixed SEW2871-micelles and PRP induced bone regeneration. Comparing with the hydrogel incorporating PRP, bone formation was observed in the defect implant with hydrogels incorporating mixed SEW2871-micelles and PRP. The size of bone regenerated in the defect with hydrogels incorporating mixed SEW2871-micelles and PRP was larger than that of gelatin hydrogels incorporating either SEW2871-micelles or PRP.

Figure 7 shows the histological images of bone regenerated in the bone defects of rats after implantation of gelatin hydrogel incorporating mixed SEW2871-micelles and PRP. New bone tissues were observed at the bone defect implanted with gelatin hydrogels incorporating mixed SEW2871-micelles and PRP. Although the collagen tissue formation was not seen at the defect implanted with PBS incorporated into gelatin hydrogels, collagen tissues were regenerated from the edge of bone defect implanted with gelatin hydrogels incorporating SEW2871-micelles or PRP. The bone defect implanted with gelatin hydrogels incorporating mixed SEW2871-micelles and PRP was completely coccupied with collagen tissues.

Figure 8 shows the quantitative results of the area percentage of matured collagen to collagen newly formed and bone mineral density. The percentage of matured collagen was significantly higher for the gelatin hydrogels incorporating mixed SEW2871-micelles and PRP than that of gelatin hydrogels (Figure 8 A). The defect implanted with gelatin hydrogels incorporating mixed SEW2871-micelles and PRP was regenerated at significantly higher bone mineral density, comparing with that of the hydrogels incorporating SEW2871-micelles or PRP.

4. Discussion

The process of bone healing can be classified into four histologically distinguishable stages: inflammation, fibrous, callus, and bone formation. Inflammation is the initial and the most vital stage from the viewpoint of bone regeneration [4, 30-32]. Macrophages are typical immune cells which infiltrate into defected sites and have a key role in the modulation of the inflammatory response [5, 33, 34]. The hypothesis of this study is that a dual release of SEW2871 and PRP from gelatin hydrogels stimulates macrophage recruitment and modulates inflammation, resulting in enhanced bone regeneration. SEW2871 has an inherent ability to enhance the recruitment of macrophages [15, 17]. PRP has a potential to promote tissue regeneration because it contains various growth factors [18, 20]. The present study experimentally demonstrates that the enhancement of macrophages recruitment promoted the bone regeneration.

Our previous researches have demonstrated that the gelatin hydrogels could controlled release various growth factors of TGF- β_1 , SDF-1, bFGF, and BMP-2, [24, 35-37] or low molecular weight and water-insoluble drugs in a controlled fashion [38-42]. In this study, water-insoluble SEW2871 was solubilized into water by the incorporation into LAO-grafted gelatin micelles. The micelles of SEW2871 water-solubilized were homogeneously incorporated into the gelatin hydrogels. PRP was also incorporated into the gelatin hydrogels. Most of growth factors in PRP are positively charged at the physiological pH. The hydrogels of negatively charged gelatin with an IEP of 5.0 can electrostatically interact with the growth factors of positive charge. The doses of SEW2871-micelles and PRP were selected according to the appropriate dose demonstrated by other researches [15, 17, 20]. The time profile of SEW2871 and PRP release from gelatin hydrogels was similar to that of degradation of gelatin hydrogels (Figure 1). This indicates that the gelatin hydrogel is a proper carrier for the control release of PRP growth factors and SEW2871-micelles. Among various types of the growth factors, PRP, TGF- β_1 and SDF-1 were representatively selected to evaluate the release, because TGF- β_1 is the highest concentration in PRP, and SDF-1 is one of important factors for cells recruitment. Compared with the data of our previous researches, the release rate of growth factors from the gelatin hydrogels was somewhat fast and degradation of gelatin was also rapid (Figure 2). It

may be explained that macrophages accelerated the degradation of biomaterials [43, 44]. Mixed SEW2871-micelles and PRP incorporated in gelatin hydrogels showed the synergistic capacity of migration and recruitment of macrophages compared with SEW2871-micelles or PRP alone (Figures 3 and 4). The SEW2871-micelles and PRP alone also enhanced the macrophages movement. However, the mechanism of macrophages recruitment enhancement by PRP is not clear yet at present. The macrophages recruitment may be explained by the paracrine factors secreted from mesenchymal stem cells. Chen et al. [45] have demonstrated that bone marrow-derived mesenchymal stem cells secrete high levels of several chemokines such as macrophage inflammatory proteins (MIP)-1, 2 and macrophage chemoattractant protein (MCP)-5. MIP and MCP are major chemoattractants for macrophages and play a key role in macrophage recruitment. When implanted into the back subcutis of mice, MSC incorporated glass cylinders induced high recruitment of macrophages compared with the original cylinder [46]. Thus, it is possible that mesenchymal stem cells are associated with macrophage recruitment by effective factors released. It is demonstrated that mesenchymal stem cells affect the macrophage recruitment. The bone marrow-derived mesenchymal stem cells, together with inflammatory cells can be recruited by SDF-1 chemoattractant [47, 48]. Taken together, several chemokines present in PRP and the paracrine factors secreted from mesenchymal stem cells recruited by SDF-1 of PRP would modify the macrophages recruitment. In the study, mixed SEW2871-micelles and PRP are released. It is highly conceivable that the controlled release enhanced the action of SEW2871 on macrophages and PRP on mesenchymal stem cells for factors secretion, resulting in the synergistic enhancement of macrophages recruitment.

The recruitment of macrophages has been studied by many researchers in terms of tissue regeneration. The deficiency of macrophages at tissue defects delayed the process of the tissue regeneration because macrophages play an important role in the phagocytosis or removal of pathogen and initiation of relative gene expression for tissues repairing [11, 49]. In terms of bone regeneration, few papers have been published on the macrophage recruitment. Xing et al. [50] have demonstrated

that macrophages recruited by C-C chemokine receptor (CCR) 2 modulated the function of osteoclasts and affected the subsequent bone fracture healing.

Inflammatory responses are associated with pro-inflammatory and anti-inflammatory cytokines. Pro-inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, are known as yhr mediators of foreign body reactions and cause further tissue damage and cell death. On the other hand, anti-inflammatory cytokines, IL-4, IL-10, and TGF- β_1 assist tissue repairing. Thus, the production of pro-inflammatory and anti-inflammatory cytokines is a key to modify inflammation response and to predict the regeneration ability. The gene expression level of TNF- α at the tissue around the implantation site of gelatin hydrogels incorporating mixed SEW2871-micelles and PRP 3 days after implantation was significantly higher than that of SHAM (no inflamed bone) and PBS groups. TNF- α is mainly expressed by macrophages and other inflammatory cells [32]. It is demonstrated that the high number of macrophages recruited may contribute to the high level of TNF- α expression. The levels of anti-inflammatory cytokines, such as TGF- β_1 and IL-10, were similar between hydrogels incorporating mixed SEW2871-micelles and PRP group and only PBS group 3 days after implantation. However, the levels of pro-inflammatory cytokine were decreased, whereas that of anti-inflammatory cytokines was increased in gelatin hydrogels 10 days after implantation. The levels of anti-inflammatory cytokine, TGF- β_1 compared with hydrogels incorporating SEW2871-micelles or PRP. In addition, the expression level of osteoprotegerin, osteoclastogenesis inhibitory factor, increased. On the other hand, collagen newly formed was observed around the implantation site of hydrogels incorporating mixed SEW2871-micelles and PRP (Figure 4 B). This indicates that the modulation of inflammation responses by macrophage recruitment and PRP release can induce granulation tissue formation and accelerate the bone formation.

The production of pro- and anti- inflammatory cytokines are associated with tissue regeneration [10, 33]. Pro-inflammatory cytokines secreted that macrophages can play a key role for foreign body reactions, such as phagocytosis of dead cells and pathogen killing [51]. On the other hand, ant-inflammatory cytokines, such as IL-4, IL-10, and TGF- β_1 , affect tissue regeneration of myfibrobalst activation, collagen formation, and vessel formation [4, 52]. If there is case where the

pro-inflammation period is shortened and subsequently the anti-inflammation environment can be provided, it is possible that regeneration step will be accelerated [7, 53]. Ratanavaraporn et al. [42] have demonstrated that the controlled release of mixed anti-inflammatory drug and BMP-2 from gelatin hydrogels enhanced bone regeneration to a significantly great extent compared with that of BMP-2. On the other hand, inappropriate concentration of pro-inflammation and anti-inflammation drugs induce delayed the bone regeneration [7, 54]. Thus, the timely production of anti-inflammatory cytokines at a suitable concentration is important to enhance bone regeneration.

Conceptually, macrophages contribute to the process of bone resorption because of their osteoclast differentiation, but their roles depend on the biological environments [55-57]. The depletion of macrophages in osteoarthritic models reduced osteophytes formations, suggesting that macrophages play a critical role in bone deposition [58, 59]. Additionally, macrophages are associated with pathologic bone loss and osteoporosis [60, 61]. Taken together, it is well recognized that macrophages modify both bone disease and repair.

The present study showed that hydrogels incorporating SEW2871-micelles alone did not enhance bone formation at the bone defect site. On the other hand, hydrogels incorporating mixed SEW2871-micelles and PRP showed excellent bone regeneration (Figures 6 and 7). This suggests that macrophages recruited and PRP growth factors affected bone regeneration. Dapeng et al. [62] and Lacombe et al. [41] demonstrated that TGF- β_1 activates macrophages and subsequently produces anti-inflammatory cytokines from them. Likewise, various chemokines and TGF- β_1 are associated with the modulation of inflammation and tissue repair [63]. Another possibility is that the mutual communication of mesenchymal stem cells and macrophages recruited by SDF-1 of PRP and SEW2871. The soluble factors generated by the interaction of mesenchymal stem cells and macrophages resulted in the immunosuppression and enhancement of tissue regeneration [64, 65]. Thus, we can say with certainty that PRP not only stimulates the recruitment of macrophages, but also triggers their activation and the production of anti-inflammatory cytokines which are associated with tissue regeneration. In addition, the dual release of SEW2871 and PRP in a controlled fashion

from gelatin hydrogels modifies the macrophages recruitment and the modulation of inflammation,
resulting in enhanced bone regeneration.

5. Conclusions

The present study experimentally demonstrated that the dual release of a macrophage recruitment agent, SEW2871 and PRP in a controlled fashion from the gelatin hydrogels enhanced the bone regeneration at a bone defect. The higher number of macrophages recruited was observed around gelatin hydrogels incorporating mixed SEW2871-micelles and PRP compared with those incorporating either SEW2871-micelles or PRP. In addition, the hydrogels decreased the production of pro-inflammatory cytokine, but increased that of anti-inflammatory cytokines. The controlled release of SEW2871 and PRP from the gelatin hydrogels recruited macrophages into the bone defect and modulated inflammatory responses thereat, resulting in promoted bone regeneration.

Acknowledgement

We gratefully thank Dr. M. Matsui for their technical assistance. The research was supported by a grant from Japan Society for the Promotion of Science (JSPS).

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Figure captions

Figure 1. (A) In vitro release profiles of SEW2871 (open marks) from gelatin hydrogels incorporating mixed SEW2871-micelles and PRP and hydrogels degradation (solid marks) in PBS with or without collagenase at 37 °C. (B) In vitro release profiles of TGF-β1 (open marks) and SDF-1 (Solid marks) from gelatin hydrogels incorporating mixed ¹²⁵I-labeled TGF- β1 and SDF-1 in PBS with or without collagenase at 37 °C. The PBS solution was changed to that with collagenase at the time indicated with an arrow.

Figure 2. (A) In vivo release profiles of SEW2871 (open marks) from gelatin hydrogels incorporating mixed SEW2871-micelles and PRP and hydrogel degradation (solid marks) after implantation into the back subcutis of mice. (B) In vivo release profiles of TGF-β1 (open marks) and SDF-1 (Solid marks) from gelatin hydrogels incorporating mixed ¹²⁵I-labeled TGF- β1 and SDF-1 after implantation into the back subcutis of mice.

Figure 3. In vitro macrophages migration by mixed SEW2871 and/or PRP 4 hr after incubation. *p < 0.05; significant against the value of control group without SEW2871 nor PRP, †p < 0.05; significant against the value of PRP group alone.

Figure 4. In vivo macrophages recruitment by gelatin hydrogels incorporating mixed SEW2871-micelles and/or PRP into critical-size bone defects 3 days after implantation. Then, cells were fixed with 4% paraformaldehyde and stained with (A) H&E (B) Masson's trichrome, and (C) CD68, a macrophage surface marker (scale bar = 100 μm, C: collagen). The number of recruited macrophage was counted (D). *p < 0.05; significant against the value of gelatin hydrogels incorporating PBS. †p < 0.05; significant against the value of gelatin hydrogels incorporating PRP and 7.5 μg of SEW2871 group.

Figure 5. Gene expression of TNF- α , IL-10, TGF- β_1 , and OPG of cells in gelatin hydrogels incorporating mixed SEW micelles and/or PRP (A) 3 days and (B) 10 days after implantation into the bone defects. * $p < 0.05$; significant against the value of SHAM group, † $p < 0.05$; significant against the value of PBS group, ‡ $p < 0.05$; significant between the two experimental groups.

Figure 6. (A) Soft X-ray images of bone regenerated at the defects 6 weeks after implantation of gelatin hydrogels incorporating SEW micelles and/or PRP. (B) Three-dimensional μ CT images of bone regeneration at the defects 6 weeks after implantation of the same hydrogels. The bone parts regenerated were indicated with arrows.

Figure 7. Histological images of bone regenerated in the ulna critical-sized defects of F344 rats implanted with the gelatin hydrogels. The sections were cut at the center of samples and stained with (A) hematoxylin and eosin (H&E) and (B) Masson's trichrome. (C: collagen, M: muscle, Scale bar = 1 mm).

Figure 8. (A) Percentage of matured collagen to collagen newly formed. (B) Bone mineral density (BMD) of bone regenerated 6 weeks after implantation, analyzed by pQCT. * $p < 0.05$; significant against the value of gelatin hydrogels incorporating PBS group, † $p < 0.05$; significant against the value of gelatin hydrogels incorporating SEW2871 group, ‡ $p < 0.05$; significant between the two experimental groups.

Table 1 Primers used in quantitative real-time PCR analysis;

mRNA	Forward	Reverse
TNF- α	5'-TGAAC TTCGGGGTGATCG-3'	5'-GGGCTTG TCACTCGAGTTTT-3'
IL-10	5'-TGAAC TTCGGGGTGATCG-3'	5'-TCATGGCCTTG TAGACACCTT-3'
TGF- β 1	5'TGAAC TTCGGGGTGATCG-3'	5'GGGCTTG TCACTCGAGTTTT-3'
OPG	5'-TGAGGTTTCCAGAGGACCAC-3'	5'-GGAAAGGTTTCCTGGGTTGT-3'
GAPDH	5'-TGTTGAAGTCACAGGAGACAAACCT-3'	5'-AACCTGCCAAGTATGATGACATCA-3'

IL, interleukin; TNF- α , tumor necrosis factor- α ; OPG, osteoprotegerin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 1.

(A)

(B)

Figure 2.

(A)

(B)

Figure 3.

Figure 4.

Figure 5

(A)

(B)

Figure 6

(A)

(B)

Figure 7.

Figure 8

(A)

(B)