Original Article

Controlled release of pioglitazone from biodegradable hydrogels to modify macrophages phenotype

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Macrophages play a key role in the inflammatory reaction of body defense. It has been demonstrated that there are various types of macrophages and their function greatly affects the progressive conditions of inflammation process. The objective of this study is to develop a drug delivery system (DDS) which can modify the biological function of macrophages. Pioglitazone (Pio) of water-insolubility was water-solubilized by the micelle formation with gelatin grafted with L-lactic acid oligomer (Pio-micelles). The Pio water-solubilized was incorporated into a biodegradable gelatin hydrogel. The experiments of Pio release and hydrogel degradation revealed that pio was released from the hydrogel incorporating Pio-micelles *in vitro* and *in vivo*. The Pio-micelles and Pio allowed macrophages to enhance the mRNA expression level of arginase1 which is a measurement of type 2 macrophages. When applied to a skin defect model of mice, the hydrogels incorporating Pio-micelles enhanced the arginase1 level around the site implanted to a significant great extent compared with the Pio solution. The Pio release technique is promising to modify the phenotype of macrophages.

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Introduction

Macrophages have remarkable plasticity in physiology and change their phenotype in response to the environmental cues, resulting in the generation of various populations with different biological functions. It has been recognized that there are, at least, two phenotypes of macrophages, called as M1 and M2. M1 macrophages with classically proinflammatory functions are typical in inflammatory reactions and pathogen defense[1-3]. Generally, M1 macrophages produce interlukin (IL)-12, IL-23, inducible nitric oxide synthase (iNOS), toxic reactive oxygen, nitric oxygen intermediates, and inflammatory cytokines, such as IL-1β, IL-6, and tumor necrosis factor (TNF)-α. On the other hands, M2 macrophages with alternatively non-inflammatory functions promote the responses of type 2 helper T cells associated with tumor progression[2], parasite infections, tissue repair[3, 4], and debris removal[4-7]. The M2 macrophages produce IL-10, high levels of scavenger, mannose, and galactose receptor, and arginase in the place of arginine[4], subsequently producing ornithine and polyamines.

The concepts and paradigms of macrophages polarization into M1 and M2 phenotypes have been noted in terms of inflammatory host responses to pathogens and cancer[3, 4, 8]. The macrophages phenotype also modify the host response in disease pathogenesis, tissue injury, and the implantation of biomaterials[9]. The findings give us the idea that the inflammatory host responses can be modified by changing the macrophages phenotype.

Peroxisome proliferator-activated receptor γ (PPARγ) is one of the key factors to modify the M1/M2 phenotype ratio[10-12]. It is demonstrated that the activation of PPARγ potentiates the polarization of circulating monocytes to macrophages of M2 type, and reduces the functions of M1 macrophages and attenuates macrophages-induced inflammatory reactions[13]. Among the activators of PPARγ pioglitazone of a thiazolidinedione (TZDs) is commercially available, and well-known as an agonist for PPARγ and modulator of inflammatory responses[14].

Gelatin is a biodegradable material and has been extensively used for food, pharmaceutical, and medical purposes. The biosafety has been proven through their long practical applications. Biodegradable hydrogels prepared from gelatin were effective as the release carrier of water-soluble[15-18] and water-insoluble drugs[19] and augmented their therapeutic activities.

The objective of this study is to investigate the possibility whether or not Pio can modify the phenotype of macrophages. The gelatin hydrogel was prepared for the controlled release of Pio to enhance the *in vivo* biological activity. The Pio was solubilized in water by the micelle formation with a derivative of gelatin grafted with L-lactic acid oligomer (Pio-micelles). Next, the Pio-micelles of Pio water-solubilization were incorporated into the biodegradable gelatin hydrogel. The ability of hydrogels incorporating Pio-micelles to modify the macrophage phenotype was evaluated in terms of arginase 1 and iNOS gene levels. We examine the controlled release of Pio from the hydrogel incorporating Pio-micelles.

Materials and Methods

1)Materials

Gelatin with an isoelectric point (pI) of 5.0 (weight-average molecular weight (MW) = 5,000, 10,000, 20,000, 60,000, and 100,000), prepared via an alkaline process of bovine bone (pI5 gelatin) or with a pI of 9.0 (Mw = 100,000), prepared via an acid process of pig skin (pI9 gelatin) and collagenase L, were kindly supplied from Nitta Gelatin Co., Osaka, Japan. Collagenase D, purchased from Roche diagnosis Inc., was used in the *in vivo* study because of its lower cytotoxicity compare to collagenase L. Disuccimidyl carbonate (DSC) and 4-dimethylaminopyridine (DMAP) were purchased from Nacalai Tesque Inc., Kyoto Japan. 1-dodecanol (DoOH), and other chemicals were purchased from Wako Pure Chemical industries, Ltd., Osaka, Japan. Pioglitazone hydrochloride was purchased from LKT laboratory Inc., and macrophage colony stimulating factor (M-CSF) was purchased from Tebu-Bio/Peprotech, Offenbach, Germany.

2)Synthesis of L-lactic acid oligomer

L-lactic acid oligomers (LAo) with weight-average molecular weights of 1000, 2000, and 3000 were synthesized as reported previously[19]. Briefly, LAo with the number-average molecular weight of 1000 was synthesized from L-lactide monomer by ring-opening polymerization with the stannous octate of catalyst and 1-dodecanol (DoOH) as an initiator. L-lactide (20 g, 138.8 mmole) was melted at 130 °C in a nitrogen atmosphere, followed by addition of toluene (5.6 ml) containing DoOH (1.38 mmole) and stannous octate (0.56 g, 1.38 mmole). After mixing for 4 hr at 130 °C, the reaction product was poured into ethanol (EtOH) for precipitation. The precipitate was dissolved in chloroform. The EtOH was evaporated to obtain the white solid of lactic

acid oligomer. The weight-averageal molecular weight of LAo prepared was determined by ^{1}H NMR spectroscopy (JNM-EX, JEOL, Ltd., Tokyo, Japan) to be 1,000.

3)Synthesis of gelatin grafted with LAo

Gelatin grafted with LAo was synthesized as reported previously[19]. Briefly, 1.0 g of PI5 gelatin with Mw of 5,000, 10,000, 20,000, 60,000 and 100,000 was dissolved in anhydrous dimethyl sulfoxide (DMSO) at room temperature. DSC and DMAP were added to the LAo solution in DMSO at the molar ratio to LAo of 3, followed by stirring at room temperature for 3 hr to activate the hydroxyl groups of LAo. Next, the mixed solution was slowly added to the gelatin solution in DMSO to give the LAo/gelatin molar ratios of 1, 3, 5, and 7, and then stirred overnight at room temperature to allow LAo to graft to gelatin. The resulting solution was dialyzed with dialysis tube against double-distilled water (DDW) for 72 hr at room temperature, and freeze-dried to obtain LAo-grafted gelatin samples (LAo-g-gelatin).

4)Water-solubilization of Pio by LAo-g-gelatin

Pio solution (1.0 mg/ml) in DMSO (15 ml) was added to 30 ml of LAo-grafted gelatin DMSO solution (1.0 mg/ml), followed by 3 hr stirring at room temperature. Then, the mixed solution was dialyzed with Spectra/Por® Dialysis Membrane MWCO: 3,500 (Spectrum Laboratories, Inc, CA, US) against DDW for 72 hr at room temperature. The dialysate obtained was centrifuged (8,000 rpm, 10 min, 25 °C) to exclude the water-insoluble fraction, and freeze-dried to obtain the Pio water-solubilized by LAo-grafted gelatin micelles (Pio-micelles). The amount of Pio was measured on high performance liquid chromatography (HPLC, HPLC prominence LC-20AT, Shimadzu, Japan). The Pio-micelles were dissolved by ethanol, followed by the HPLC measurement to assess the Pio amount by referring the calibration curve of Pio amount and HPLC peak area.

5)Activity evaluation of Pio-micelles

To evaluate the biological activity of Pio-micelles, the conventional bioassay of *in vitro* cell culture was carried out[10, 12, 13]. Briefly, bone marrow-derived macrophages (BMM) were isolated from the tibia and femur of mice, and seeded into each well of 12-multi-well culture plate (Corning Incorporated, NY, U.S.) at a density of 1×10^{6} cells/ml with Iscove's Modified Dulbecco's Medium (IMDM) containing 20 vol % fetal bovine serum (FBS), 1 wt % penicillin-streptomycin (PS), and 50 ng/ml M-CSF. The medium was changed to the IMDM containing 5 vol % FBS and 1 wt % PS 6 days later. The water-soluble Pio-micelles containing 100 nM of Pio were added into the medium, and the cells were incubated further for 24 hr. As a control, Pio ethanol solution (100 nM, 100 µl) was used. Then, the cells were washed by 10 mM phosphate-buffered saline solution (PBS, pH 7.4) two times, followed by lysing in RLT buffer (Qiagen Inc., Osaka, Japan), and then the cell RNA was isolated using an RNeasy Mini Kit (Qiagen Inc., Osaka, Japan) according to the instructions of manufacturer. Total RNA was reverse transcribed using superscript VILO cDNA synthesis kit (InvitrogenTM , CA.).

6)Preparation of gelatin hydrogels incorporating Pio-micelles

The gelatin hydrogel incorporating Pio-micelles were prepared through the dehydrothermal crosslinking reaction of gelatin[20]. Briefly, Pio-micelles (10 mg/ml) were added to 10 wt% of PI9 gelatin aqueous solution. Then, the solution was mixed, cast into a mold (2 cm x 2 cm) and freeze-dried. The samples freeze-dried were dehydrothermally crosslinked at 160 °C for 24 hr to obtain hydrogels incorporating Piomicelles (Pio-hydrogels).

7)*In vitro* evaluation of Pio release from Pio-hydrogels and Pio-hydrogels degradation

The Pio-hydrogel was placed in 1 ml of PBS with or without collagenase. The release test for the initial 24 hr was carried out in PBS at 37 °C, and thereafter in PBS containing 100 µg /ml collagenase L. At different time intervals, the PBS or that containing collagenase was exchanged. The supernatant collected was freeze-dried, followed by ethanol addition for Pio dissolution. After the sonication during 2 min on the ice (UR-21P Tomy Seiko Co., Ltd., Tokyo, Japan) and centrifugation (12,000 rpm, 10 min), the Pio amount in the supernatant was determined on HPLC as described above. For the *in vitro* degradation of Pio-hydrogels, the Pio-hydrogels were placed in 1 ml of PBS with or without collagenase L. The degradation test was performed in the condition similar to that of release test. The supernatant collected was diluted by PBS at appropriate ratios, and the amount of gelatin protein in the diluted solution was determined by Micro BCA protein Assay kit (Thermo scientific, USA) to evaluate the amount of gelatin water-solubilized during the degradation test. The tests were performed independently for 3 samples.

8)*In vivo* evaluation of Pio release from Pio-hydrogels

Following animal experiment was approved and performed according to guidelines of Institute for Frontier Medical Sciences, Kyoto University. Mice were anesthetized by an intraperitoneal injection of ketamine (75 mg/kg). A small slit was made on the midline of dorsal skin, and the Pio-hydrogel was subcutaneously implanted followed by surgically suturing. The mice were sacrificed 12 hr or 1, 3, and 7 days after implantation, and the remaining hydrogel was collected from the back of mice (4 mice / each time point). The hydrogel was degraded completely in 1.0 mg/ml collagenase L solution, and then the solution was freeze-dried. Pio present in the freeze-dried sample was dissolved in ethanol. Similarly to the *in vitro* tests, after sonication and centrifugation, the amount of Pio was determined on HPLC.

9)*In vivo* evaluation of Pio bioactivity

Pio-hydrogels or Pio-free hydrogels were implanted as described above. Mice were sacrificed 1, 3, and 7 days later, and the implanted hydrogels were collected from the mice (4 mice / each time point / each group). The hydrogel collected was minced thoroughly with a scissor in 500 µl PBS, followed by the addition of collagenase D (2 mg/ml) to the solution. After 1 hr incubation at 37 °C, the resulting solution was filtered by a cell strainer of 40 µm opening (REF352340 BD Falcon, USA) to obtain cells dissociated. The cells were washed twice with PBS containing 0.1 vol % bovine serum albumin (BSA), and 2 mM ethylenediaminetet raacetic acid (EDTA) to prepare the single cell suspension.

10)Animal experiment with skin defect model

C57BL/6CrSlc male mice (8 weeks old) were purchased from Shimizu Laboratory Supplies Co., Kyoto, Japan. All animal experimentation was conducted in accordance with the guidance of the Institute for Frontier Medical Sciences, Kyoto University.

To prepare a mouse model of skin defect, mice were anesthetized by the intraperitoneal injection of ketamine (75 mg/kg). The dorsal surface was shaved with an electric clipper, followed by the treatment of a depilatory agent to remove hair completely. After the skin washing with DDW, an 8-mm punch biopsy tool was used to prepare two skin wounds on each side of midline, followed by the scissor excision to complete full-thickness wounds. The skin wounds were treated with saline (Cont), saline of solution Pio-micelles containing 20 µg of Pio, Pio-free hydrogels, and Pio-hydrogels containing 20 µg of Pio (4 mice / each group). After that, the wound was covered with an occlusive dressing (Tegaderm, Sumitomo 3M, Ltd., Tokyo, Japan), and then the trunk of mouse body was fixed with an elastic adhesive bandage (Silkytex, ALCARE, Co, Ltd, Tokyo, Japan). The photographs of wounds were taken at different time intervals to view the area of the wound defect and the extent of epithelization. The area was evaluated as the percentage of wound area to the initial defect area by sue of Image J (http://rsb.info.nih.gov/ij/).

11)Gene expression assay

Mice were sacrificed 7 days after treatment, and the tissue including the skin wound (1 cm x 1 cm) was taken minced, and digested in the PBS containing collagenase D (2 mg/ml). After 1 hr incubation at the 37 °C, tissue digested was treated similarly to obtain the single cell suspension. A $CD11b^+$ cell fraction was then sorted using Dynabeads Biotin Binder (Invitrogen™, CA.), according to the manufacturer's protocol. Briefly, the cells in the suspension were labeled with a biotin-conjugated rat anti-mouse CD11b (Biolegend, CA.). The incubation of cells was carried out at 4 °C for 15 min. After the cells were centrifuged at 4 °C, 3,400 rpm for 5 min, and the supernatant was discarded. Then superparamagnetic polystyrene beads coated with a recombinant streptavidin were applied. The bead-bound cells were purified using DynaMag™-2 (Invitrogen™, CA.). Total RNA was prepared from $CD11b^+$ cells purified with TRIzol reagent (Life Technologies Corporation, Carlsbad, CA). The RNA concentration was assessed on a NanoDrop-1000 spectrophotometer (NanoDrop Technologies). The cDNA samples were prepared from 1000 ng of total RNA was performed using superscript VILO cDNA synthesis kit (Invitrogen™, CA., U.S.). The following primers were used: (GAPDH, forward 3'-TGAAGCAGGC ATCTGAGGG-5' and reverse 3'-CGAAGGTGGAAGAGT GGGAG-5'; Arginase1, forward 3'-CTCCAAGCCAAAGT CCTTAGAG-5' and reverse 3'-AGGAGCTGTCATTAGG GACATC-5'; iNOS, forward 3'-CTCTGAGGGCTGACAC AAGG-5' and reverse 3'-CCAAGCCCTCACCTACTTCC 5'). The gene expression was determined by quantitative polymerase chain reaction (qPCR) ABI7500 system (Applied Biosystems) using the power SYBR Green kit (Life Technologies corporation, Carlsbad, CA). The data were analyzed using Science Detection Software 2.2.2 and the relative expression was calculated as ddCT using GAPDH as a reference gene.

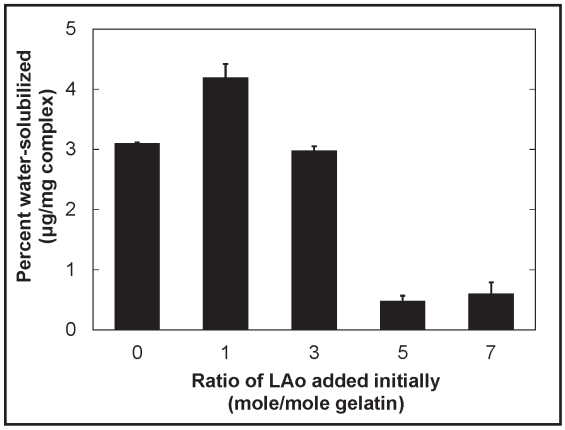

Fig.1 Percentage of Pio water-solubilized by LAo-g-gelatin micelles prepared at different molar ratios of LAo added in preparation. The molecular weight of gelatin to prepare the LAo-g-gelatin is 100,000.

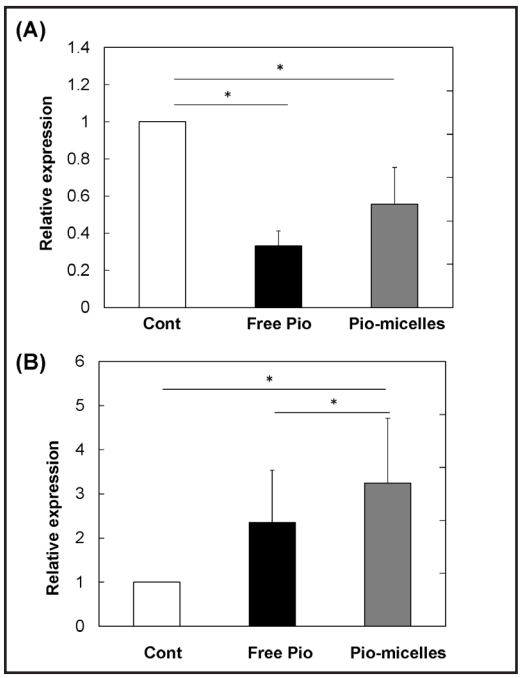

Fig.2 mRNA expression level of iNOS (A) and arginase 1 (B) for BMM cultured with 100 nM free Pio and Pio-micelles containing 100 nM Pio or without Pio (cont). *p<0.05; significant between the two groups.

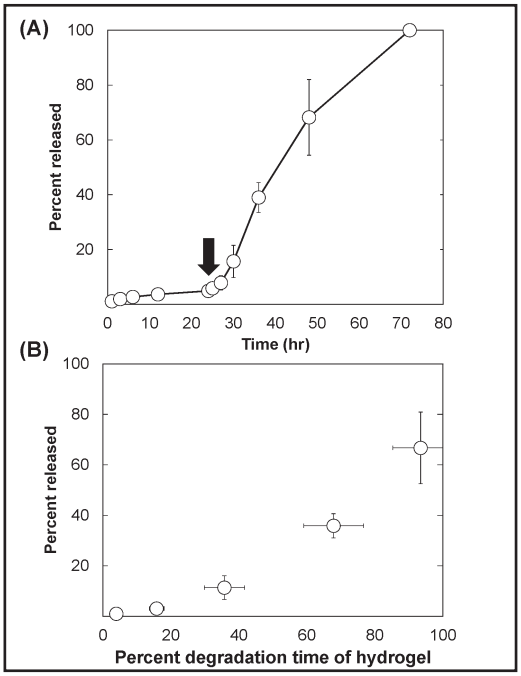

Fig.3 (A) *In vitro* release profile of Pio from gelatin hydrogels incorporating Piomicelles. PBS was changed by that containgin collagenase (100 μg/1ml) 24 hr later indicated by the arrow.
(B) Relationship of percentage between the Pio release and gelatin hydrogel degradation. The molecular weight of gelatin and the molar ratio of LAo added to prepare the LAo-g-gelatin are 20,000 and 1.0, respectively. Hydrogels incorporating Pio-micelles are prepared by dehydrothermal treatment at 160 °C for 24 hr.

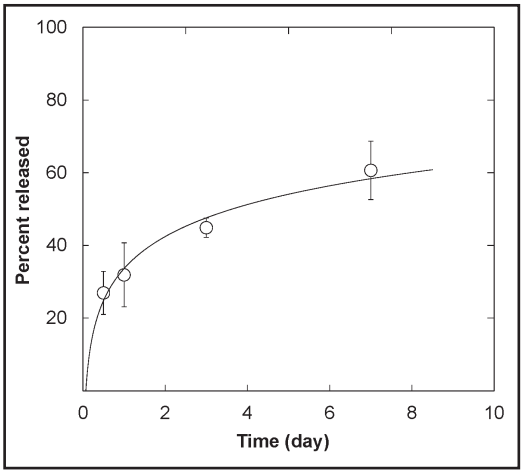

Fig.4 *In vivo* release profile of Pio from gelatin hydrogels incorporating Pio-micelles prepared by dehydrothermal treatment at 160 °C for 24 hr.

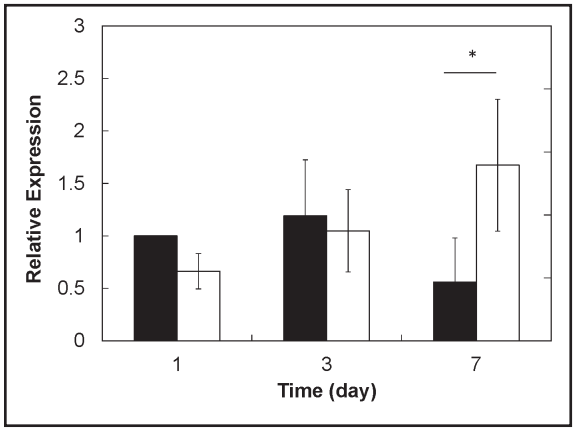

Fig.5 Time course of gene expression of arginase1 in CD11b$^+$ cells around the site of gelatin hydrogels with (white bars) or without Pio-micelles incorporation (black bars) implanted in dorsal of mice. The molecular weight of gelatin and the molar ratio of LAo added to prepare the LAo-g-gelatin are 20,000 and 1.0, respectively. Hydrogels incorporating Pio-micelles are prepared by dehydrothermal treatment at 160 °C for 24 hr. *p<0.05; significant between the two groups

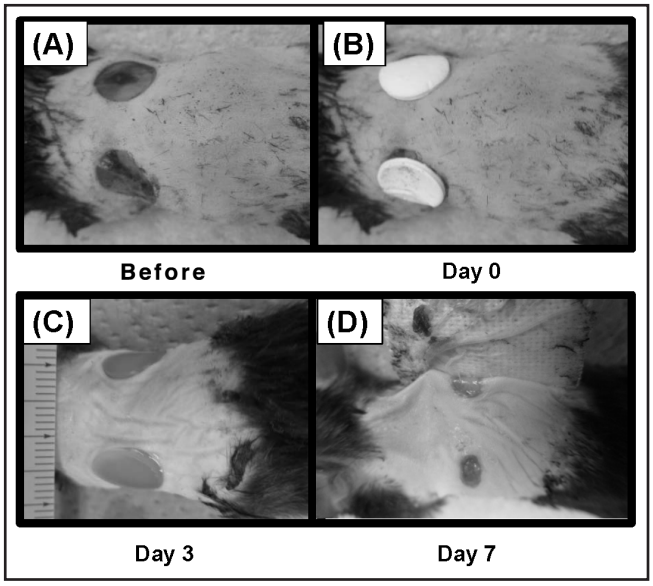

Fig.6 Appearance of wound defect and Pio-hydrogels degradation before (A) and 0 (B), 3 (C) or 7 days (D) after treatment with Pio-hydrogels (left) or Pio-free hydrogels (right).

12) Statistical analysis

All the data were statistically analyzed by using Tukey-Kramer tests for multiple comparisons, and the statistical significance was indicated by a p value less than 0.05. The experimental results were expressed as the mean ± the standard deviation.

Results

1)Water-solubilization of Pio by LAo-g-gelatin and bioactivity

Figure 1 shows the percentage of Pio water-solubilized by various LAo-g-gelatin samples. Irrespective of the LAo grafted ratio, Pio was solubilized in water by mixing with the LAo-g-gelatin. However, the percentage decreased with an increase in the ratio of LAo grafted.

2)*In vitro* bioactivity of Pio-micelles

Figure 2 shows the mRNA level of iNOS and Arginase1 for BMM cultured with free Pio and Pio-micelles. Similarly to free Pio, the Pio-micelles significantly suppressed the iNOS expression by a factor of 2 (Fig. 2A). The Pio-micelles and free Pio significantly, enhanced the arginase 1 expression. And the enhancement by the Pio-micelles was significantly high compared with that by free Pio.

3)Pio release profiles of Pio-hydrogels

Figure 3A shows the time profile of Pio release from Pio-hydrogels. Pio release from the time Pio-hydrogels was hardly observed in PBS. However, Pio was released with time in the presence of collagenase. The time profile of Pio release was in a good accordance with that of hydrogel degradation (Fig. 3B).

Figure 4 shows the *in vivo* time profiles of Pio release from Pio-hydrogels. The Pio was released from the Pio-hydrogels *in vivo*.

4)*In vivo* biological activity of Pio-hydrogel

Figure 5 shows the time course of arginase1 gene expression on the CD11b$^+$ cells of tissues around Pio-hydrogels implanted. There is no significance difference in the level between the Pio-hydrogels and Pio-free hydrogels 1 and 3 days after implantation. However, significant higher expression of arginase1 was observed for the Pio-hydrogels 7 days later.

Figure 6 shows appearance of wound defect and Pio-hydrogels degradation. The hydrogel with or without Pio

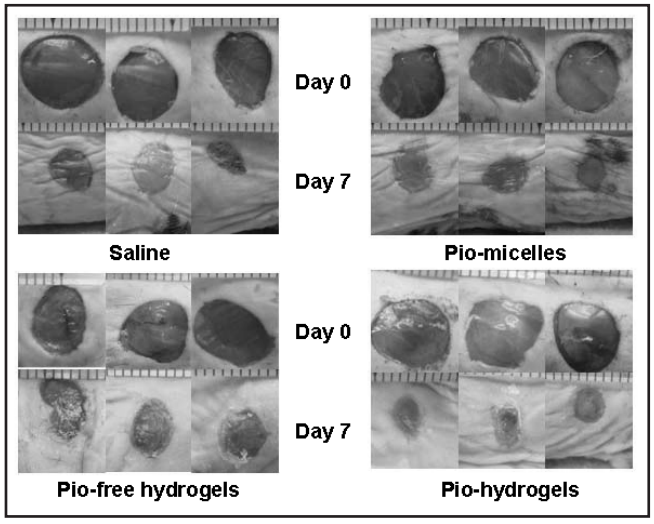

Fig.7 Time course of wound skin defect size 0 day (upper-panel) and 7 days (lowerpanel) after treated with saline (cont), Pio-micelles, Pio-free hydrogels, and Pio-hydrogels.

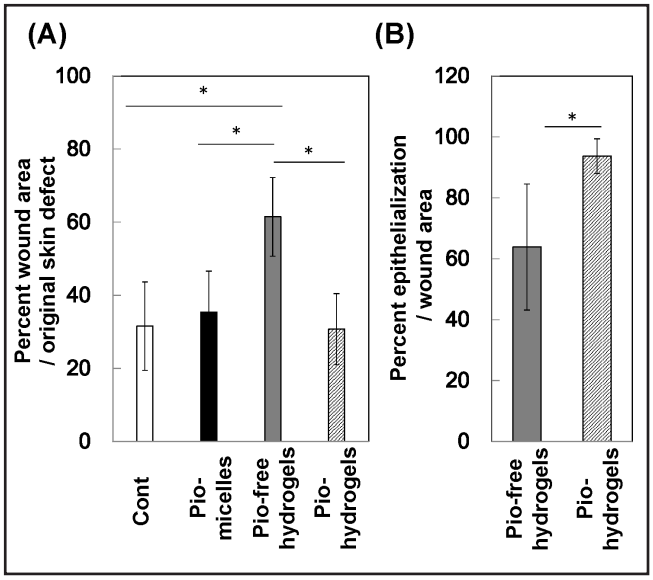

Fig.8 Percent wound area (A) and epithlialization (B) at the skin defect of mice 7 days after treatment with saline (cont), Pio-micelles, Pio-free hydrogels, and Pio35 hydrogels. *p<0.05; significant between the two groups.

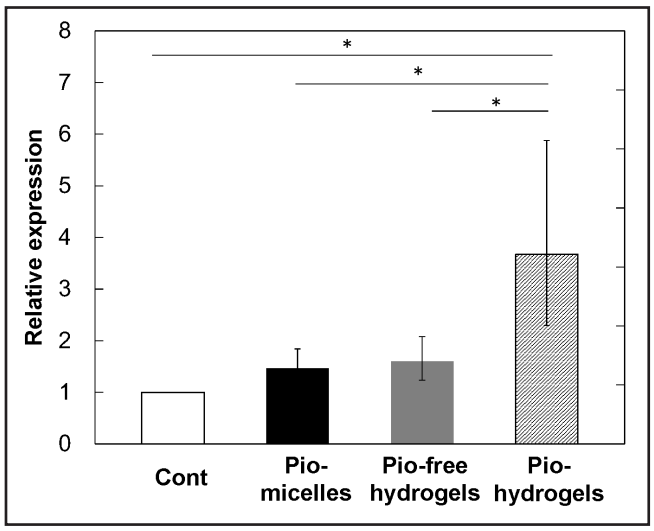

Fig.9 Gene expression of arginase 1 in CD11b$^+$ cells around the mouse skin defect of mice 7 days after treated with saline (Cont), Pio-micelles, Pio-free hydrogels, and Pio-hydrogels. *p<0.05; significant between the two groups.

incoporration were degraded 7 days after implantation. Figure 7 shows the time course of wound skin defect size 0 and 7 days after treated with saline (cont), Pio-micelles, Pio-free hydrogels, and Pio-hydrogels. A wound closure was observed for the skin defects treated with saline and free Pio-micelles, but no epithelialization was seen. For the Pio-free hydrogels group, neither around closure nor epithelialization were observed. On the contrary, the Pio-hydrogels accelerated the wound closure accompanied with epithelialization. Figure 8 shows percent wound area and epithlialization at the skin defect of mice 7 days after treatment with saline, Pio-micelles, Pio-free hydrogels, and Pio-hydrogels. The percent wound area was significantly suppressed by the Pio-hydrogels and Pio-micelles, in contrast with Pio-free hydrogels. On the other hand, the Pio-hydrogels significantly enhanced the percent epithelialization of skin defects.

Figure 9 shows the arginase1 gene expression of CD11b$^+$ cells in the tissue treated with pio-hydrogels and others. The gene level for the Pio-hydrogels group was significant higher than that of Pio-micelles, Pio-free hydrogels, and control groups.

Discussion

The present study demonstrates that Pio released from the gelatin hydrogels modified the profile of gene expression for macrophages *in vitro* and *in vivo*. The Pio of a water-insoluble drug was solubilized in water by making use of LAo-g-gelatin micelles. Then, the Pio water-solubilized (Pio-micelles) was crosslinked with gelatin to prepare the gelatin hydrogel incorporating Pio-micelles (Pio-hydrogels). No Pio release was observed from the Pio-

hydrogels in PBS where the hydrogels are not degraded (Fig. 3A). Gelatin is normally degraded by enzymes, but not by a simple hydrolysis. In the presence of collagenase, it is likely that the hydrogels are degraded enzymatically to generate gelatin water-soluble fragments, resulting in the hydrogel degradation-based Pio release. There was a good correlation in the time profile between the Pio release and the hydrogel degradation (Fig. 3B). This strongly indicates that the Pio release is governed only by the degradation of hydrogels as the release carrier. The *in vivo* Pio release was observed (Fig. 4).

In this study, we focused on macrophages which play a key role in the regulation of inflammation process, and tried to artificially modify their functional phenotype by a drug. To enhance the drug activity, the controlled release was tried. However, for that purpose, the material of drug release carrier should be biodegraded and biocompatible, because the material remaining often causes inflammation. Many biodegradable materials have been investigated as the carrier of drug release[15-21]. However, most of them remain even after the role of drug release completes, and consequently the remaining often induces inflammation responses to the material. As one trial to tackle this problem, the material of drug carrier used must be degraded to disappear accompanied with the drug release in a synchronized manner. From this point, the gelatin hydrogel is advantageous over other release materials. The drug is release from the gelatin hydrogel as a result of hydrogel degradation[22]. When the drug release completes, the hydrogel disappears, which does not cause the material-induced inflammation.

To induce the phenotype modification of macrophages, we used the pioglitazone of a PPARγ agonist which has an ability to change the macrophages function. It is demonstrated that the activation of PPARγ signaling plays a protective role in the tissue remodeling and wound repair by reducing oxidative stress and inflammation[23]. The local administration of rosiglitazone of another PPARγ agonist induced antiinflammatory responses[24] and increased the expression of M2-specific marker[25]. Among the PPARγ agonists, Pio is commercially available and one of the strong modulators for PPARγ. It has been clinically used as a diabetic's drug. Based on that, Pio is a good candidate to experimentally confirm the function to modify the macrophages M1/M2 ratio aiming at the clinical application. As expected, this study demonstrates that this agonist enabled macrophages to modify the cell phenotype.

To evaluate the effect of Pio on the macrophages phenotype, BMM were cultured with Pio-micelles to evaluate the level of iNOS and arginase1 mRNA expression. The iNOS and arginase1 are known as a M1 and M2 markers, respectively. Generally, M1-type macrophages exhibit an increased NOS activity which promotes the L-hydroxyarginine, L-citrulline, and NO production contributing to the anti-microbial activity[26]. On the contrary, for M2-type macrophages, the arginase1 activity increases while the NOS activity decreases[27]. L-arginine is converted by arginase1 to Lornithine of an important substance to produce polyamines for cell proliferation, or proline of a collagen building block, to promote tissue repair[28]. When the BMM were cultured with free Pio and Pio-micelles, the level of iNOS expression significantly decreased. On the contrary, the arginase1 expression level increased significantly (Fig. 2). Some researches have demonstrated that the activation of PPARγ inhibited the expression of pro-inflammatory genes including cytokines and iNOS[29, 30]. The arginase1 expression is regulated by PPARγ[31]. These results reported are similar to the findings of this study. The Pio-micelles have a potential to allow BMM to enhance the arginase1 activity of M2 macrophages measure.

When the Pio-hydrogels were subcutaneously implanted into the mouse skin, the arginase1 expression level of $CD11b^+$ cells increased 7 days after implantation, to a significantly great extent compared with that of Pio-free hydrogels (Fig. 5). Pio was released *in vivo* (Fig. 4). We can say with certainty that Pio released from Pio-hydrogels could modify the $CD11b^+$ cells of macrophage to show the M2 phenotype even *in vivo*. The similar trend of macrophages phenotype modification was observed for the skin wound model of mice. A significantly higher arginase1 expression was detected for $CD11b^+$ cells in the tissue around the wound after the implantation of Pio-hydrogels than for the same dose of free Pio and Pio-free hydrogels (Fig. 9). It is possible that the controlled release of Pio enabled macrophages to make the phenotype M2-type more efficiently compared with the injection of free Pio. The Pio local release would increase the possibility of Pio action to macrophages, resulting in an enhanced Pio-induced phenotype change.

The wound area was significantly reduced by the application of Pio-hydrogels (Fig. 7). The Pio-hydrogels accelerated the wound closure accompanied with epithelization (Fig. 8). Free Pio-micelles promoted the wound closure, although the extent was significantly small

compared with that of Pio-hydrogels. It is apparent from Figure 9 that the Pio-hydrogels enhanced the level of arginase1 gene expression significantly. The Pio release would enable macrophages to modify their phenotype, which assists the wound healing process positively. This positive effect results in the promoted wound closure. However, the mechanism is not clear at present. There have been reported by some researches to support our data. The macrophages of M2a phenotype are generated *in vitro* by the exposure to IL-4 or IL-13 to increase expression of CD 206, arginase, and TGF-β through the common receptor IL-4Rα. The production of TGF-α by M2a macrophages is partly responsible for the enhancement of fibroblasts proliferation and the collagen production which are observed in macrophages/fibroblasts co-cultures[4, 32, 33]. Macrophages phenotype can also be altered by the phagocytosis of apoptotic or necrotic cells[34-36]. Wound-healing or tissue repair functions are physiologically modified by macrophages of an M2a or M2 phenotype[3, 37, 38]. Macrophages during the process of skin wound healing exhibit a heterogeneous and temporally regulated phenotype that does always classify into M1/M2 categorization[39]. The macrophages phenotype related to the skin wound healing is determined by the microenvironment more complex than treatment with one or a few cytokines in a cellculture system. Therefore, it is not surprising that the phenotype of *in vivo* tissue repairing macrophages do not always correspond to that of *in vitro* defined categories. We cannot observe significant different in the gene expression of iNOS, CD206, CD36, and TNF-α by RT-PCR (data not shown). The gene expression may increase or decrease in earlier stage. TZDs inhibit the expression of various inflammatory proteins such as iNOS, TNF-α, and MMP-9 in macrophages[40]. TZDs were found to be effective in treating type 2 diabetes as they directly reduced the systemic insulin resistance of peripheral tissues[41, 42]. A potential therapeutic action of PPARγ agonists improves the strongly impaired angiogenic process in diabetic wound tissue[43]. We revealed that the diabetic wound healing was accelerated by Pio-hydrogels, in contrast to free Pio-micelles and Pio-free hydrogels (data not shown). PPARγ is a member of the nuclear receptor family that includes 48 human transcription factors whose activity is regulated by direct binding of steroid and thyroid hormones, vitamins, lipid metabolites and xenobiotics[44]. It is demonstrated that TZDs function by the activation of nuclear hormone receptor PPARγ[45]. The PPAR family of transcription factors consists of two additional members: PPARα and PPARβ/δ, and all members heterodimerize with 9-cis-retinotic acid retinoid X receptors[46, 47]. Epidermal keratinocytes express all three PPAR subtypes[48]. Keratinocytes are well known produce vascular endothelial growth factor (VEGF) at the wound site[5] and *in vitro*[49]. Angiogenesis is induced by the VEGF production which is one of the keys contributing to the skin repair[50, 51]. Therefore, it is possible that the mechanism of *in vivo* wound healing is not only due to the change in macrophages phenotype, but also to the function of PPARγ. Considering the present data and these reported previously, it is highly conceivable that the local release of Pio induces the production of various factors and angiogenesis in addition to the modification of macrophages phenotype, resulting in an enhanced wound healing.

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Conflict of interests

None

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