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Basic research on aw-AC/PLGA composite scaffolds for bone tissue engineering

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Abstract Recently, it has become important to develop effective material to be used as scaffolds for bone tissue engineering. Therefore, we fabricated new three-dimensional (3D) scaffolds consisting of biodegradable poly(D,L-lactide-co-glycolic acid)(PLGA)(75/25) with anti-washout type AC (aw-AC) particles. The aim of this study was to evaluate this new scaffold concerning its basic properties and biocompatibility.The obtained scaffolds were observed with scanning electron microscopy (SEM), and measured for porosity, shrinkage and biaxial compressive strengths. It was shown that PLGA with aw-AC composite scaffolds (aw-AC/PL) showed a greater strength and stability than PLGA scaffolds (PL). Also, the mass reduction of aw-AC/PL during incubation decreased compared to that of PL. The number of MC3T3-E1 cell in PL and aw-AC/PL was counted at 5 h, 1 week, and 2 weeks after cell seeding. As a result, aw-AC/PL exhibited a superior performance in terms of attachment and proliferation compared to PL. Histologically, aw-AC/PL showed an excellent response toward soft tissues. Therefore, it was shown that aw-AC/PL was more biocompatible than PL. In conclusion, it was strongly suggested that aw-AC/PL was more useful for cell transplantation than PL in bone tissue engineering.

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Introduction

Bone tissue engineering requires materials that are biocompatible, well vascularized, mechanically suited for bone function, integrated with the host skeleton, and support osteoinduction of the implanted cells that form new bone [1].

In recent years, biodegradable polymers have often been utilized to fabricate porous scaffolds for three-dimensional (3D) cell culture to regenerate tissue-based artificial organs. Highly porous scaffolds with interconnected pore structures are desirable in many cases to facilitate cell seeding and adhesion, secretion of extracellular matrices, and eventual regeneration.

Poly(D,L-lactic-co-glycolic acid)(PLGA) is one of the most widely used biodegradable polymers as scaffolds in bone tissue engineering since it is biocompatible and its degradation rate and mechanical properties can be easily controlled by varying the copolymer ratio of lactic to glycolic acid [2]. Though it is known to have excellent biocompatibility, it has some disadvantages. One is that PLGA typically does not demonstrate the mechanical properties of trabecular bone [3]. Another is that PLGA is hydrophobic, porous scaffolds fabricated with these polymers float in cell culture medium. When cells in culture medium are plated on top of a porous scaffold or injected into its interior for seeding, the majority of its pores remain empty since the scaffold does not absorb the culture medium [4]. Therefore, it has a limitation concerning its ability to promote cell attachment and proliferation. Moreover, it has been reported that PLGA scaffolds induced a slight inflammatory response after implantation in vivo [5]. Therefore, we fabricated new three-dimensional (3D) scaffolds which consisted of biodegradable poly(D,L-lactide-co-glycolic acid)(PLGA)(75/25) with anti-washout type AC (aw-AC) particles to solve theses problems in this study.

Many bioactive cements that form HAp were reported based on the fundamental investigation of Brown and Chow [6–16]. They found that an equimolar mixture of tetracalcium phosphate [TTCP:$Ca_4(PO_4)O_2$] and dicalcium phosphate anhydrous (DCPA:$CaHPO_4$), hereafter called conventined apatite cement (c-AC), set to form HAp upon mixing with an aqueous solution [17]. However, c-AC has three problems: Firstly, c-AC takes a long time to set; Secondly, the paste of c-AC crumbles since in the initial stage since it has no mechanical strength to resist the pressure; Thirdly, c-AC was implanted subcutaneously in rats immediately after mixing to evaluate the response to soft tissue. One week after implantation, we found that c-AC had crumbled and caused a severe inflammatory response [18]. In order to solve these problems, we developed aw-AC [17–21]. aw-AC has a much higher mechanical strength in the initial stage compared with c-AC and can maintain its original shape at implantation while setting. Moreover, aw-AC is superior to c-AC with respect to the soft tissue response. In this study, we developed PLGA with aw-AC composite scaffolds (aw-AC/PL) by a solvent casting/particulate leaching method. The aim of the present study was to fabricate new 3D scaffolds and investigate their biocompatibility, mechanical strength, shrinkage, and ability to promote cell attachment and proliferation.

Materials and methods

Materials

Poly(D,L-lactide-co-glycolic acid)(PLGA)(75/25) with a copolymer ratio of 75:25 in mass, prepared from lactide and glycolide using stannous octoae as the initiator. PLGA(75/25), having an inherent viscosity of 0.68 dL/g at –10 °C in $CHCl_3$, was obtained from Corefront Corporation (DURECT Corporation: Birmingham Division, USA). Tetracalcium phosphate (TTCP) was obtained from Taihei Chemical Industrial Co. Dicalcium phosphate anhydrous (DCPA) was obtained from J.T, Baker (Divison of Mallinckrodt, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma Chemical Company. Sugar particles were purchased from Taito Jointstock Company. Sugar particles were sieved through varying pore sizes: 2 mm, 1 mm, and 0.6 mm.

Preparation of aw-AC powder

The powder phase of AC was prepared as described previously [22–30]. In brief, an equimolar mixture of TTCP and DCPA was mixed using a speed mill (SK-M2, Kyoritsuriko, Tokyo, Japan). The mixture was sterilized by exposure to 20 kGy of gamma radiation and kept in a vacuum desiccator at 60 until use. For the liquid phases, c-AC used carbon-free double distilled water. For the preparation of aw-AC, 0.5% sodium alginate containing 0.2 mol/l neutral sodium hydrogen phosphate ($Na_{1.8}H_{1.2}PO_4$) solution was used as the liquid phase [27, 31]. The powder and liquid phases were mixed at a P/L ratio of 2.0. The obtained paste was allowed to harden at the room temperature for 24 h. Afterward, it was crushed into powder and sieved through a 100 μm sieve. The obtained powder was poured and mixed with PLGA solution as follows.

Preparation of aw-AC/PL composite scaffold

The aw-AC/PL composite scaffolds were fabricated by the solvent casting/particulate leaching method. In brief, PLGA(75/25) was dissolved in 10% DMSO solution. The PLGA solution was mixed with sugar crystals (both 2 mm) and aw-AC powder in aw-AC:PL ratio of 1:0.5 [aw-AC/PL(0.5/2)], 1:1 [aw-AC/PL(1/2)], and 2:1 [aw-AC/PL(2/2)]. At the same time, PLGA solution with no aw-AC was mixed with sugar crystals using each of 2 mm [P(2)], 1 mm [P(1)], and 0.6 mm [P(0.6)]. Then, all the dispersion was cast into a mold. Afterward, it was frozen immediately at –18 °C. The polymer was precipitated in deionized distilled water (ddH_2O) and the sugar crystals were leached out of the aw-AC/PLGA mixture. After that, the produced scaffolds were dried for 2 days. Each time, a 3.0 × 3.0 × 5.0 cm scaffold was produced.

Scanning electron microscopy (SEM)

The surface and cross-sectional morphologies of porous PL and aw-AC/PL were observed on a JSM-5300 (JEOL, Tokyo, Japan) at a 15 kV acceleration voltage. Before observations, the scaffolds were sputter-coated with 10 nm of gold under vacuum condition.

Pore size and porosity analysis

The mean pore diameter was evaluated with SEM images of cell free scaffolds. Linear measurements from the most distant points of pore openings were recorded (n = 10/scaffold) [1]. Porosity was determined using a method similar to that reported previously [34]. A cube (5 × 5 × 7 mm) was cut out of a scaffold sample. The volume was calculated from measured dimensions, and the mass was measured with an analytical balance. Five specimens of each scaffold were measured to calculate an average density. Porosity was calculated from the scaffold density (P) and the polymer skeletal density (P_0) using the following equation:

$$\text{Porosity}\,(\%) = (1 - P/P_0) \times 100$$

Shrinkage test analysis

The samples were cut into 3 × 3 × 10 mm columns for the shrinkage test. The mass of each scaffold was measured before and after incubation in α-MEM. The % decrease in mass was calculated from the following equation [33]:

$$\text{Mass loss}\,(\%) = 100 \times (M_0/M_t)/M_0$$

where M with inferior 0 and t indicates the mass at the immersion time of 0 and t, respectively. All values presented are the average of specimens.

Mechanical strength measurement

The mechanical strength of PL and aw-AC/PL was evaluated in terms of diametral tensile strength (DTS). The samples were cut at a diameter of 8.5 mm and a height of 10 mm per column. Testing was performed under three conditions: dry, wet /37 °C/1 week and 3 weeks. The samples were crushed at a cross-head speed of 1 mm min^{-1} using a universal testing machine (AGS-500A, Shimadzu, Kyoto, Japan).

X-ray diffraction (XRD) analysis

The compositional change in the aw-AC/PL upon setting or gamma irradiation was analyzed with the use of powder X-ray diffraction (XRD; Rint 2000, Rigaku, Tokyo). In the case of XRD, measurements used a vertically mounted diffractometer system with Ni filtered CuKα radiation (0.154 nm) generated at 30 kV and 16 mA. The specimens were scanned from 3 to 60° (where θ is the Bragg angle) to determine the reaction product in continuous mode.

Initial cell attachment and proliferation of MC3T3-E1 cells

In this study, MC3T3-E1 cells were used. The culture medium was α-MEM (Dainihonseiyaku, Osaka, Japan) supplemented with 10% fetal bovine serum (Whittaker Bioproducts Inc., Walkerville, MO, USA). Each scaffold was placed in a plastic well (48 well cell culture cluster, Corning, NY, USA). The porous prepared scaffolds were cut into 5 × 5 × 7 mm cubes and prewetted in phosphate-buffered saline (PBS) for 2 h. MC3T3-E1 cells (1×10^4) were resuspended in 100 µl media, and the concentrated cell suspensions were pipetted onto scaffolds. After that, they were incubated in an atmosphere containing 5% CO_2 at 37 °C. Cell culture in plastic wells was used as a control. Cells were allowed to adhere to the scaffolds for 5 h and 1 ml media was added to the 48-well plate (48 well cell culture cluster, Corning, NY, USA). Culture media was changed every 3 day. After cell culture for 5 h, 1 week, and 2 weeks, the number of MC3T3-E1 cells was measured by cell-count.

Histological preparations

Eight-week-old male rats of the Wistar strain, obtained commercially (Charles River, Japan) and fed standard pellets and water ad libitum, were used for the soft tissue response to scaffolds without cells. The subcutaneous tissue of rats was selected for this experiment, because it has been used widely for assessing the response of biomaterials in soft tissue. In this surgery, PL and aw-AC/PL in all conditions cut into 5 × 5 × 7 mm cubes were implanted into the subcutaneous tissue of the rat abdomen. The soft tissues containing scaffolds were removed from three rats each at 3 days and 3 weeks after surgery. The specimens were fixed in 10% neutral-buffered formalin and embedded in MMA (osteoresin; Wako, Japan). They were then sliced into serial 5 µm thick longitudinal sections, stained with hematoxylin-eosin, and subjected to light microscopic examination.

Results

Figure 1 shows the highly interconnected macroporous structure of all scaffolds. Enlarging the sugar particles resulted in a reduction of both the small pore size and the degree of interconnectivity. When more aw-AC particles were added, the small pores decreased after incorporation of more aw-AC, and consequently, the level of interconnection decreased. Figure 2 shows the pore sizes and porosities of scaffolds. The average pore diameters of PL(2), PL(1), and PL(0.6) were 1015 µm, 615 µm, and 437 µm, respectively. The average porosities of PL(2), PL(1), and PL(0.6) were 96.9%, 92.1%, and 91.4%, respectively. aw-AC/PL had pores ranging in average diameter from 798 to 823 µm. The average porosities of aw-AC/PL(0.5/2), aw-AC/PL(1/2), and aw-AC/PL(2/2) were 94.7%, 93.2%, and 90.9%, respectively. The pore size and porosity of PL(2) were significantly larger than those of PL(1) and PL(0.6). When larger sugar particles were added, the pore size and porosity increased. When more aw-AC particles were added, the porosity decreased; however, the pore size did not. Figure 3 shows XRD patterns of aw-AC/PL(0.5/2), aw-AC/PL(1/2), and aw-AC/PL(2/2) with those for the powder phase of PLGA and aw-AC for comparison. No difference among aw-AC/PL(0.5/2), aw-AC/PL(1/2), and aw-AC/PL(2/2) was observed. The mechanical strength of each scaffold is shown in Fig. 4. The DTS of PL(2) was significantly lower compared with those of PL(1) and PL(0.6) under all testing

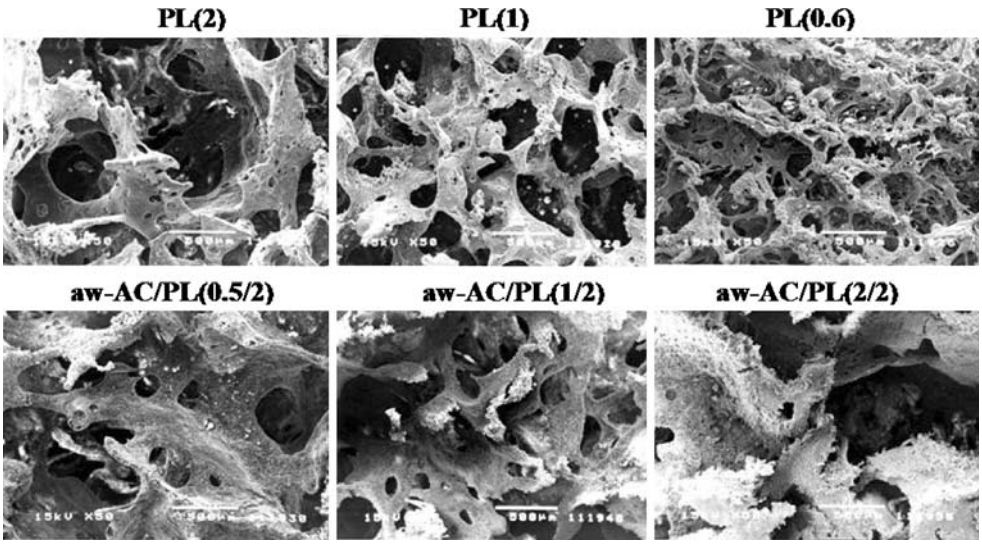

Fig. 1 SEM images of the scaffolds. Cell-free scaffolds are imaged at 50× original magnification to demonstrate the macro-porosity of samples

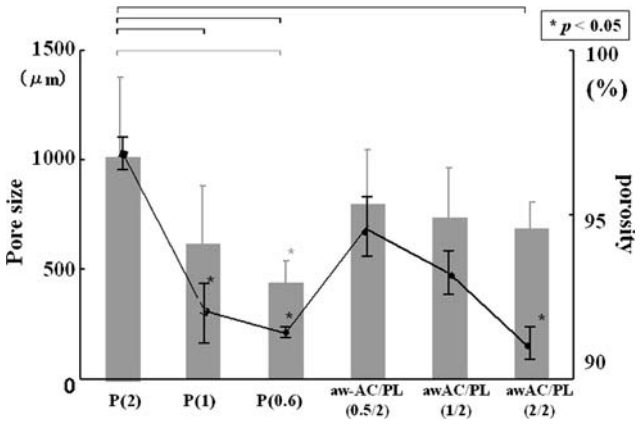

Fig. 2 Pore size and porosity of the scaffolds. SEM images of cell-free scaffolds were evaluated to determine the mean pore diameter. Linear measurements from the most distant points of pore openings were recorded (n = 10/scaffold). Descriptive statistics were calculated and comparisons of the mean pore diameters among the scaffolds were evaluated using analysis of variance (α = 0.01). * and bars indicate a significant difference as compared with PL(2) and others on porosity and pore size, respectively

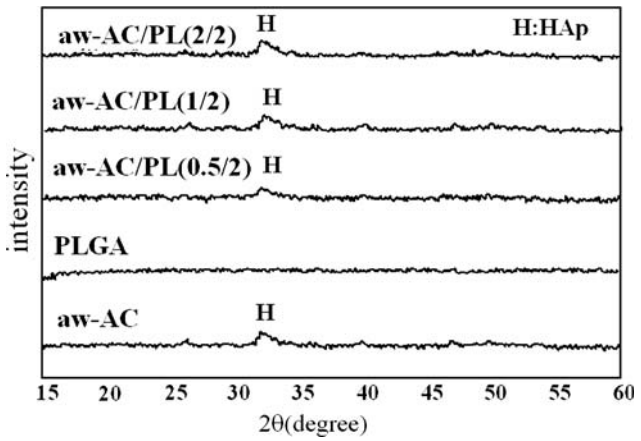

Fig. 3 Powder X-ray diffraction patterns of PLGA, aw-AC/PL(0.5/2), aw-AC/PL(1/2), and aw-AC/PL(2/2). The powder phase of calcium phosphate cement and poorly crystallized aw-AC are shown for comparison

conditions. In the case of aw-AC/PL, the DTS was significantly higher when compared with those of PL(2), PL(1), and PL(0.6). Higher aw-AC/PLGA ratios produced better mechanical properties under all conditions. Figure 5 shows that the mass reduction of PL(2) was larger than in PL(1) and PL(0.6) at the same time, and was significantly larger than in aw-AC/PL(0.5/2), aw-AC/PL(1/2), and aw-AC/PL(2/2) at all period. Adding aw-AC particles to the scaffolds resulted in a significant reduction in shrinkage. Figure 6 shows that the attachment of MC3T3-E1 cells to aw-AC/PL(0.5/2), aw-AC/PL(1/2), and aw-AC/PL(2/2) was significantly greater than with PL(2), PL(1), and PL(0.6). Adding aw-AC particles to the scaffolds resulted in a significant increase in initial cell attachment.

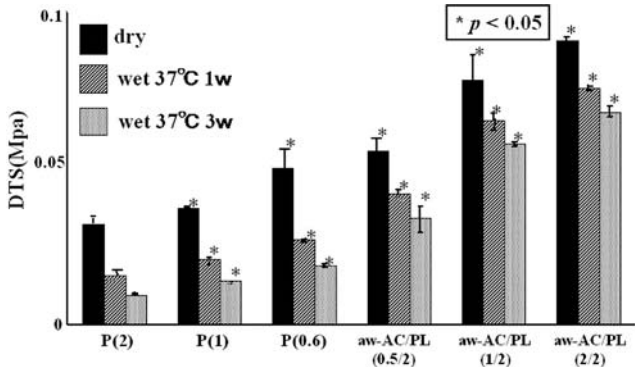

Fig. 4 DTS value of PL and aw-AC/PL under three conditions: dry, wet /37 °C/1 week and 2 weeks. This was evaluated in terms of diametral tensile strength. The samples were cut at a diameter of 8.5 mm and a height of 10 mm column. The results are shown as the mean ± standard deviation (n = 3). * and bars indicate a significant difference as compared with PL(2) and others, respectively

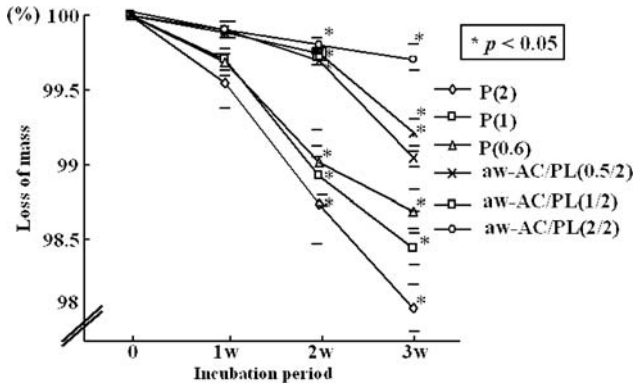

Fig. 5 Shrinkage test results with scaffolds before and after incubation in α-MEM. The results are shown as the mean ± standard deviation ($n = 5$). * and bars indicate a significant difference as compared with PL(2) and others, respectively

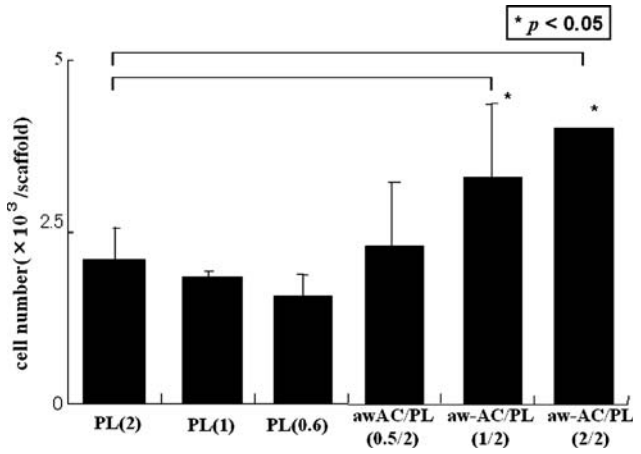

Fig. 6 Initial attachment of MC3T3-E1 cells on scaffolds. The numbers of MC3T3-E1 cells on scaffolds were measured after 5 h of culturing. The results are shown as the mean ± standard deviation ($n = 5$). * and bars indicate a significant difference as compared with PL(2) and others, respectively

Figure 7 shows that the proliferation of aw-AC/PL(2/2) significantly increased when compared with aw-AC/PL(1/2) and aw-AC/PL(0.5/2). Especially, the number of cells proliferating in aw-AC/PL(1/2) and aw-AC/PL(2/2) significantly increased compared to that in aw-AC/PL(0.5/2) at 2 weeks. Adding aw-AC particles to the scaffolds resulted in a significant increase in cell proliferation. Figure 8 summarizes the histological appearances of the soft tissue apposed to PL(1), aw-AC/PL(0.5/2), and aw-AC/PL(2/2). aw-AC/PL(0.5/2) at 3 days after implantation showed slight inflammation consisting of a smaller number of lymphocytes, plasma cells, and macrophages than that of PL(1). On the other hand, histological appearances of both PL(1) and aw-AC/PL(2/2) at 3 weeks showed that the inflammatory response was resolved compared to that at 3 days. This means that aw-AC/PL had an excellent

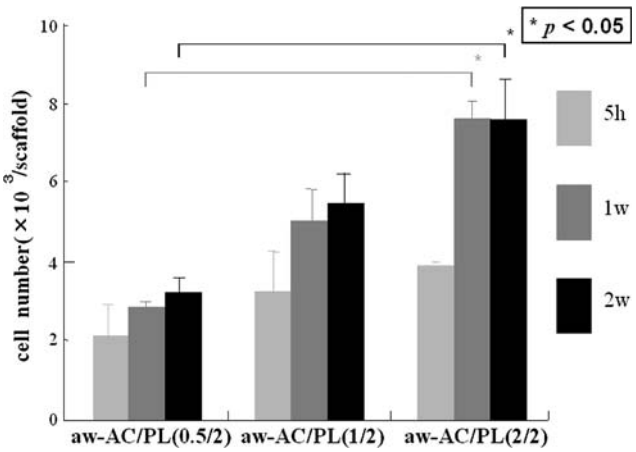

Fig. 7 Proliferation of MC3T3-E1 cells on scaffolds evaluated by cell count. The results are shown as the mean ± standard deviation (n = 5). * and bars indicate a significant difference as compared with PL(2) and others, respectively

response toward soft tissues initially when compared with PL(1).

Discussion

Bone grafts have been used to fill bone defects caused by disease or trauma, such as bone fractures, infections, and tumors. Autografts have the distinct advantage of histocompatibility without the risks of disease transfer and are still the best material for bone repair. However, their limited availability necessitates the development of alternative bone substitutes. In recent years, the main goal of bone tissue engineering has been to develop biodegradable materials to be used as bone graft substitutes for filling large bone defects. These materials should maintain adequate mechanical strength, be osteoconductive, and degrade at a controlled rate to provide space for the formation of new bone. There has been widespread use of calcium phosphate bioceramics, such as HAp and tricalcium phosphate (TCP), for bone regeneration [34, 35]. Their biocompatibilities are thought to be due to their chemical and structural similarity to the mineral phase of native bone. The interactions of osteogenic cells with bioceramics are important for bone regeneration. Bioactive ceramics are known to enhance osteoblast differentiation as well as osteoblast growth. However, their clinical applications have been limited because of their brittleness, difficulty of shaping [36], and extremely slow degradation rate in the case of HAp [37]. At the same time, PLGA did not demonstrate the mechanical properties of trabecular bone [4] and was hydrophobic. Therefore, it is limited in its ability to promote cell attachment and proliferation [4]. Moreover, it was reported that PLGA scaffolds induce slight

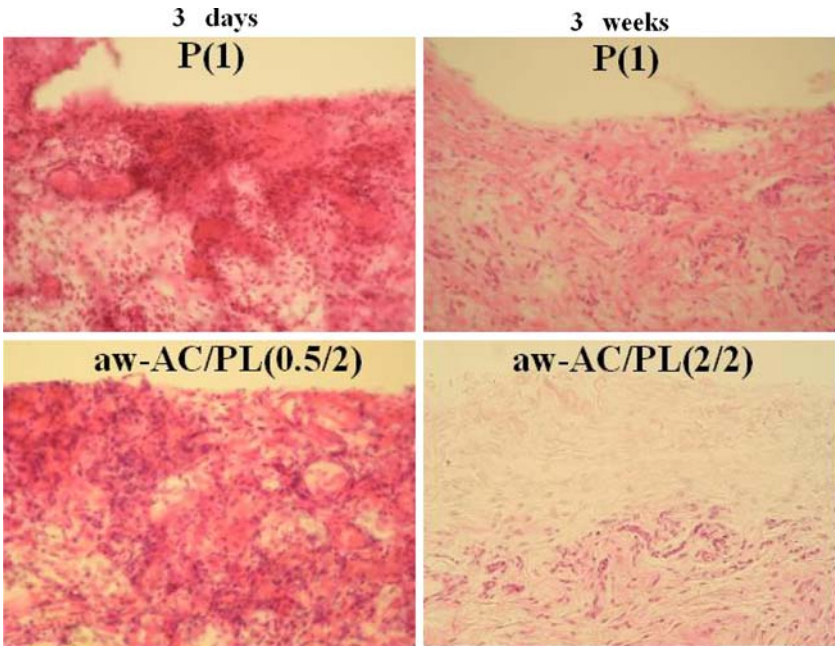

Fig. 8 Photomicrograph of subcutaneous tissue surrounding a PL and aw-AC scaffold 3 days and 3 weeks after surgery. (Original magnification ×200; hematoxylin-eosin stain)

inflammatory responses after implantation in vivo [5]. The development of biodegradable polymer/bioceramic composites could be a solution to these problems. The addition of biodegradable PLGA to HAp would allow for better manipulation, biocompatibility, and control over both the macro and microstructure in shaping composites to fit bone defects. In addition, PLGA could be used as a binder for HAp to reduce the brittleness of ceramics [35, 38–40]. PLGA/HAp composites are promising materials for bone grafts, and have been extensively investigated [40–44]. Most of the previous methods for fabricating polymer/bioceramic composite scaffolds including PLGA/HAp composites involved solvent casting/particulate leaching method, the gas forming/particulate leaching method, and the phase separation method [45–47]. In this study, we selected the solvent casting/particulate leaching method to fabricate highly porous aw-AC/PL for bone tissue engineering. SEM images of the scaffolds showed the highly interconnected macroporous structure of the scaffold, and increasing the sugar particle size resulted in a reduction of both the small pore size and the degree of interconnectivity. When more aw-AC particles were added, more micropores and membranes could be seen, and the small pores dramatically decreased. This method would efficiently fabricate the highly interconnected macroporous structure of the scaffold. In the case of aw-AC/PL(0.5/2), aw-AC/PL(1/2), and aw-AC/PL(2/2), no significant difference was observed in XRD patterns. Thus, XRD patterns of aw-AC/PL showed that the presence of HAp was confirmed in aw-AC/PL regardless of the presence or absence of PLGA. Mechanical strength testing was performed under physiological conditions. The dry and wet scaffolds showed significant differences in mechanical properties. PLGA acted as a matrix and aw-AC particles acted as fillers in the PLGA network. Therefore, it was confirmed that a higher aw-AC/PL ratio would produce superior mechanical properties. Moreover, it has been reported that the DTS value decreased logarithmically with an increase in porosity [21, 48]. The scaffolds for bone tissue engineering should maintain their structural integrity during cell culture. However, it was reported that PLGA undergoes bulk degradation by simple hydrolysis into lactic and glycolic acids [49]. Moreover, the degradation acidic products reduced the local pH and accelerated the degradation rates. It has been reported that the pH value of PLLA/HAp composite is more stable than that of PLLA and HAp scaffolds [50]. aw-AC/PL decreased the acidic degradation by products from PLGA during incubation and maintained its structure and pores for new bone formation. As a result, the shrinkage of aw-AC/PL was superior to that of PL in this study. The attachment and proliferation of MC3T3-E1 cells with aw-AC/PL(0.5/2), aw-AC/PL(1/2), and aw-AC/PL(2/2) were significantly higher than with PL(2), PL(1), and PL(0.6). Cell attachment and proliferation were affected by the hydrophilic property, structure, and chemical composition of the scaffold surface. Adding AC particles to the scaffolds resulted in a significant increase in initial cell attachment and proliferation because HAp in aw-AC improved these factors of PLGA scaffolds. The amount of proteins including cell adhesive proteins in aw-AC, such as fibronectin and vibronectin, subsequently dictated the cell adhesion behavior [51, 52]. This could be attributed to the effect of the increased initial cell attachment of aw-AC/PL compared with PL, thereby increasing

cell proliferation. The reason was that most cells attached onto appropriately modified surfaces far better than onto hydrophobic surfaces including less proteins [53–56]. Figure 8 showed the histological appearances of the soft tissue apposed to PL(1), aw-AC/PL(0.5/2), and aw-AC/PL(2/2). In the case of aw-AC/PL(0.5/2), slight inflammation consisting of a smaller number of lymphocytes, plasma cells, and macrophages was observed PL(1) at 3 days after implantation. On the other hand, the histological appearances of both PL(1) and aw-AC/PL(2/2) at 3 weeks showed thin fibrous tissue which includes a few giant cells and presents slight inflammatory cell infiltration. In the case of PL(1), the scaffolds crumbled in the initial stage because they did not have sufficient mechanical strength to resist the pressure [26, 57]. Moreover, PLGA degraded to acidic products and caused pH changes. Crumbling scaffolds and acidic products of PLGA caused inflammation in vivo [49]. We demonstrated that aw-AC/PL had an excellent response toward soft tissues initially when compared with PL.

Finally, it was suggested that aw-AC/PL could be a better material than PL as a scaffold for bone tissue engineering. However, further studies are needed to examine that aw-AC/PL could enhance the bone regeneration efficacy of osteogenic cell transplantation in vivo. The promising characteristics of aw-AC/PL as a scaffold encourage further investigations of this material for its use in bone tissue engineering.

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