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A Method of Cell-Sheet Preparation Using Collagenase Digestion of Salmon
Atelocollagen Fibrillar Gel

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ABSTRACT

We prepared a cell sheet by using collagenase treatment to digest salmon atelocollagen fibrillar gel (SAC gel) on which human periodontal ligament (HPDL) cells had been cultured. The SAC gel was found to be digested completely within 2 h at a concentration of 50 U of collagenase per mg of collagen. The SAC gel on which HPDL cells were cultured for 10 d was treated with collagenase, resulting in the formation of a detached and shrunken cell sheet. Immunostaining results showed that the cytoskeleton and fibronectin matrix level of the cell sheet were maintained after collagenase treatment. In addition, collagenase treatment had almost no effect on the activities of HPDL cells.

Repair and regeneration of tissues by tissue engineering is one of the important techniques in regenerative therapy. Tissue engineering is dependent on the use of biodegradable polymer scaffolds, such as collagen (1), gelatin, poly-lactic acid and poly-glycolic acid (2). In this technique, the scaffolds are gradually degraded and replaced with the extracellular matrix (ECM) that is produced by the cells, resulting in regeneration of tissues (3). In the case of the cells cultured in porous scaffolds, however, the density of cells is low and the connective tissue may become excessive when the scaffolds are absorbed in the living body (4). In addition, it has been pointed out that the inflammation reaction originating in the biodegraded scaffolds may be increased after a transplant (5).

In order to overcome the above-mentioned problems, Shimizu *et al.* proposed a means of reconstructing tissue by layering the cell sheets without using a scaffold (4). Confluent cardiomyocytes cultivated on a culture plate grafted with the poly (*N*-iso-propylacrylamide) can be harvested as a viable contiguous cell sheet by lowering the temperature without any enzymatic digestions. This cell-sheet engineering without the use of artificial scaffolds resolved the problems, including the inflammatory reactions, that arose from the materials biodegraded from scaffolds. Cell-sheet engineering thus has the potential to allow the construction of various tissues *in vitro*

and to greatly advance tissue-engineering research.

As another method for preparing cell sheets, we attempted to detach the cells cultured on collagen gel as a sheet by digesting the collagen with collagenase. Collagen is more cytocompatible than synthetic polymers because it is derived from living tissue (6). This cytocompatibility allows the cultivation of various cells derived from various species, and it is expected that the cell sheet obtained from collagen will be more viable and differentiated than those derived from synthetic polymers.

As a collagen sample, we used salmon atelocollagen (SAC). We have reported the successful preparation of a collagen fibrillar gel stable at 37°C from SAC for which the denaturation temperature was 18.6°C (7). The SAC fibrillar gel (SAC gel) was prepared by cross-linking with carbodiimide during fibril formation under specific conditions; concentrations of collagen, carbodiimide, NaCl, and Na-phosphate buffer were 0.25%, 50 mM, 35 mM, and 15 mM, respectively. The melting temperature of the SAC gel was found to be 47°C, which was equivalent to porcine atelocollagen fibrillar gel (PAC gel) (7). In addition, the active proliferation of human periodontal ligament (HPDL) cells was observed on the SAC gel. We expect that the SAC gel has the potential to be used in the development of cellular matrices and tissue engineering. Here, we show the methods of detachment of HPDL cells as a sheet by digesting the SAC gel with

collagenase, and layering the cell sheet on other cells.

SAC was prepared from the fresh skin of chum salmon (*Oncorhynchus Keta*) by a procedure reported previously (8). The lyophilized SAC was added to diluted HCl (pH 3) to a concentration of 0.5% (w/v) to give a SAC solution. The SAC gel was prepared by mixing the SAC solution and the 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Dojindo, Tokyo) solution according to a method reported previously (7).

PAC was purchased as a 0.3% (w/v) solution in diluted HCl (Cellmatrix Type I-P; Nitta Gelatin, Osaka). The PAC gel was prepared using a mixture of 0.3% (w/v) PAC solution and one-sixth volume of Na-phosphate buffer (pH 6.8, 90 mM) containing 210 mM NaCl at 4°C. The mixture was incubated at 37°C for 24 h, resulting in a PAC gel.

Sensitivity to collagenase was evaluated as solubility in collagenase solution at 37°C. The SAC gel was washed with phosphate-buffered saline (pH 7.4) containing 1 mM CaCl₂ and 0.33 mM MgCl₂ (PBS). Then, collagenase (*Clostridium histolyicum* for collagen analysis; Wako Pure Chemical Industries, Osaka) was dissolved in PBS at a concentration of 1000 U/ml, and the solutions were filtered through a 0.22- μ m filter unit (Millex GP; Millipore, Tokyo). The collagenase solutions were poured on the SAC gels at concentrations of 10, 25, 50, or 100 U/mg of collagen, and incubated at 37°C. The supernatants were collected and the protein contents of supernatants were evaluated

using a bicinchoninic acid protein assay kit (Sigma Aldrich Japan K.K., Tokyo) according to a method reported previously (8).

HPDL cells were isolated and cultured by the method reported previously (8). Before cell cultivation, the SAC gel was rinsed with PBS, and then with Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo) containing 10% fetal bovine serum (FBS; Gibco BRL, Life Technologies, Rockville, MD, USA). HPDL cells were seeded on the SAC gel at a density of 5×10^3 cells/cm², and cultured at 37°C in air containing 5% CO₂. The medium was changed every 2 d.

A schematic illustration of the method for preparing the cell sheet is shown in Fig.

1. The SAC gel on which HPDL cells were cultured was detached from the culture dish with tweezers and floated in DMEM (Fig. 1A). Then, collagenase solution was added to the medium at a concentration of 50 U of collagenase per mg of collagen (50 U/mg), and incubated for 2 h at 37°C. The method for reattachment of a cell sheet is schematically shown in Fig. 1B. The SAC gel was transferred to a 60-mm culture dish (Asahi Techno Glass, Tokyo) so that the cell surface of the gel might attach to the new culture dish. The gel was incubated for 1 h at 37°C. Then, DMEM and collagenase solution were added to the culture dish, and it was incubated for 2 h at 37°C.

The reattached cell sheet was fixed with 4% paraformaldehyde in PBS for 20 min.

The sheet was permeabilized with 0.5% Triton X-100 in PBS for 2 min. Then, the sheet was blocked with 0.1% BSA in PBS for 90 min and reacted with a 1 : 200 dilution of mouse anti-human fibronectin (FN) monoclonal antibody (Takara, Osaka) for 2 h. Finally, the sheet was treated with a 1 : 200 dilution of FITC-conjugated goat antibody against mouse IgG (Beckman Coulter, France) for 1 h. To examine the maintenance of the cytoskeleton, F-actin of the cell sheet was stained with a 1 : 100 dilution of rhodamine-conjugated phalloidin instead of antibodies. The immunostained sample was observed with a fluorescence microscope (BX40; Olympus, Tokyo).

The SAC gel on which HPDL cells were cultured was laid a top of HPDL cells cultured on a 60-mm culture dish, as shown in Fig. 1C. After incubation for 1 h at 37°C, collagenase solution was added to the medium, and incubated for 2 h at 37°C. The double-layered cell-sheet was observed by an inverted light microscope (CK2; Olympus).

To evaluate the side effect of collagease treatment, HPDL cells were cultured in the wells of a 24-well culture plate (Asahi Techno Glass, Tokyo) for 3 d, and then collagenase solution was added to the medium at a concentration of 50 U/ml. After incubation for 2 h, the wells were rinsed with PBS and then cultured with DMEM. To assess the cell viability and the differentiated cell function of HPDL cells after the

collagenase treatment, a cell viability assay and alkaline phosphatase (ALP) activity assay were performed according to methods reported previously (8).

Sensitivity to collagenase is shown in Fig. 2. The SAC gel was completely digested at a concentration of 50 U or more of collagenase per mg of collagen for 2 h, although three hours were necessary to digest the SAC gel at a concentration of 25 U or less of collagenase per mg of collagen. On the other hand, the PAC gel was not digested completely at 50 U /mg for 2 h, and took 4 h to be digested completely (data not shown). It was found that the SAC gel exhibited higher sensitivity to collagenase compared with the PAC gel although the SAC gel was cross-linked by carbodiimide. The reason why the SAC gel was highly sensitive to collagenase is unclear. It was determined that collagenase treatment should be performed at a concentration of 50 U/mg for 2 h to prepare a cell sheet.

The SAC gel on which HPDL cells were cultured for 10 d was treated with collagenase at a concentration of 50 U/mg for 2 h according to the scheme shown in Fig. 1A, resulting in the formation of a detached and shrunken cell sheet (Fig. 3B). One of the reasons for the successful preparation of the cell sheet was that the density of cells cultured on the SAC gel was high. Yunoki *et al.* reported that the proliferation rate of HPDL cells cultured on a SAC gel was higher than that of cells cultured on a PAC gel

(7). The enhanced proliferation of HPDL cells cultured on the SAC gel resulted in high cell density and successful detachment as a contiguous cell sheet. On the other hand, a cell sheet was not obtained from the PAC gel at a cultivation time of 10 d (data not shown). Cells cultured on a collagen fibrillar gel show low proliferative activity (9), which would result in low cell density, causing the cells to separate after detachment from the PAC gel. It is currently under investigation why the SAC gel enhanced cell proliferation.

The obtained cell sheet shrank, its diameter decreasing from 35 mm to 8 mm (Fig. 3B). This result was in agreement with a previous report in which the cell sheet shrank after detachment from a culture surface (10). When the cells detached from the ECM, the interaction between the cells and the ECM was lost, and the cell sheet shrank due to active cytoskeletal reorganization. The cell-sheet shrinkage might indicate that the cytoskeleton and cell-to-cell connections were maintained in the cell sheets obtained in our study.

To preserve cell morphology without any shrinkage, the cell sheet was reattached to a new culture surface according to the scheme shown in Fig. 1B. After digesting the SAC gel by collagenase treatment at a concentration of 50 U/mg for 2 h, the reattached cell sheet was left on the surface (Fig. 3C). In this case, the cell sheet did not shrink, and

the change in diameter was only 5 mm (from 35 mm to 30 mm). In cell-sheet engineering using a temperature-responsive culture surface, support membranes, such as a poly(vinylidene difluoride) membrane (11), were used to support the cell sheet and prevent cell shrinkage. In our experiments, the SAC gel played the role of a support membrane due to the high mechanical strength of the SAC gel. It was found that the mechanical strength of the SAC gel was higher than that of the PAC gel due to the stabilization by cross-linking during fibril formation (data not shown). In the case of the PAC gel, it did not act as a support due to the low mechanical strength of the gel.

The degree of invasiveness of the collagenase treatment into the cytoskeleton and the FN matrix of HPDL cells was examined by immunohistochemical analysis. Stress fibers were observed in the cell sheet (Fig. 4A), indicating that the cytoskeleton was maintained. The FN matrix was preserved after collagenase treatment and spanned several cells in the cell sheet (Fig. 4B). FN is one of the major matrix proteins synthesized by cultured cells, and plays an important role as a cellular adhesive protein. Preservation of FN enabled cells to maintain their differentiated functions, and to reattach on other culture surfaces or cells. These results show that collagenase treatment was not invasive to either the cytoskeleton or the FN matrix of HPDL cells.

The cell sheet was layered on other cultured cells according to the scheme shown

in Fig. 1C. The left side of the photograph shown in Fig. 3D is the layered cell sheet. This layering method would allow the construction of a three-dimensional tissue *in vitro* based on the concept of cell-sheet engineering. The layering of three or more cell sheets remains to be investigated in future studies.

The effect of the collagenase treatment on the activities of HPDL cells was determined. The viability of HPDL cells after collagenase treatment was slightly decreased compared to that of non-treated cells (Fig. 5A). However, the proliferation rate of collagenase-treated cells was almost the same as that of non-treated cells. ALP activity is one of the differentiated cell functions of HPDL cells. Figure 5B shows that HPDL cells maintained their ALP activity after collagenase treatment. These results indicate that collagenase treatment had almost no effect on the cell activities.

This is the first report of cell-sheet preparation from HPDL cells using a collagen gel. We have already confirmed that human gingival fibroblasts (HGF) and human osteoblasts (HOst) proliferate on SAC gels in a manner similar to HPDL cells. Periodontal tissue is considered to be the construct in which cell sheets of HPDL cells, HGF and HOst are layered through the ECM. We are trying to construct periodontal tissue *in vitro* by layering these cell sheets. In conclusion, we prepared a cell sheet from a SAC gel using collagenase digestion at a concentration of 50 U/mg for 2 h. In addition,

a cell-sheet was obtained from a SAC gel on which HPDL cells were cultured for 10 d. The characteristics of the SAC gel, such as high sensitivity to collagenase, high mechanical strength, and the enhancement of cell proliferation, were found to be important factors for our cell-sheet preparation.

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Figure captions

FIG. 1. Schematic illustration of the methods for preparing the cell sheet.

FIG. 2. Collagenase sensitivity of the SAC and PAC gels. The solubility values are the means \pm SD (n=3).

FIG. 3. Photographs of a SAC gel before collagenase treatment (A), cell sheet obtained after collagenase digestion (B), and the reattached cell sheet (C). (D) Phase-contrast microscopy of the layered cell sheet ($\times 40$). The left side of the photograph is the layered cell sheet.

FIG. 4. Fluorescence microscopy of cell sheet reattached on the new culture surface ($\times 400$). Reattached cell sheet was fixed and double-stained with rhodamine-conjugated phalloidin for F-actin (A) and with anti-FN antibody (B).

FIG. 5. Effects of collagenase treatment on cell viabilities (A) and ALP activities (B) of HPDL cells. (A) Cell viabilities of HPDL cells after collagenase treatment (circles)

and non-treatment (squares), expressed as the absorbance determined from an MTS assay. Bars represent the SD (n = 4). (B) ALP activities of HPDL cells cultured for 10 d after collagenase treatment and non-treatment, expressed as the ALP activity normalized by the cell viability. Bars represent the SD (n = 4).

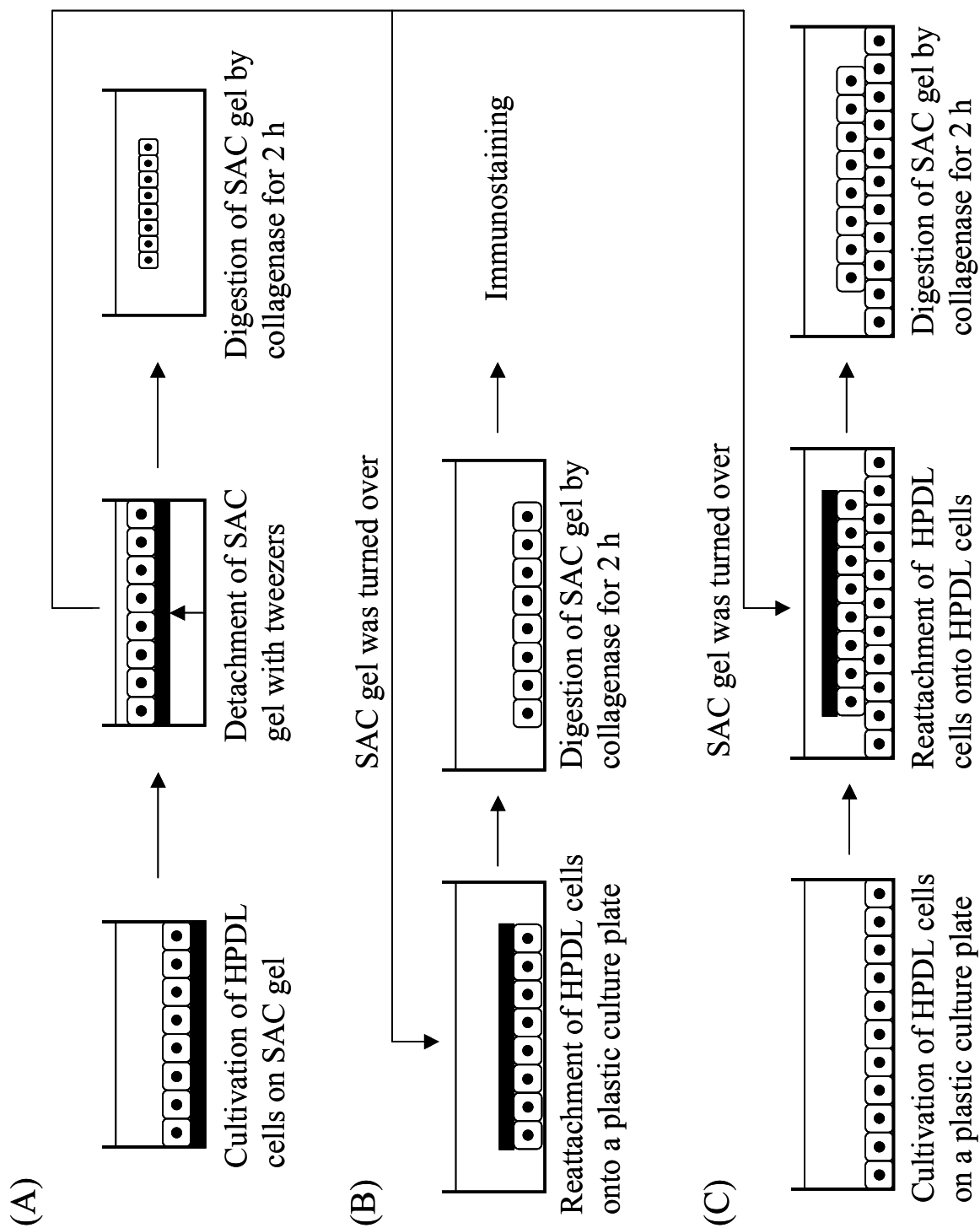


Fig.1

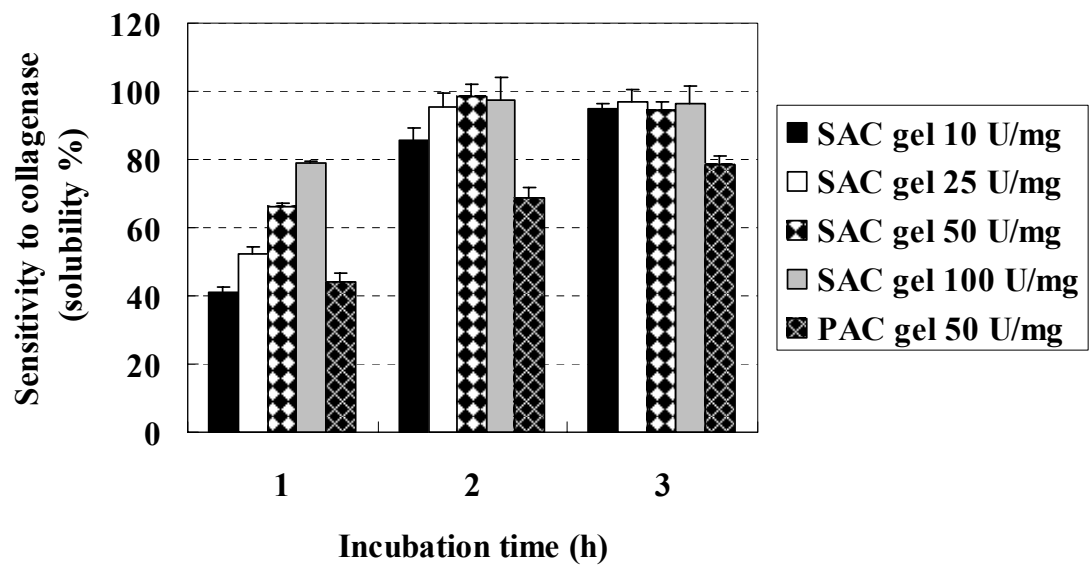


Fig.2

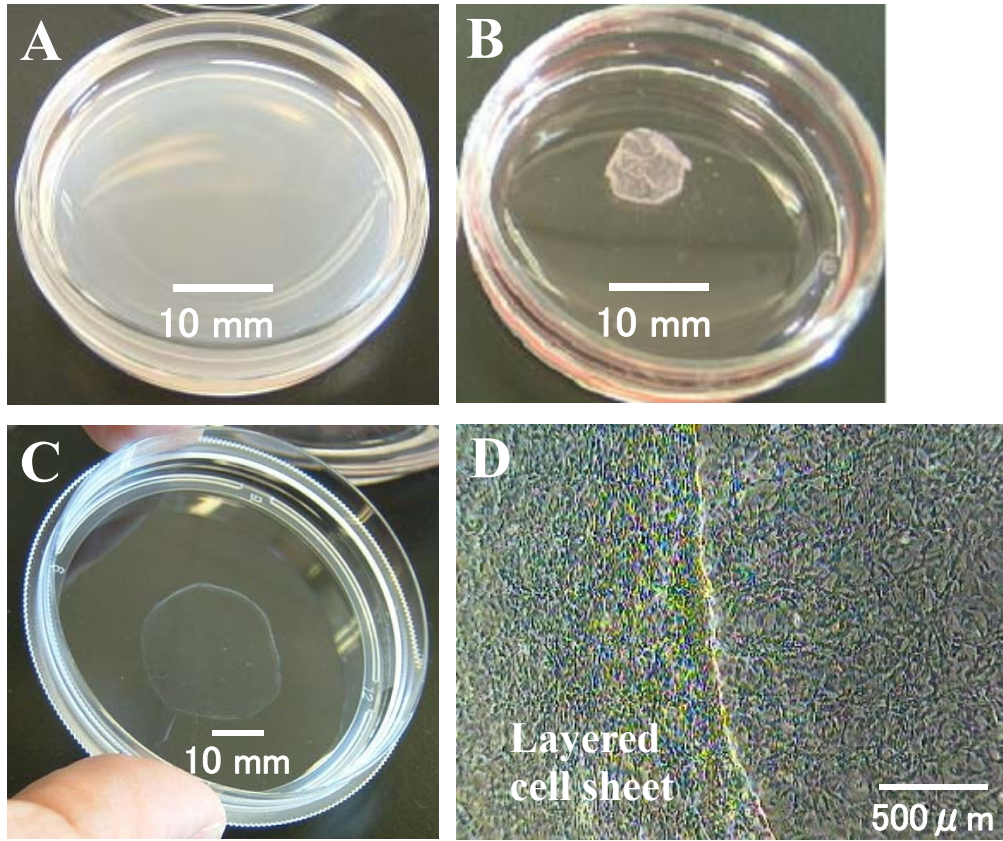


Fig.3

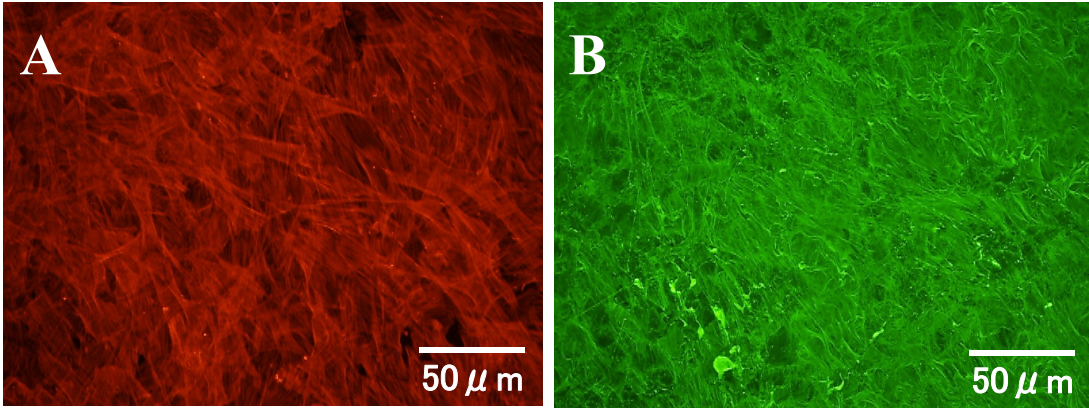


Fig.4

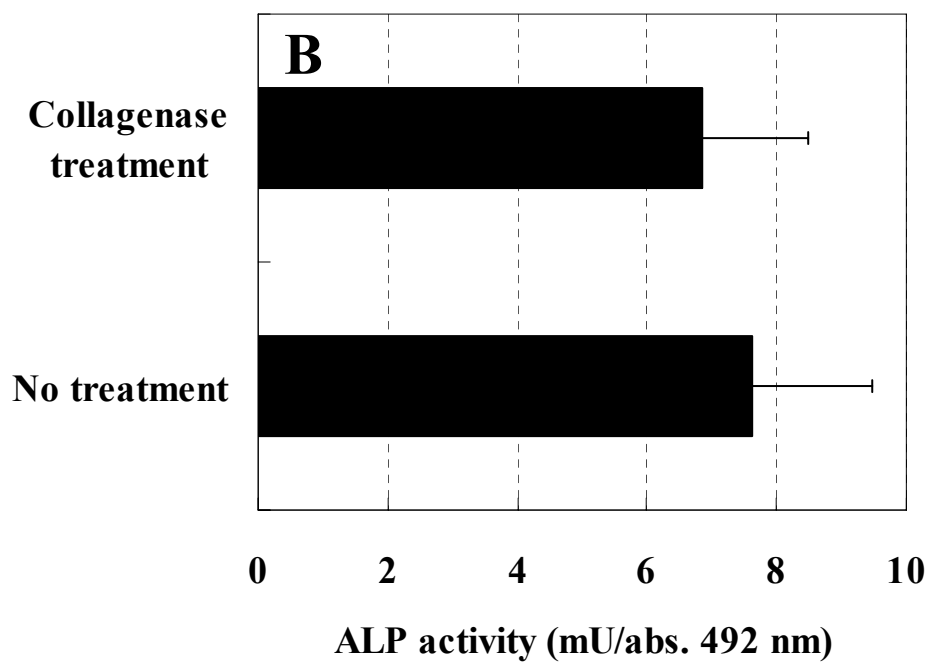
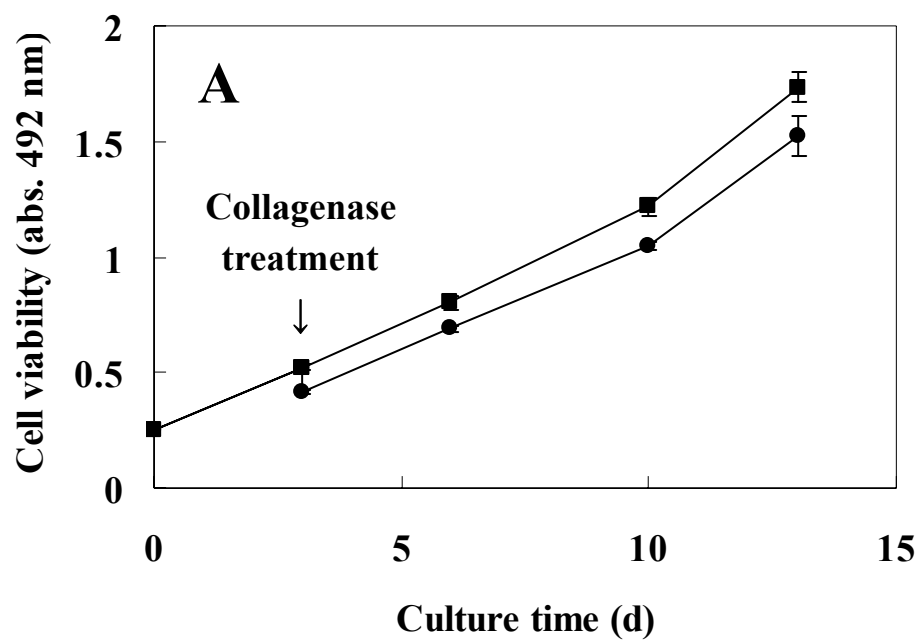


Fig.5