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ORIGINAL ARTICLE

Multiple-type dynamic culture of highly oriented fiber scaffold for ligament regeneration

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Abstract The ruptured anterior cruciate ligament does not heal spontaneously as it has a low capacity for healing. Therefore, the development of new healing techniques employing tissue engineering is vital. As a potentially new approach for ligament regeneration, this study used a highly oriented fiber scaffold made of elastin and collagen (the mean diameters were 1.7 ± 0.4 μm and 0.5 ± 1.4 μm, respectively), which comprise the extracellular matrix of the ligament. In addition, a multiple-type dynamic culture consisting of a combination of pressure and twist stimulation was performed to examine the influence of mechanical force on the functional maintenance of ligament cells and on the differentiation of ligament cells to osteoblast-like cells. Our results show that a pressure stimulation and elastin A upregulated the expression of alkaline phosphatase (ALP) (a marker of osteogenic differentiation) and promoted the osteogenic differentiation of ligament cells. In addition, the twist stimulation upregulated the expression of type III collagen (the main component of ligament tissue). Furthermore, the combination of pressure and twist stimulation promoted the expression of type III collagen and ALP protein depending on the portion of scaffold.

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Introduction

Ligaments are dense connective tissues that join bone to bone and play a role in stabilizing joints and enabling complex movement. In general, ligaments are elastic tissues that are composed of highly oriented collagen, elastin, and fibroblasts [1–5]. The anterior cruciate ligament (ACL) that joins the femur to the tibia is one of the most important ligaments in the knee joint. The ACL provides stability to the knee joint [6–8]. The ACL is easily damaged and it has a low capacity for healing because of its poor vascularization. Therefore, it will not heal itself when ruptured [6, 8–10]. Current surgical ACL reconstruction approaches are not ideal. The most commonly used method is patellar tendon autografting [6], which requires a long recovery time, often resulting in donor site morbidity, and does not necessarily prevent degenerative diseases such as osteoarthritis [6]. Tissue engineering is a widely known approach for the regeneration of functional tissues without the risks associated with current surgical reconstruction methods. Biocompatibility and re-absorbability are necessary factors in ligament tissue engineering. In addition, a scaffold that can withstand mechanical loads in the living body and provide a substrate for cell engraftment is vital. In this study, a highly oriented fiber scaffold made of elastin and collagen (ECM fiber scaffold) was prepared, and its availability was estimated.

Mechanical stress plays an important role in ligament homeostasis [3, 11, 12]. Many reports have examined the influence of mechanical stress such as cyclic stretch on the ligament cells and found that mechanical stress induces cell

proliferation, ECM synthesis, and cell differentiation [3, 4, 11–13]. Ligaments in physiological environments are subjected to mechanical stress, including stretch stimulation [3], torsion (twist) strain [12, 14], and compression stimulation [15]. In general, the length of the long axis of a human ACL is approximately 32 mm, and it has been reported that the ACL is damaged by a strain of approximately 6 % (2 mm) on the long axis [16]. Thus, the human ACL is not stretched more than 6 % in daily life, and stretch stimulation is not considered to be important for the ACL. Therefore, twist strain—for which there are few reported examples—was examined in this study. In addition, pressure stimulation, which influences osteogenic differentiation, was examined [13]. Ligaments join bone to bone; therefore, it is useful to mimic the interface structure between ligament and bone by differentiating ligament cells to osteoblasts.

The purpose of this study was to examine the influence of the ECM fiber scaffold and to investigate the effect of mechanical stresses (pressure, twisting, and a combination of pressure and twisting) on the phenotype of ligament cells.

Materials and methods

Materials

Preparation of insoluble and soluble elastin and type I collagen

Insoluble elastin was extracted from porcine arteries obtained from a slaughtering center (Matsusaka, Mie, Japan) using the method of Miyamoto et al. [5]. Soluble elastin was then prepared from the insoluble elastin by a previously described method [5]. Briefly, insoluble elastin was mixed with 0.25 M oxalic acid and maintained at 100 °C for 1 h. The resulting clear yellow solution was cooled at 4 °C and centrifuged at 2000×*g* for 30 min. The remaining insoluble fraction was mixed with 0.25 M oxalic acid and maintained at 100 °C for 1 h. These procedures were repeated eight times until complete dissolution occurred. The first and second fractions were classified as elastin C; the third, fourth, and fifth fractions were classified as elastin B; and the sixth fraction and beyond were classified as elastin A. Solutions of each of the three classes were dialyzed in deionized water for one week using a cellulose acetate tube (cut-off molecular weight of 12–16 kDa; Sanko Junyaku Co., Ltd., Tokyo, Japan) at 4 °C. The soluble elastin powders were obtained by freeze-drying. Among the extracted soluble elastins, elastin A [average molecular weight (Mn): 25.6 kDa] [17] was used in this study. Type I atelocollagen derived from porcine arteries was obtained from the ECM Laboratory Co. (Mie, Japan).

Preparation of ECM fiber scaffold by electrospinning

The original electrospinning device for the ECM (elastin or collagen) fiber scaffold was fabricated in this laboratory, as previously reported. The ECM fiber was prepared using a previously reported method [17]. Briefly, the ECM solution and crosslinker were mixed and placed in a plastic syringe attached to stainless steel needles with an inner diameter of 21 G (Terumo Corp., Tokyo, Japan). The flow rate of the ECM solution jet from the syringe was 1.2 mL/h with a voltage of 15 kV. The distance between the target electrode and the nozzle was 10 cm. After electrospinning, the obtained ECM fiber was heated for crosslinking at 121 °C for 20 min.

Dynamic culture

Cell culture and seeding

Normal human periodontal ligament fibroblasts (HPdLFs) and the stromal cell growth medium (SCGM) BulletKit were obtained from Lonza Walkersville Inc. (Walkersville, MD, USA). Subculturing of HPdLFs was performed according to the manufacturer's protocol. Periodontal ligament cells between passages 4 and 6 were used for seeding onto the ECM scaffold. The ECM scaffold (2 cm × 2.5 cm × 80 μm) was sterilized at 121 °C for 30 min and placed on a 35 mm cell culture dish (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). HPdLFs were seeded onto the ECM scaffold at a density of 5×10^4 cells/scaffold and incubated at 37 °C for 1 h in a humidified atmosphere of 5 % CO_2 and 95 % air. After 1 h, SCGM containing 5 % fetal bovine serum (FBS) was added to the culture dish, and the cells were incubated for one week.

Pressurized dynamic culture

The original device for the dynamic culture with water pressure was fabricated in this laboratory (Fig. 1a) and includes the solution-sending pump. The HPdLFs on the ECM scaffold were cultured in SCGM containing 5 % FBS for one week. After one week, the cells were washed with PBS, and the culture medium was replaced with Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich Co., St. Louis, MO, USA) containing 0.1 % FBS. The samples were placed on the device and subjected to a pressure of 0.8 MPa for 12 h. In the control group, the culture medium was replaced with DMEM containing 0.1 % FBS and cells were cultured in the static condition for 12 h. SCGM that contained 0.1 % basic fibroblast growth factor (bFGF) was used as the cell growth medium, and DMEM that did not contain bFGF was used to eliminate the effect of bFGF when the dynamic culture was applied.

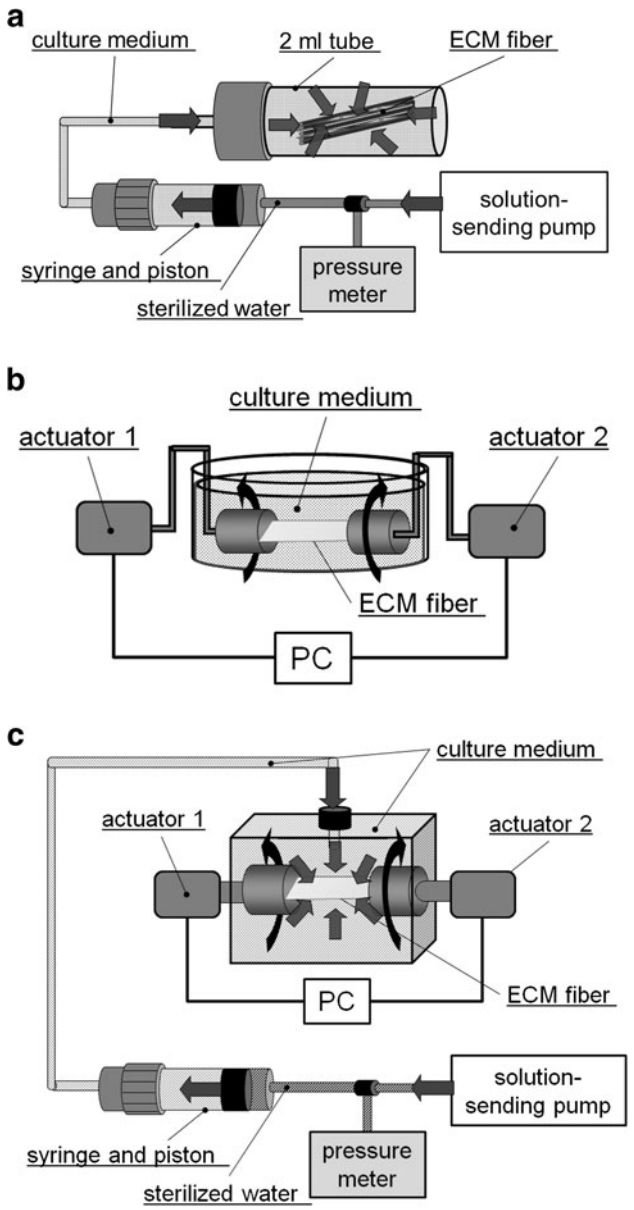

Fig. 1 Apparatus used for the dynamic cultures described in this study. **a** Apparatus used for pressurization dynamic culture. The syringe piston was moved by sending sterilized water from a solution-sending pump. At the same time, the other space filled with culture medium was pressurized by the syringe piston movement. The pressure level was maintained at 0.8 MPa by the pressure meter. **b** Apparatus used for twist-type dynamic culture. The ECM fiber scaffold was placed on the actuators (which were controlled by a computer). The actuators made a twist motion of 50° by leaning 25° to the right and left of each other. **c** Apparatus used for multiple-type dynamic culture. This device was a combination of the devices used for pressurization culture and twist-type culture

Twist-type dynamic culture

The original device for the twist-type dynamic culture was fabricated in this laboratory (Fig. 1b). This device included two computer-controlled actuators (Keyence, Osaka, Japan) to generate the twisting motion. The HPdLFs on the ECM scaffold were cultured in SCGM with 5 % FBS for one week. After one week, the cells were washed with PBS, and the culture medium was replaced with DMEM containing 0.1 % FBS. The samples were placed on the device and cultured for 12 h at a twist angle of 50° at 5 cycles per minute. In the control group, the culture medium was replaced with DMEM containing 0.1 % FBS and the cells were cultured in the static condition for 12 h.

Multiple-type dynamic culture

The original device for the multiple-type dynamic culture, which is a combination of pressurized and twist-type cultures, was fabricated in this laboratory (Fig. 1c). The HPdLFs on the ECM scaffold were cultured in SCGM with 5 % FBS for one week. After one week, the cells were washed with PBS, and the culture medium was replaced with DMEM containing 0.1 % FBS. The samples were placed on the device and cultured for 12 h at 0.8 MPa at a twist angle of 50° at 5 cycles per minute. In the control group, the culture medium was replaced with DMEM containing 0.1 % FBS and cells were cultured in the static condition for 12 h.

Assessment methods

Elastic modulus measurement

The elastic moduli of the bovine nuchal ligament, bovine tendon, and ECM fiber scaffold were measured. Bovine nuchal ligaments and tendons obtained from a slaughtering center were washed repeatedly in phosphate-buffered saline (PBS), and fat tissues were scraped off to leave the core portion, which was used in the measurement of the elastic modulus.

Tensile testing was performed using an original elastic modulus measurement device run by computer-controlled actuators. The elastic modulus was determined as follows:

$$\text{Elastic modulus (Pa)} = (F/A)/\{(L - Lo)/Lo\},$$

where F is the tension (N), A is the cross-sectional area (m^2) of the specimen, L is the length (m) of the specimen, and Lo is the initial length (m) of the specimen.

Scanning electron microscopy (SEM)

To prepare the samples for SEM observation, the ECM scaffolds were placed into 2.5 % glutaraldehyde and fixed for 2 h at 4 °C. After being washed with phosphate buffer, the samples were immersed in 1 % osmium tetroxide for 2 h at room temperature. The fixed samples were washed with phosphate buffer and dehydrated in a graded ethanol series. After dehydration, *t*-butyl alcohol was substituted

for ethanol, and the samples were dried in a freeze dryer (VFD-21S; Vacuum Device Inc., Ibaraki, Japan). After drying, the samples were coated with gold ions using an ion sputter (E-1010; Hitachi High-Technologies Corporation, Tokyo, Japan), and the coated samples were observed using SEM (S4000; Hitachi High-Technologies Corp.).

Immunofluorescence assay

HPdLFs were fixed in cold acetone:ethanol (1:1) for 20 min after being cultured in static conditions for one week or after the multiple-type dynamic culture. After washing, the cells were blocked with 1 % bovine serum blocking solution for 90 min and then incubated at 4 °C overnight with a 1:200 dilution of primary antibody [monoclonal mouse anti-human smooth muscle actin clone1A4 (Dako Japan Co., Ltd., Tokyo, Japan), affinity purified anti-collagen type III (Rockland Immunochemicals Inc. Gilbertsville, PA, USA), and monoclonal anti-human/mouse/rat alkaline phosphatase antibody (R&D Systems, Inc., Minneapolis, MN, USA)]. The cells were then incubated at room temperature for 90 min with a 1:100 dilution of the secondary antibody [DyLight™ 649-labeled antibody to rabbit IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD, USA) and DyLight™ 649-labeled antibody to mouse IgG (Kirkegaard and Perry Laboratories, Inc.)] in the dark room. Fluorescent-stained cells were observed with a confocal laser microscope (Fluoview FV 100; Olympus, Tokyo, Japan). The conditions of observation (e.g., laser strength, scan speed) were standardized for each protein to evaluate the strength of protein expression.

Alkaline phosphatase assay

Protein extracts of HPdLFs were prepared using 0.1 % Triton X-100/20 mM-HEPES–NaOH. The alkaline phosphatase (ALP) assay was performed with LabAssay™ ALP (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the manufacturer's protocol. The obtained data were normalized against the amount of protein measured by the bicinchoninic acid method. The ECM scaffold without seeded cells was prepared as a negative control.

Reverse transcription polymerase chain reaction (RT-PCR)

The expression of ALP, type I collagen, and type III collagen mRNA in HPdLFs was measured by RT-PCR, as described elsewhere [18]. The PCR protocol and primer oligonucleotide sequences are listed in Table 1. Agarose gel electrophoresis (3 %) was performed with one-fifth of the PCR product and analyzed by densitometry with the FAS III (Toyobo Co., Ltd., Tokyo, Japan). Density values for PCR products were normalized to the values for GAPDH. The resulting data were then normalized to the control (static) value and expressed as a ratio with respect to the control value.

Data analysis

All data are expressed as the mean value ± standard deviation (SD). For the analysis of covariance, SAS

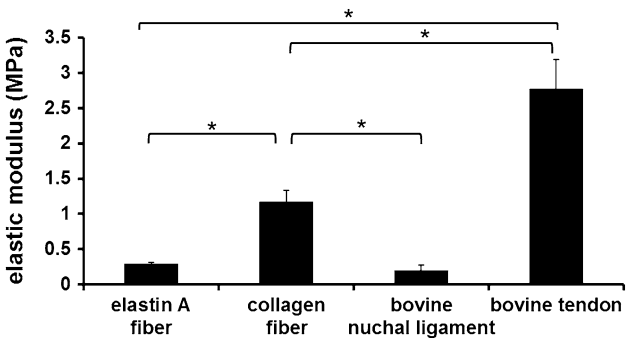

Fig. 2 Elastic modulus of the ECM fiber scaffold. The elastic modulus of the elastin A scaffold, which was similar to the value for a bovine nuchal ligament, was lower than that of the collagen scaffold (elastin A fiber, collagen fiber: $n = 4$; bovine nuchal ligament and bovine tendon: $n = 3$). $*p < 0.05$

Table 1 RT-PCR primers for gene expression analysis

Gene	Sequence (5′–3′)		Size (bp)	Annealing temperature (°C)	Amplification cycle number
GAPDH	Sense	GAT GTC ATC ATA TTT GGC AGG TT	322	60	27
	Antisense	CCT GCA CCA CCA ACT GCT TAG CCC			
Alp	Sense	TGG AGC TTC AGA AGC TCA ACA CCA	454	60	32
	Antisense	ATC TCG TTG TCT GAG TAC CAG TCC			
Type I collagen	Sense	TTT GTG GAC CTC CGG CTC	244	60	28
	Antisense	AAG CAG AGC ACT CGC CCT			
Type III collagen	Sense	AAC GGT CTC AGT GGA GAA CG	869	60	28
	Antisense	CCA CTC TTG AGT TCA GGA TGG			

software for statistical analysis (StatView version 5.0; SAS Institute Inc., Cary, NC, USA) was used. Dunnett's test and the Tukey–Kramer test were used. $p < 0.05$ indicated statistical significance.

Results

Elastic modulus of the ECM fiber scaffold

The measurement of the elastic modulus of the ECM scaffold by tensile testing indicated that the elastic modulus of the elastin A scaffold (0.3 MPa), which was similar to that of the bovine nuchal ligament (0.2 MPa), was lower than that of the collagen scaffold (1.2 MPa) (Fig. 2).

Observation of the HPdLFs on the ECM fiber scaffold

Under dry conditions, the mean diameters of the elastin A and collagen scaffolds were 1.7 ± 0.4 and 0.5 ± 1.4 μm, respectively (Fig. 3a, d). Observation by SEM and immunofluorescence staining indicated that cells adhered to elastin A (Fig. 3a) and collagen (Fig. 3d) fibers. The cells stretched along the direction of the ECM scaffold (Fig. 3b, c, e, and f).

Effect of pressurized dynamic culture

Measurement of ALP activity after pressurized dynamic culture indicated that the ALP activity of the cells on the ECM scaffold was approximately 1.6 times higher than that of cells maintained in static culture conditions (Fig. 4a). The highest ALP activity was observed in cells on the elastin A scaffold compared with the collagen scaffold.

In addition, ALP mRNA expression in cells with applied pressure stimulation was approximately 1.2 times higher than that in the cells in the static condition (Fig. 4b). In contrast, the expression level of types I and III collagen mRNA in the cells with applied pressure stimulation was similar to the expression level in cells in the static condition (Fig. 4c, d).

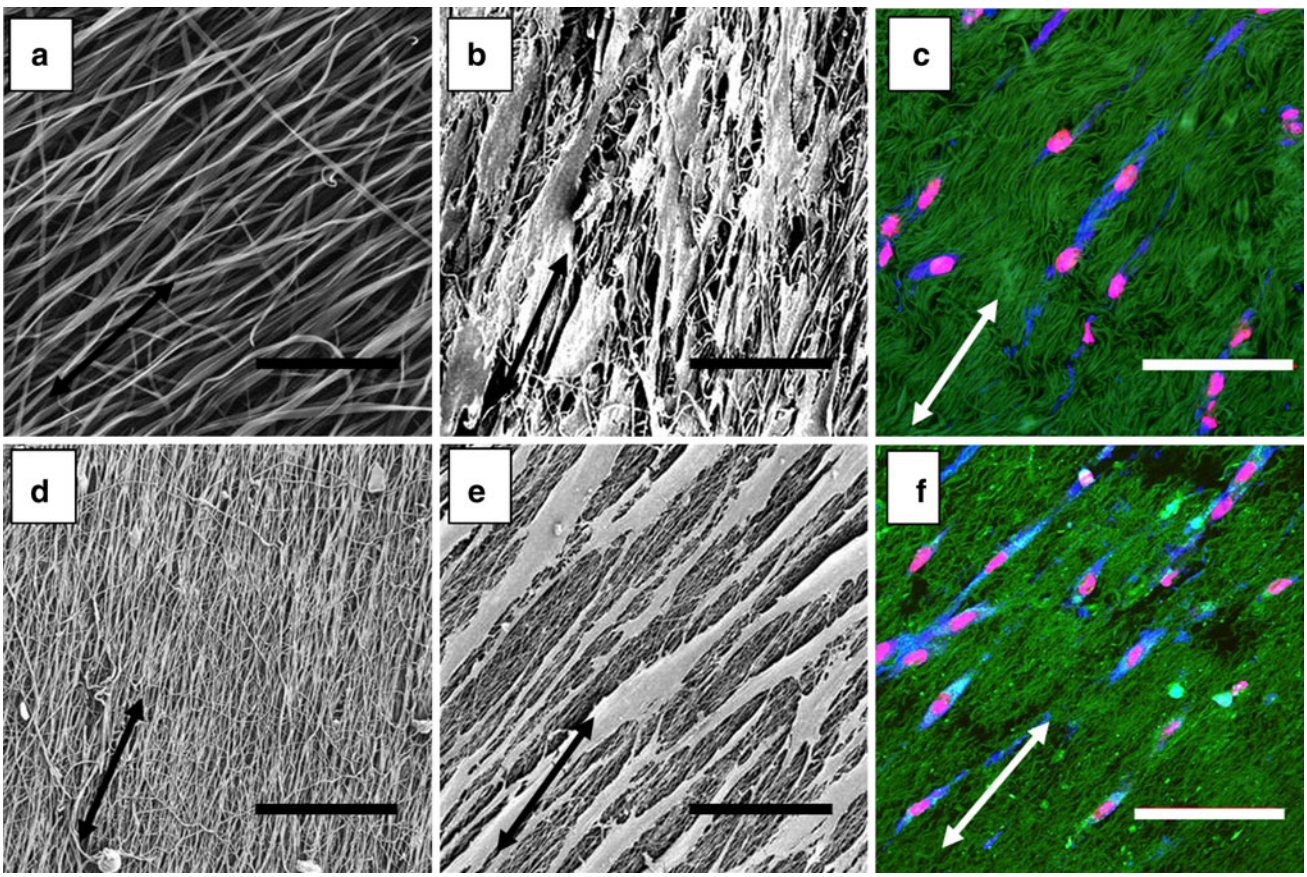

Fig. 3 SEM and immunofluorescence staining images. **a** Elastin A fiber scaffold (SEM, ×200). **b** HPdLFs on the elastin A scaffold (SEM, ×200). **c** HPdLFs on the elastin A scaffold (immunofluorescence staining, ×400). **d** Collagen fiber scaffold (SEM, ×200). **e** HPdLFs on the collagen scaffold (SEM, ×200). **f** HPdLFs on the collagen scaffold (immunofluorescence staining, ×400). In **c** and **f**, the *red*, *blue*, and *green fluorescence* show the cell nucleus, smooth muscle actin, and autofluorescence of the ECM scaffold, respectively. The *double-headed arrow* indicates the direction of the oriented fiber scaffold. *Scale bar* 90 μm (**a**, **b**, **d**, **e**), 100 μm (**c**, **f**)

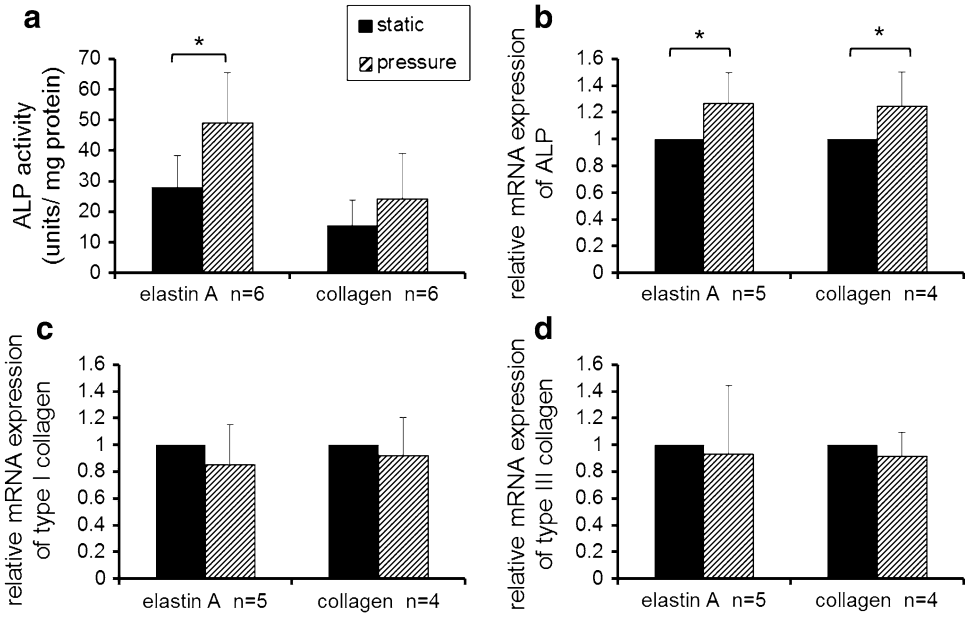

Fig. 4 Effect of pressurized stimulation on ALP activity and mRNA expression. **a** ALP activity. ALP activity was increased by pressurized stimulation ($n = 6$). **b** ALP mRNA expression. **c** Type I collagen mRNA expression. **d** Type III collagen mRNA expression. ALP mRNA expression of HPdLFs in pressurized culture was higher than that of cells in static culture (elastin A: $n = 5$; collagen: $n = 4$). The data are expressed as a ratio to the control (static) value. $*p < 0.05$

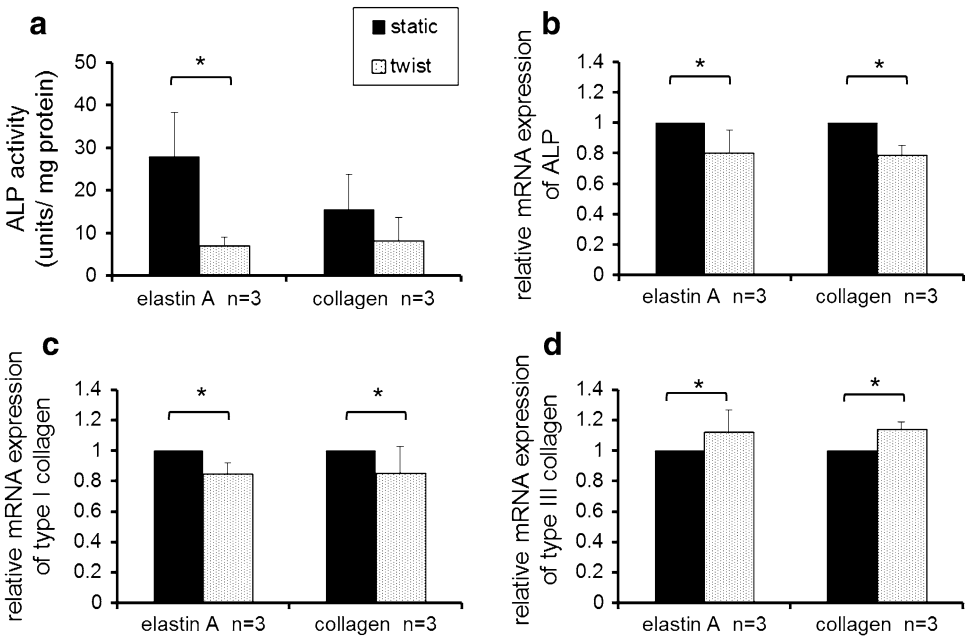

Fig. 5 Effect of twist-type stimulation on ALP activity and mRNA expression. **a** ALP activity. ALP activity was decreased by twist stimulation ($n = 3$). **b** ALP mRNA expression. **c** Type I collagen mRNA expression. **d** Type III collagen mRNA expression. ALP mRNA and type I collagen mRNA expression in the HPdLFs in twist culture were lower than that of the cells in static culture. However, type III collagen mRNA expression was increased by twist stimulation (vs. static culture, $n = 3$). The data are expressed as a ratio to the control (static) value. $*p < 0.05$

Effect of twist-type dynamic culture

Measurement of the ALP activity after twist-type dynamic culture indicated that the ALP activity of cells on elastin A and collagen scaffolds decreased by approximately 75 and 50 %, respectively, compared with the activity of cells in the static culture condition (Fig. 5a). Furthermore, ALP mRNA expression in cells on the ECM scaffold was decreased by approximately 20 % (Fig. 5b). Although type I collagen mRNA decreased in cells by approximately 20 % compared with the cells in the static condition, type III collagen mRNA expression increased by approximately 10 % in the cells following twist stimulation (Fig. 5c, d).

Effect of multiple-type dynamic culture

The expression levels of ALP and types I and III collagen mRNA were similar in cells that had undergone simultaneous twisting (50°) and pressure stimulation (0.8 MPa) as the cells were maintained in static conditions (Fig. 6).

Immunofluorescence staining indicated that ALP protein expression levels differed in the cells under static culture conditions on the elastin A scaffold and on the collagen scaffold (Fig. 7); cells on the elastin A scaffold showed ALP protein expression (Fig. 7a1–3), whereas the cells on the collagen scaffold had negligible ALP protein expression (Fig. 7c1–3). When twist and pressure were applied as multiple-type dynamic culture, the expression levels of ALP and type III collagen differed between the center and edge portions of the ECM scaffold (ALP: Fig. 7a4–6, c4–6; type III collagen: Fig. 7b4–6, d4–6). The expression of ALP protein in the cells at the center portion was low (Fig. 7a5, c5), whereas that of type III collagen protein was high (Fig. 7b5, d5). In contrast, the expression of ALP protein in cells at the edge portion was high, whereas that of type III collagen protein was low. These results were observed in cells on both elastin A and collagen scaffolds.

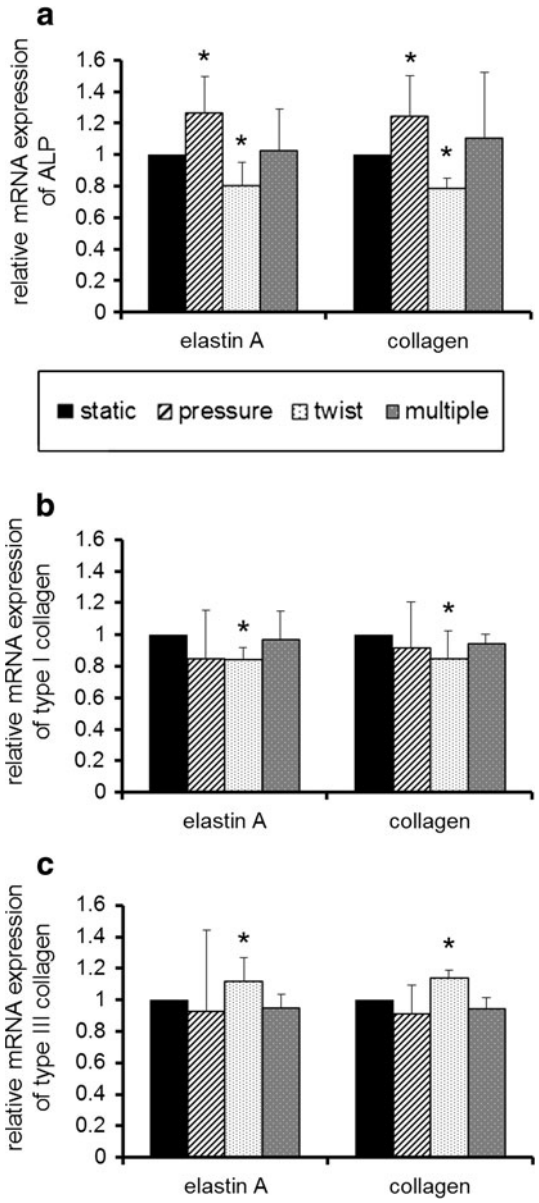

Fig. 6 Effect of multiple-type stimulation on ALP activity and mRNA expression. **a** ALP mRNA expression. **b** Type I collagen mRNA expression. **c** Type III collagen mRNA expression. The expression level of each mRNA in HPdLFs in multiple-type stimulation was at the same level as that of cells in static culture (elastin A: $n = 6$; collagen: $n = 3$). The data are expressed as a ratio to the control (static) value. $^*p < 0.05$

Discussion

Characterization of HPdLFs

In this study, HPdLFs were used as a ligament cell line. The expression of surface antigens on the HPdLFs is similar to that of ACL cells (positive: CD105, CD166, CD146, CD29, and CD44; negative: CD31, CD34, and CD45) [19–22]. In addition, many reports have suggested that HPdLFs can differentiate to form cells such as osteoblasts [13, 23, 24]. Therefore, the results of this study using HPdLFs would be generalized to ACL cells.

Elastic modulus of the elastin A and collagen scaffold

As shown in Fig. 2, the elastic modulus of the elastin A scaffold was comparable to that of the bovine nuchal ligament (0.2 MPa), which is possible because the nuchal ligament is mainly composed of elastin [25]. Because of their high initial strength, tendon autografts are used in current surgical ACL reconstruction. As the elastic moduli of the collagen scaffold (1.2 MPa) and the elastin A scaffold (0.3 MPa) were lower than that of bovine tendons (2.8 MPa), these ECM scaffolds are an incomplete substitute for ACL replacement. Although the strength of the elastin A scaffold is thought to be enhanced by combining it with the collagen scaffold prepared in this study, a higher initial strength is the main issue for future applications.

ALP expression on the ECM scaffold under the static condition

ALP activity and ALP mRNA expression were measured to evaluate the osteogenic differentiation in this study. It is well known that ALP is a typical osteoblast marker and a good marker for osteogenic differentiation [26]. Because it has been reported that HPdLFs express ALP in the process of osteogenic differentiation [13], the measurement of ALP expression as a marker of osteogenic differentiation is appropriate. The ALP activity of HPdLFs cultured for one week on the elastin A scaffold was significantly higher (27 U/mg protein) than that of the cells on the collagen scaffold (15 U/mg protein) (Fig. 4a). A similar tendency

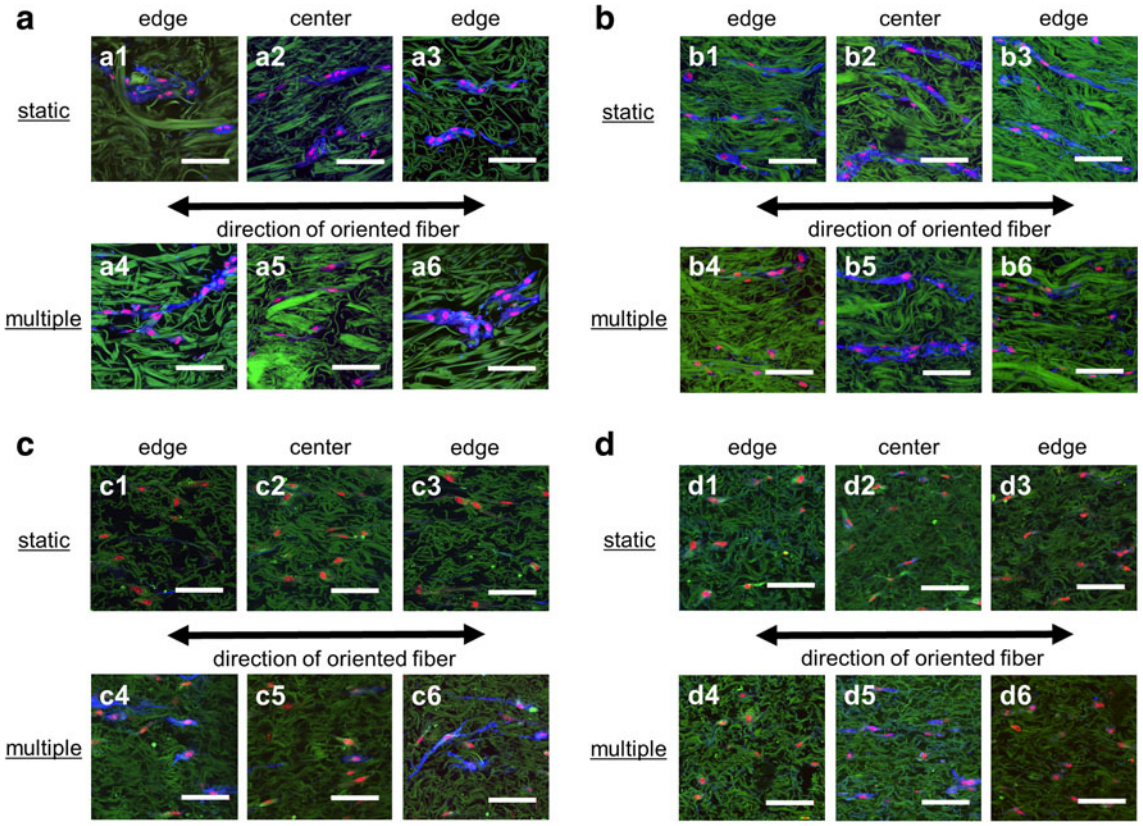

Fig. 7 Immunofluorescence staining after the multiple-type dynamic culture. **a** ALP staining of the HPdLFs on the elastin A scaffold. ALP expression of HPdLFs was observed in static culture (**a1**–**3**). Although ALP expression was observed in the cells in multiple-type dynamic culture, there was a difference in its expression level between cells in the center of the scaffold and at the edge of the scaffold (**a4**–**6**). The expression level in the cells at the edge of the scaffold (**a4**, **6**) was higher than that in the cells in the center of the scaffold (**a5**). The expression level in the cells at the edge portion (**a4**, **6**) was similar to that in the cells in static culture (**a1**–**3**). **b** Type III collagen staining of HPdLFs on the elastin A scaffold. Type III collagen expression was observed in cells both in static culture (**b1**–**3**) and in multiple-type culture (**b4**–**6**). In multiple-type culture, the expression level of the cells on the center portion (**b5**) was higher than that of the cells on the edge portion (**b4**, **6**). **c** ALP staining of HPdLFs on the collagen scaffold. Although a high level of ALP expression was not observed in cells in static culture (**c1**–**3**), high expression was observed in cells from the multiple-type culture, particularly in the cells on the edge of the scaffold (**c4**, **6**). **d** Type III collagen staining of HPdLFs on the collagen scaffold. Type III collagen expression was undetectable in cells in static culture (**d1**–**3**). Cells in multiple-type culture showed high type III collagen expression in the center region of the scaffold (**d5**) and lower expression at the edge portion of the scaffold (**d4**, **6**). The *red*, *blue*, and *green fluorescence* show the cell nucleus, target protein, and the autofluorescence of the ECM scaffold, respectively. *Double-headed arrows* show the oriented direction of the ECM fiber scaffold. *Scale bar* 100 μm

was observed for the mRNA expression of ALP. Thus, elastin A is more likely than collagen to induce the differentiation of the HPdLFs to osteoblast-like cells. Under the static condition, it can be speculated that the observed difference in the response of HPdLFs between the elastin A scaffold and the collagen scaffold may be the result of binding to different receptors such as elastin binding protein [27, 28] in the ligament cells. Further study is needed to understand this difference.

Matrix expressions on the ECM scaffold under the mechanical stress

Mechanical stress (pressure stimulation, twist-type stimulation, and multiple-type stimulation) was applied to the ligament cells on the ECM scaffold to mimic the mechanical stress applied to the ACL. The following conditions were examined: stimulation time of 12 h, pressure of 0.8 MPa, and a twist angle of 50°.

Pressure stimulation

It has been reported that the expression of osteogenic genes (e.g., ALP and osteopontin) increases when pressure stimulation is applied to HPdLFs [13]. In agreement with the previous findings, in the present study, ALP activity and ALP mRNA expression increased when the pressure stimulation was applied to HPdLFs on the ECM scaffold (Fig. 4a, b). Therefore, pressure stimulation would promote the osteogenic differentiation of ligament cells.

Twist-type stimulation

In the twist dynamic culture, both edges of the ECM scaffold were fixed on the twist device (Fig. 1b). A twisting motion of 50° was then applied to the ECM scaffold by rotating both edges 25° in the opposite direction to each other. According to our estimation, the tensile strain gradually increases from the axis of rotation to the outer edge. Furthermore, in addition to the tensile strain, the fluid shear stress increases from the center line, which is in the vertical direction of the axis of rotation, to the fixed edge portions. Thus, the cells on the ECM scaffold would sense different mechanical strains depending on which portion of the scaffold (center or edges) they are on.

When the twist stimulation was applied to HPdLFs on the ECM scaffold, the mRNA expression of type III collagen—which is the main component of ligament tissue—was increased (Fig. 5d), and the expression of ALP—which is an osteogenic induction marker—was reduced (Fig. 5a, b). The twist stimulation decreased the ALP expression, which agrees with the findings of van der Pauw et al. [29] and Yamaguchi et al. [30] regarding HPdLF exposed to fluid shear stress and cyclic tension forces, respectively.

We found that type I collagen expression differed upon twist stimulation and stretch stimulation. Tetsunaga et al. [3] and Kim et al. [11] demonstrated that both type I and III collagen expression of ACL cells was induced by stretch stimulation, but our twist stimulation induced only type III collagen. This suggests that mechanisms governing matrix synthesis may differ according to the type of mechanical stimulation. Further investigation of these mechanisms is needed.

Multiple-type stimulation

To mimic the physiological environment, it is not sufficient to examine only a single stimulation type. Therefore, a multiple-type dynamic culture composed of a combination of pressure and twist stimulation was used. The results for the multiple-type dynamic culture were different from those of the single stimulation (Fig. 6). The mRNA expression of ALP, type I, and type III collagen in the cells from the multiple-type culture was statistically similar to that in the cells in the static culture. However, it is possible that the effects of twist stimulation and pressure stimulation may cancel each other out. To confirm this speculation, immunofluorescence staining was performed on the cells after the multiple-type dynamic culture. As shown in Fig. 7, the expression level of type III collagen is increased at the center portion, while its expression level is decreased at the edge portions. Just the opposite was observed for ALP expression. It seems that the expression levels of these proteins completely cancel each other out. As a result, the mRNA expression in the cells in the multiple-type culture may be similar to that in the cells in the static culture. From these results, according to the multiple-type dynamic culture, the phenotype of the ligament cells would be affected by their localization in the scaffold. However, as many of the biomolecular mechanisms driving these cellular responses are not known, future studies should focus on determining these mechanisms.

Conclusion

From our results, it is suggested that the elastin A scaffold upregulates the expression of ALP and induces the osteogenic differentiation of ligament cells. In addition, the application of multiple-type dynamic culture promotes the expression of type III collagen (which is the main component of the ligaments) at the center portion of the scaffold and the expression of ALP (an osteogenic marker) at the edge portions. Thus, it is expected that the ECM scaffold and the mechanical stimuli presented in this study would be useful for promoting phenotypical alteration of the ligament cells.

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