New Scaffolds for Tissue Engineering using Adipose-Derived Stem Cells

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Adipose-derived stem cells (ADSCs) have attracted attention as a useful cell source for tissue engineering, even in the clinical setting. However, it is difficult to regenerate tissues with complex structures in the absence of a three-dimensional matrix, such as an artificial scaffold. Namely, scaffolds are required to allow implanted ADSCs to regenerate tissues with three-dimensional structures. Although non-woven PGA (poly (glycolic acid)) fabric has been used widely as an artificial scaffold, ADSCs poorly adhere to and grow on PGA fabric due to its hydrophobicity. To improve cell adhesion and growth on PGA fibers, we devised a PGA fabric coated with collagen.

We isolated ADSCs from the inguinal adipose tissue of a rat and characterized the cells using flow cytometry with both CD29- and CD90-positive markers and CD11b- and CD45-negative markers.

Our cell growth examination revealed that ADSC growth was improved on all of the collagen-coated fabrics compared with that observed on the corresponding non-coated fabrics. This may be because ADSCs are able to adhere and grow more readily on the hydrophilic surface of collagen-coated PGA fabrics.

Key words：ADSCs, non-woven PGA fabric, collagen-coating, scaffold, change to hydrophilicity

1. Introduction

Adipose-derived stem cells (ADSCs), as well as bone marrow stem cells, have been reported to exhibit pluripotency to differentiate into mesenchymal cells. Since a large amount of ADSCs can be collected more readily than bone marrow stem cells, ADSCs are currently considered to be a more useful source of stem cells for tissue engineering[1-3]. Therefore, ADSCs have attracted attention as a cell source, one of the three major important components of tissue engineering. In addition, ADSCs have been reported to secrete various growth factors[4,5], another major important component of tissue engineering. Thus, ADSCs act as both a cell source and a supplier of growth factors, two of the three major important components of tissue engineering. For this reason, new trials implanting ADSCs have been recently performed[6,7]. However, it is difficult to regenerate tissues with complex structures in the absence of a three-

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dimensional matrix, such as an artificial scaffold[8, 9].

To address this problem, it is necessary to supply an appropriate scaffold, the third important element of tissue engineering. Appropriate scaffolds should be flexible enough to be easily formed into complex structures and durable enough to maintain their structure and strength for the required amount of time. These cells can more easily adhere to and grow on the scaffold if a scaffold has been provided[10]. Namely, an appropriate scaffold with these characteristics can induce ADSCs to regenerate more complex and stable tissue.

Non-woven PGA (poly (glycolic acid)) fabric has been widely used as an artificial scaffold[11] because PGA fabric can be easily formed into complex shapes and maintains its shape and strength for an adequately long time, as compared with natural scaffolds such as collagen[12]. However, PGA fabric exhibits a lower capacity for cell adhesion than natural scaffolds due to its hydrophobicity[12]. To improve cell adhesion and cell growth on PGA fibers, we newly developed a PGA fabric coated with collagen. The present paper reports that a new scaffold has been developed using a collagen-coated PGA fabric that improves adhesion and growth of ADSCs *in vitro*.

2. Materials and Methods

2.1 *Materials*

In this study, we used four kinds of non-woven PGA fabric (NEOVEIL, fabric-5, fabric-2 and fabric-0.9) without a collagen coating and four other kinds of non-woven PGA fabric (c-NEOVEIL, c-fabric-5, c-fabric-2 and c-fabric-0.9) with a collagen coating. Fabric-5, fabric-2 and fabric-0.9 were composed of fibers with a mean diameter of 5, 2 and 0.9 μm, respectively.

2.1.1 *Preparation of non-woven PGA fabrics*

NEOVEIL purchased from GUNZE Ltd. (Kyoto, Japan) was created using a needle punch method[13]. The needle punch method is a two-step process. PGA resin was extruded into the form of fiber, which was then stretched and crimped. The PGA fibers were carded, needle punched, then thermally interlocked.

Fabric-5 and fabric-2 were created using the melt-blowing method[14]. PGA polymers were extruded through dies with small nozzles, attenuated with heat, and high-velocity air streams and spun into fibers. The fibers were then deposited on to a collector in a random manner and to form a non-woven fabric.

Fabric-0.9 was created using the electrospinning method[15]. PGA was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Sigma Chemical Co.). For the electrospinning process, the polymer solution was placed in a syringe fitted with a needle. Voltage was provided by a high voltage power supply employed at a distance of approximately 30 cm between the grounded target (cathode) and the needle tip (anode). The polymer solution was drawn from the syringe, forming a pendant drop at the tip of the needle by combining the force of gravity and an electrostatic charge. A positively charged jet ejected from the drop was splayed onto the negatively charged target. A structure was formed on the collecting plate and then carefully removed for subsequent use.

2.1.2 *Collagen-coated non-woven PGA fabrics*

The collagen-coated non-woven PGA fabrics were created as follows. NEOVEIL, fabric-5, fabric-2 and fabric-0.9 were dipped in a 5mg/ml collagen solution for one hour, removed from the solution and air dried overnight. Whether the fabrics were coated successfully with collagen was confirmed with the ninhydrin color reaction[15]. Finally, the materials were treated in a vacuum oven (4VO-250N, As One, Osaka, Japan) at 140 ℃ for 12 hours so that the collagen became thermally cross linked. Therefore, four kinds of collagen-coated non-woven PGA fabrics (c-NEOVEIL, c-fabric-5, c-fabric-2 and c-fabric-0.9) were created.

2.1.3 *The Fiber diameter and spacing size of non-woven PGA fabrics*

The non-woven PGA fabrics were analyzed

morphologically using a scanning electron microscopy (SEM) and a Poromer 3G. The diameter of the fiber (fiber diameter) was determined using SEM by measuring 100 fibers per sample at random. The distance from the fiber-edges to the edges between neighboring fibers on the same plain was defined as the size of the fiber spacing (spacing size). The spacing size was determined using Porometer 3G (BEL Japan, lnc., Osaka, Japan). For the collagen-coated PGA fibers, the state of collagen coating was also observed using SEM.

2.2 *Preparation of ADSCs*

2.2.1 *Isolation of ADSCs from rat adipose tissue*

The animal experiments were approved by the Doshisha University Animal Experimentation Committee. All surgical and anesthetic procedures were performed in accordance with the animal care guidelines of Doshisha University.

In the present animal experiment, a male rat (purchased from SHIMIZU Laboratory Supplies Co., Ltd., Kyoto, Japan; F344 strain, five weeks of age, 200 g in body weight) was used. The rat was maintained under specific pathogen-free conditions at a room temperature of 19-22 ℃ with free access to water and standard pellets. The animal was housed in the laboratory for one week before the experiment.

The rat was given Isoflurane (Escain®, Mairan Seiyaku, Inc., Osaka, Japan) inhalation as a basic anesthesia. Sodium pentobarbital (Somnopentyl, Kyoritsu Seiyaku, Inc., Tokyo, Japan) at a dose of 6.48 mg diluted in 1 ml of saline solution was administered intraperitoneally to the rat with a tuberculin syringe and a 23 G injection needle.

Under the above-mentioned anesthesia, adipose tissue was removed from the inguinal region of the rat. The adipose tissue was washed with phosphate-buffered saline (PBS, PH 7.4) to remove blood. Then, the adipose tissue was finely minced using sterilized surgical scissors. The minced adipose tissue was placed into Dulbecco's modified Eagle's medium (D-MEM) with 0.15% type-I collagenase. D-MEM containing the minced adipose tissue was kept at 37 ℃ for 45 minutes, then vigorously agitated with a vortex. It was incubated for 15 minutes at 37 ℃ with 5% CO_2. Next, D-MEM containing 10% fetal bovine serum (FBS) was added to the D-MEM containing the minced adipose tissue in order to neutralize enzymatic activity. The sample was filtered through a 40 μm mesh filter to remove tissue debris. Finally, the remnant cells, in which so-called ADSCs were contained, were collected via centrifugation at 1,200 rpm for five minutes. The ADSCs were cultured at 37 ℃ with 5% CO_2 under humid conditions in D-MEM containing 10% FBS and 1% L-glutamine.

The ADSCs were washed with PBS three times and detached from the dish wall with trypsin EDTA (0.05 w/v% Tripsin-0.53 mmol/l EDTA・4Na Solution with Phenol Red) for preparation in a single cell suspension of D-MEM containing 10% FBS and L-glutamine.

2.2.2 *Characterization using flow cytometry*

ADSCs were cultured for a total of 10 passages and used to determine the phenotype characterization using flow cytometry. Many ADSCs are considered to be CD29- and CD90-positive[17, 18]. However, fractions of CD29- and CD90-positive cells contain granulocytes and monocytes, including macrophages and lymphocytes[19-21], in which CD11b and CD45 are positive[22, 23]. Therefore, we characterized the ADSCs using CD29 and CD90 positivity and CD11b and CD45 negativity. The cells were labeled with each of the following antibodies: CD11b-FITC, CD29-FITC, CD45-FITC and CD90-FITC (CD29, CD45 and CD90 were purchased from Thermo Fisher Scientific Inc., CD11b was purchased from AbD Serotec, OX5 1GE, UK). 5×10^5cells were incubated in 100 μl of PBS with fluorescent-labeled monoclonal antibodies diluted to 1/10 at 25 ℃ for 15 minutes in the dark. The labeled cells were analyzed by flow cytometry using BD FACSAria™II equipped with the BD FACSDiva™ software program. We defined a luminescence of 900 or

more cells as positive and a luminescence of less than 900 cells as negative.

2.3 *Cell growth examination*

For the cell growth examination, non-woven PGA fabrics (NEOVEIL, fabric-5, fabric-2 and fabric-0.9) and collagen-coated non-woven PGA fabrics (c-NEOVEIL, c-fabric-5, c-fabric-2 and c-fabric-0.9) were cut into circle shaped sheets with a diameter of 15 mm. To make the weights of the fabric sheets applied in the cell growth examination uniform, fabric-2 and c-fabric-2 were formed into two-layer piled sheets and fabric-0.9 and c-fabric-0.9 were formed into four-layer piled sheets. The remaining kinds of fabrics were formed into single layer sheets. After being sterilized by dipping in 99.5% ethanol for 30 seconds and washing twice with PBS, each sheet was placed on the bottom of a 24 well culture plate for the following experiments. As a control, a 24 well culture plate without any sheets was used.

ADSCs retrieved in the form of a single cell-suspension were prepared in a concentration of 5×10^3 cells/ml of D-MEM containing 10% FBS and 1% L-glutamine. This suspension was poured at a concentration of 750 µl/well so that 3.75×10^3cells/well were seeded onto the sheet. Then, the cells were cultured with regular replacement of the culture medium every two days. The sheets seeded with ADSCs were removed from the culture wells one day after seeding and gently rinsed with PBS to remove any non-adherent cells. The ADSC-seeded sheets were transferred into new 24 well culture plates. The number of viable cells on each sheet was counted at four time points: one, three, five and seven days after seeding using the ATPlite assay[24] with an ATPlite Kit (Perkin Elmer, Inc., Waltham, MA 02451, USA) and its calibration curve. Three wells were used to determine the cell number for each kind of fabric at each time point. The cell growth curve for each type of PGA fabric was drawn based on the cell-count number.

3. Results

3.1 *The fiber diameter and spacing size of the non-woven PGA fabrics*

The fiber diameter of the non-woven PGA fabrics are summarized in Table 1. NEOVEIL has a 15.5 µm median fiber diameter and 26.2 µm spacing size. Fabric-5 has a 4.9 µm median fiber diameter and 16.5 µm spacing size. Fabric-2 has a 2.0 µm median fiber diameter and 10.5 µm spacing size. Fabric-0.9 has a 0.8 µm median fiber diameter and 3.0 µm spacing size. c-NEOVEIL has a 15.8 µm median fiber diameter. c-Fabric-5 has a 4.7 µm median fiber diameter. c-Fabric-2 has a 1.9 µm median fiber diameter. c-Fabric-0.9 has a 1.0 µm median fiber diameter (Table 1).

SEM photomicrographs are shown in Fig. 1. The SEM pictures of the collagen-coated fabrics revealed that the collagen not only coated the surface of the individual fibers, but also formed a film-shaped membrane between the neighboring fibers in some place. The fiber diameter and fiber spacing distribution are shown Fig. 2 and 3.

3.2 *Characterization determined using flow cytometry*

The immunological characterization revealed that more than 98.6% of the cells obtained as the fraction containing ADSCs were positive for CD29 and CD90, while more than 99.6% of the cell were negative for CD11b and CD45 (Fig. 4).

		NEOVEIL	fabric-5	fabric-2	fabric-0.9
PGA fabrics	fiber diameter (µm)	15.5	4.9	2.0	0.8
	fiber spacing (µm)	26.2	16.9	10.5	3.0
collagen-coated PGA fabrics	fiber diameter (µm)	15.8	4.7	1.9	1.0

Table 1. The median fiber diameter and the fiber spacing.

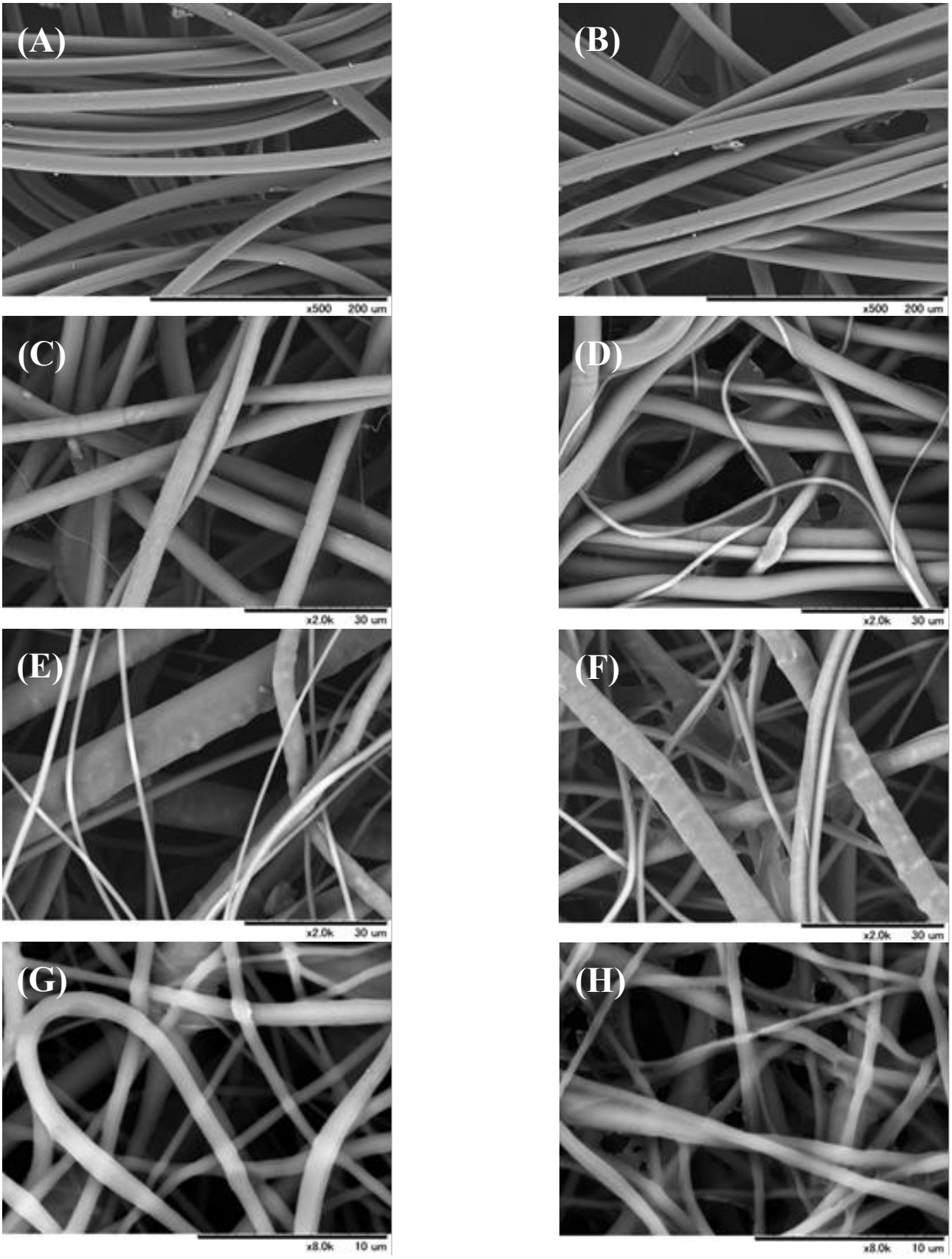

Fig.1. The SEM photomicrographs of the non-woven fabrics.

(A) NEOVEIL, (B)c-NEOVEIL, (C)fabric-5, (D)c-fabric-5, (E)fabric-2, (F)c-fabric-2, (G)fabric-0.9, (H)c-fabric-0.9

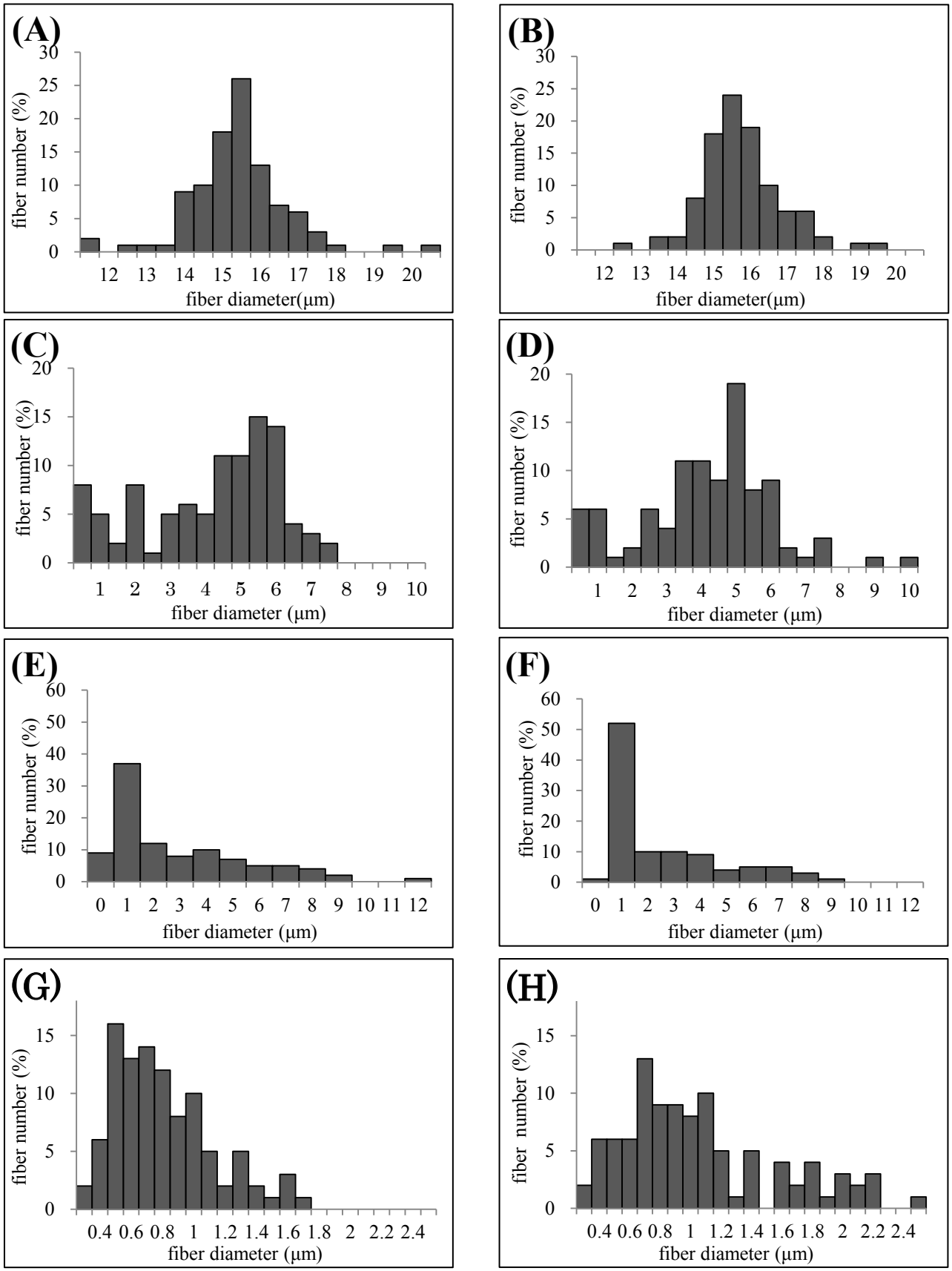

Fig.2. The fiber diameter distribution.

(A)NEOVEIL, (B)c-NEOVEIL, (C)fabric-5, (D)c-fabric-5, (E)fabric-2, (F)c-fabric-2, (G)fabric-0.9, (H)c-fabric-0.9

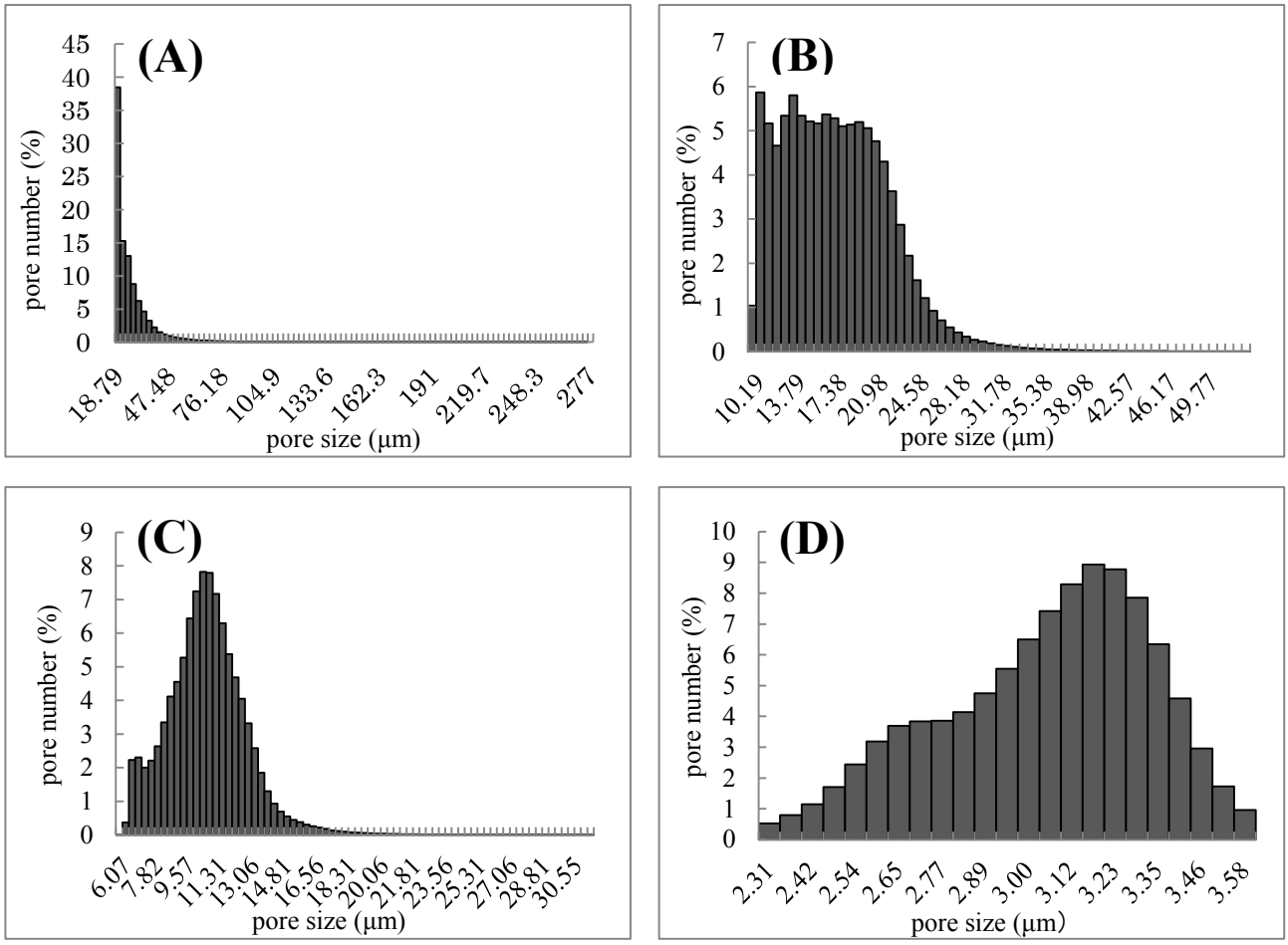

Fig.3. The fiber spacing distribution.

(A)NEOVEIL, (B)fabric-5, (C)fabric-2, (D)fabric-0.9

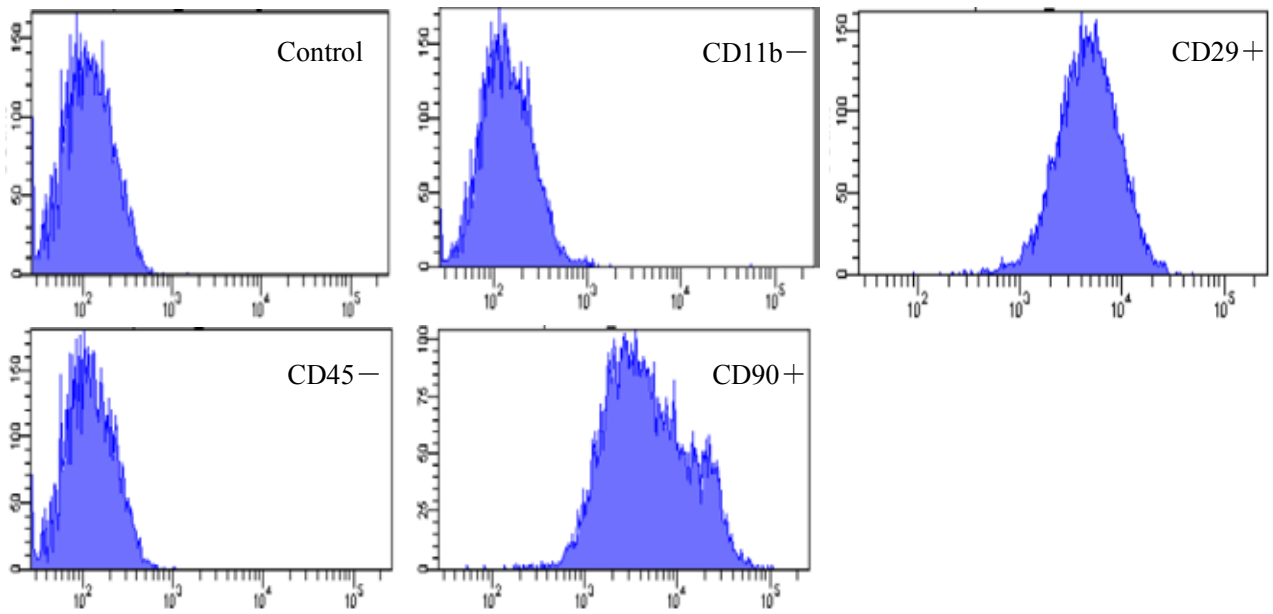

Fig.4. Characterization of ADSCs.

The horizontal axis indicates the luminescence and the vertical axis indicates the cell number. The control is the non-staining cells. ADSCs were positive for CD29 and CD90, while negative for CD11b and CD45.

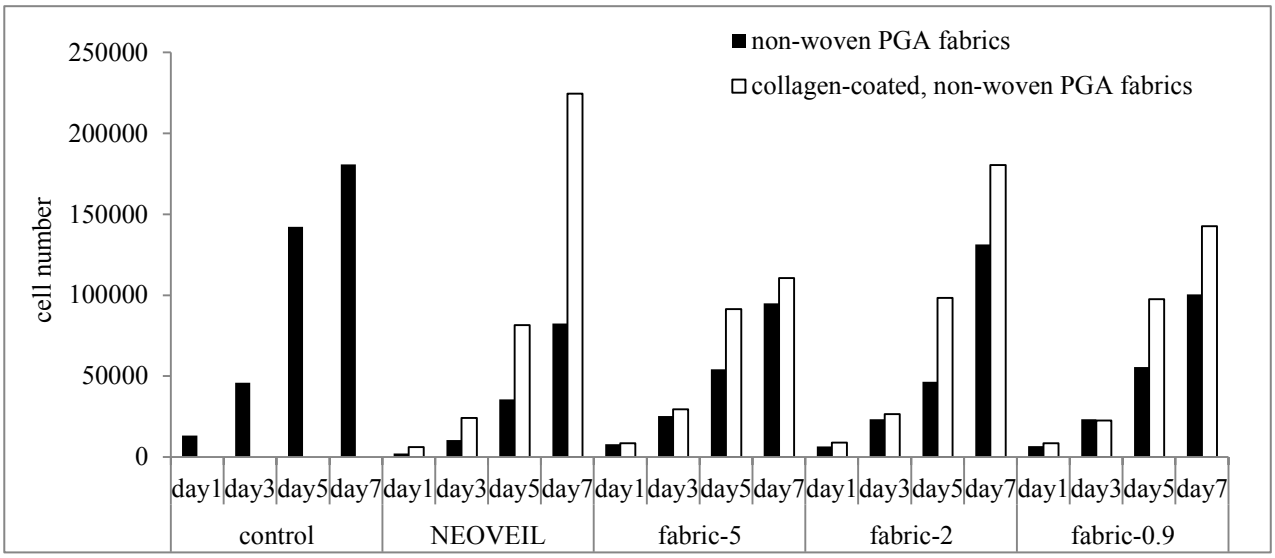

Fig.5. The cell growth examination.
The cell growth on each type of fabric either with or without a collagen-coating.

3.3 *Cell growth examination*

Our cell growth examination revealed that cell growth was improved on all of the collagen-coated fabrics compared with that observed on the corresponding non-coated fabrics. The cell growth definitely diminished in all of the non-coated PGA fabrics compared with that observed in the 24 well plate without any sheets (control). On the other hand, in all of the collagen-coated PGA fabrics, the cell growth on day 7 increased to near the level observed in the control or even surpassed that level (Fig. 5).

4. Discussion

In tissue engineering, the use of a three-dimensional scaffold is necessary to accommodate transplanted cells and guide new tissue formation[8,9]. ADSCs implanted with appropriate scaffolds are also believed to lead to more stable tissue regeneration.

So-called ADSCs are considered to be CD29- and CD90-positive[17,18]. However, fractions of CD29- and CD90-positive cells contain granulocytes and monocytes, including macrophages and lymphocytes,[19-21] in which CD11b and CD45 are positive[22,23]. Therefore, we characterized the ADSCs using CD29 and CD90 positivity and CD11b and CD45 negativity.

For exact research, from now on, CD44, a well-known marker of mesenchymal stem cells, will be used to identify cells[25]. In addition, CD31, an endothelial marker[26], should also be examined because cultured cells may contain endothelial cells. ADSCs gradually lose their differentiation capacity after being cultured many times. Therefore, the expression of CD105 should be examined to determine whether cells maintain a differentiation capacity[27].

Micro-fiber, non-woven PGA fabric has been widely used as an artificial scaffold[8] and has also been used successfully for regeneration of tissue and organs [28, 29] due to its capacity to be formed into complex shapes while maintaining its strength compared with collagen. However, it is difficult for cells to adhere to and growth on PGA fibers compared to natural scaffolds such as collagen due to PGA's hydrophobicity[12]. To address this issue, we newly developed PGA fabrics coated with collagen, which gives the hydrophobic surface of PGA fibers hydrophilic properties. In the present experiment, collagen was chosen as the coating material because it is an ingredient of the extra-cellular

matrix and cells adhere to it well[30-32].

Our cell growth examination revealed that cell growth was improved on all of the collagen-coated fabrics compared with that observed on the corresponding non-coated fabrics. This is because ADSCs are more readily able to adhere to and grow on the hydrophilic surface of collagen-coated PGA fabric.

The SEM pictures of the collagen-coated fabrics demonstrated that the collagen coated the surface of the individual fibers and formed a film-shaped membrane between the neighboring fibers. The various fiber diameter PGA fabrics used in this study showed that cell growth decreased with decreasing fiber diameter. Consequently, cell growth was best on the NEOVEIL fabric with a collagen coating. The reason for this is thought to be that NEOVEIL has the widest fiber spacing, thereby allowing wide collagen films to be formed and that provide the cells with the best conditions to proliferate effectively.

For the other fabrics with smaller diameter fibers, cell growth was worse in the present *in vitro* experiments. However, in our previous *in vivo* examination using rats, cell growth was enhanced when the fiber diameters were decreased from a micro to a smaller scale[33]. The *in vivo* results can be explained by the fact that smaller diameter fiber fabrics have relatively larger surface areas onto which many kinds of internal proteins are adsorbed *in vivo* that act to increase cell attachment and proliferation[12]. The newly developed collagen-coated fabrics with smaller diameter fibers, when used *in vivo*, may adsorb many kinds of internal proteins more easily, which may lead to more enhanced cell growth on collagen-coated smaller fibered fabrics than on c-NEOVEIL. Under these expectations, we are now planning a further examination in which collagen-coated fabrics with smaller diameter fibers will be tested for their superior *in vivo* effects to grow implanted cells on collagen-coated fabrics with larger diameter fibers.

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