Changes in Granulation-Formation Induced by Different Fiber-Diameters and Fiber-Spacing in Scaffolds for Tissue-Regeneration -Preliminary Study-

Taichi ORIKASA*, Junki IKEDA*, Ayumi TANZAWA*, Mari MATOBA*, Yoshizumi IWAME*,
Chiko YOSHIDA**, Hiroko TORII***, Yuki OZAMOTO****, Shinichiro MORITA*****,
Koichi HATAKEYAMA*****, Yuen NAKASE******, Hiroyuki TSUJIMOTO****** and Akeo HAGIWARA******

(Received January 19, 2012)

The fibers of the extracellular matrix (ECM) typically have diameters ranging from 50 to 500 nm. A scaffold should mimic the structure of the ECM as much as possible. As a result, 5 different fibers with diameters of 0.7, 0.9, 3, 7 and 20 μm were made into 5 kinds of scaffolds for tissue-regeneration. The scaffolds were made of Poly(glycolic acid) (PGA) nonwoven fabrics with fiber-spacing of 1.8 to 31.6 μm. This study investigated the influence of the 5 different scaffolds on infiltrating cells and granulation-formation.

Six female Wistar rats underwent surgery to create 5 “pockets” in the subcutaneous tissue of the back, and one of the 5 types of PGA scaffolds was implanted into each “pocket”. These implanted PGA scaffolds and surrounding tissues were excised *en block* on postoperative days 14, and then the infiltrating cells and granulation-formation were assessed microscopically.

In the scaffolds with a fiber-diameters of 0.9 and 3 μm and fiber-spacing of 10.8 and 9.0 μm, respectively, dense granulation tissue was formed over the full-thickness of the scaffolds. However, in the scaffolds with fiber-diameters of 7 and 20 μm and fiber-spacing of 15.1 and 31.6 μm, respectively, a relatively sparse granulation tissue was formed only around the fiber bundles and sparse granulation tissue was observed to form in the spaces between the bundles. In the scaffolds with a fiber-diameter of 0.7 and fiber-spacing of 1.8 μm, dense granulation tissue only formed in the superficial and shallow layers of the scaffolds, and no cell-infiltration was found in the central region of the thickness. These results suggest that scaffolds with fiber-diameters smaller than 3 μm and fiber-spacing approximately 10 μm are suitable for artificial scaffolds made with PGA fibers to induce the regeneration of dense granulation tissue over the full-thickness of the scaffold.

Key words: extracellular matrix, fiber-diameter, fiber-spacing, granulation-formation, scaffolds for tissue-regeneration

* Undergraduate Senior, Department of Medical Life System, Doshisha University, Kyoto
Telephone:+81-774-65-6878, E-mail: bmi0813@gmail.com
** Undergraduate Sophomore, Department of Medical Life System, Doshisha University, Kyoto
*** Graduate Student, Department of Medical Life System, Doshisha University, Kyoto
**** Department of Kusatsu General Hospital, Shiga
***** Bio-Medical Material Research Center, Doshisha University, Kyoto
****** Department of Medical Life System, Doshisha University, Kyoto
Corresponding Author: Akeo HAGIWARA
Telephone:+81-774-65-6878, E-mail: ahagiwar@mail.doshisha.ac.jp

1. Introduction

Many researchers have tried to develop scaffolds for tissue-engineering. These studies suggest that the scaffolds should mimic the structure of the extracellular matrix (ECM) as closely as possible, because an ECM-mimicking scaffold will play a role similar to the ECM for promoting tissue-regeneration *in vivo*. [1,2)]

Poly(glycolic acid) (PGA) is a biodegradable aliphatic polyester currently applied as scaffolds for tissue-regeneration in a variety of medical fields. [3)] PGA has high strength, and its degradation rate is very predictable. [3)] Usually, the minimal size of the PGA fiber is 10 μm in diameter, [4)] which is larger than the ECM ranging from 50 to 500 nm in diameter. [5-7)] Recently, PGA fibers can be produced with diameters of less 700 nm.

This study made scaffolds with PGA fibers similar in size to those in the ECM, and investigated the changes in infiltrating cells and granulation-formation induced by different fiber-spacing and fiber-diameters ranging from the usual PGA fibers to ECM-sizes fibers.

2. Materials and Methods

2.1 *Preparation of PGA nonwoven fabrics*

This study used 5 kinds of PGA nonwoven fabrics: fabric-0.7, fabric-0.9, fabric-3, fabric-7 and fabric-20, which had fibers with a mean diameter of 0.7, 0.9, 3, 7 and 20 μm, respectively.

Fabric-0.7 was obtained by electrospinning. [8)] The electrospinning process, in its simplest form consisted of uses a pipette to hold the polymer solution, two electrodes and a DC voltage supply in the kV range. The polymer drop from the tip of the pipette was drawn into a fiber due to the high voltage. The jet was electrically charged and the charge caused the fibers to bend in such a way that every time the polymer fiber looped, its diameter was reduced. The fiber was collected as a web of fibers on the surface of a grounded target.

Fabric-0.9, fabric-3 and fabric-7 were obtained by the melt-blowing method. [9,10)] The melt-blowing method is a one step process in which high-velocity air blows a molten thermoplastic resin from an extruder die tip onto a conveyor to form a fine fibered self-bonding web. Three kinds of PGA nonwoven fabrics were produced by controlling high-velocity air.

Fabric-20 was obtained by the needle punch method. [10)] The needle punch method is a two step process. PGA resin is extruded into fibers, and then stretched and crimped. The PGA fibers were carded, needle punched and then interlocked thermally.

2.2 *Fiber-diameter and spacing-size of PGA nonwoven fabrics*

The PGA fibers were analyzed morphologically using scanning electron microscopy (SEM). The diameter of fiber (fiber-diameter) was determined using SEM by measuring of 30 fibers at random per sample, and presented as the mean±standard deviation (SD). 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). [11)] The spacing-size was also determined using SEM by measuring of the 30 fiber-spacing-sizes at random and presented as the mean±standard deviation (SD).

2.3 *Preparation of square sheets of PGA nonwoven fabrics for animal experiment*

Each of the 5 kinds of PGA nonwoven fabrics was cut into a square-shaped sheet (square sheet) measuring 10 mm×10 mm in size and weighting 3.8 mg. The square sheets were sterilized by soaking in 99.5% ethanol for 30 seconds and rinsed twice in saline, just before the implantation into the tissues of rats.

2.4 *Animals and experimental design*

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

Six female Wistar rats ranging in weight from 200 -220 g were used in this study. All the rats were

maintained at room temperature (19-22°C) with free access to water, and were fed with standard pellets. The animals were housed in the laboratory for one week before the surgical operation. The health of all the rats was checked prior to surgery.

2.5 *Surgical technique*

The 6 rats were given ethyl ether (Diethyl Ether®, Wako, Inc., Osaka, Japan) inhalation anesthesia. Sodium pentobarbital (5 mg, Somnopentyl®, Kyoritsu Seiyaku, Inc., Tokyo, Japan) was diluted into one ml saline solution, and then the sodium pentobarbital was administrated intraperitoneally to every rat with a tuberculin syringe and 23G injection needles.

The 6 rats received the implantation of the 5 types of square sheets. All of the surgical procedures were performed by one person under sterile conditions. Five 1.5 cm lateral incisions were made on the dorsum skin per rat and the each incision was made into a 1.5×1.5 cm^2 "pocket" in the subcutaneous layer (Fig. 1). One of the 5 kinds of square sheets was implanted into each "pocket" as shown in Fig. 2. Therefore, 5 pieces, one piece from each of the 5 kinds of sheet, were implanted into different 5 "pockets" of each rat. These incisions were closed with skin sutures using 4/0 polyamide threads. The 6 rats were housed under normal conditions for 14 days.

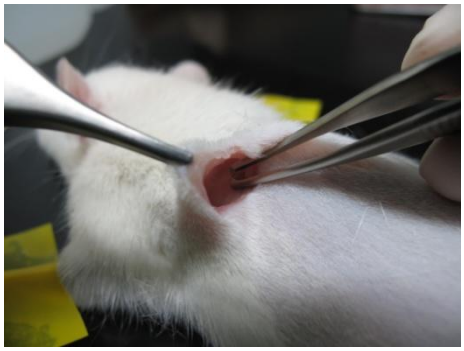

Fig. 1. "pocket" in the subcutaneous layer.

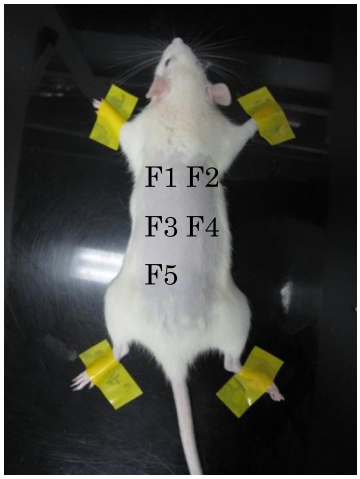

Fig. 2. Fabric-0.7 was implanted in F1. Fabric-0.9 was implanted in F2. Fabric-3 was implanted in F3. Fabric-7 was implanted in F4. Fabric-20 was implanted in F5.

2.6 *Preparation of tissue specimens for microscopic examination*

All the rats were sacrificed 14 days after the implantation: they were given ethyl ether inhalation anesthesia and then given a lethal dose of sodium pentobarbital (3.5 mg/kg of body weight) intraperitoneally. The implanted square sheets and surrounding tissues were surgically excised *en block* as the specimens for the microscopic examination. These specimens were fixed with 10% neutral formalin solution and cut prepared into thin sections (4 μm in thickness), and stained with hematoxylin-eosin using standard procedures for histological examinations.

2.7 *Microscopic evaluation*

Six tissue specimens for each kind of square sheet were assessed microscopically for the presence of infiltrating cells and granulation-formation in the scaffolds of the square sheets. The specimens contained four kinds of cells in the granulation tissue: lymphocytes, fibroblasts, macrophage and multinucleated giant cells. The quantity of the infiltrating cells on the inside of the granulation tissue was assessed using a zero-to-four grading scale with the following scores: 0: no infiltrating cells, 1: a very small number of infiltrating cells, 2: a small number of

infiltrating cells, 3: a moderate number of infiltrating cells and 4: a large number of infiltrating cells (Table 1). The granulation-formation was evaluated from two points of view: one was “dense or sparse” for the density of the granulation, and the other is “full thickness or a partial region” for the site of granulation-formation, and it was classified into 4 classes of poor, fair, good and excellent.

Table 1. Grading scale for the quantity of infiltrating cells

Score	Quantity of infiltrating cells
0	no
1	a very small number
2	a small number
3	a moderate number
4	a large number

3. Results

3.1 *Fiber-diameter and spacing-size of PGA nonwoven fabrics*

The fiber-diameter and spacing-size of PGA nonwoven fabrics are summarized in Table 2. Fabric-0.7 has a mean 0.7 μm fiber-diameter and 1.8 μm spacing-size. Fabric-0.9 has a mean 0.9 μm fiber-diameter and 10.8 μm spacing-size. Fabric-3 has a mean 3.0 μm fiber-diameter and 9.0 μm spacing-size. Fabric-7 has a mean 7.0 μm fiber-diameter and 15.1 μm spacing-size. Fabric-20 has a mean 20.0 μm fiber-diameter and 31.6 μm spacing-size.

3.2 *Infiltrating cells*

A microscopic view of each of the 5 kinds of PGA nonwoven fabrics are shown in Fig. 3. The quantity of infiltrating cells is summarized in Table 3. A small number of lymphocytes were seen infiltrating the square sheets of fabric-7 and fabric-20, whereas they were not seen infiltrating the fabric-0.9 and fabric-3. A large number of fibroblasts infiltrated both fabric-0.9 and fabric-3, the quantity of infiltrating fibroblasts were remarkably larger in fabric-0.9 and fabric-3 than in the other types of square sheets.

3.3 *Granulation-formation*

Granulation-formation is summarized in Table 4. In the fabric-0.9 and fabric-3, dense granulation tissue was observed to form all over the full-thickness of the square sheets, in comparison to the tissue observed to form on the other square sheets (Fig. 3-B,C). In the other three kinds of square sheets, no dense granulation tissue formed over the full-thickness of the square sheets. In the fabric-0.7, dense granulation tissue only formed in the superficial and shallow layers of square sheets, and no cell-infiltration was found in the central region of the thickness (Fig. 3-A). The fabric-7 and fabric-20 only showed relatively sparse granulation tissue to form around the fiber bundles, and sparse granulation was observed to form in the spaces between the bundles (Fig. 3-D,E).

4. Discussion

The ECM, which acts as a native scaffold during regeneration of injured tissue, is composed of fibers with diameters ranging from 50 to 500 nm. [5-7] The scaffolds artificially made from PGA for tissue regeneration are designed to mimic the structure of

Table 2. Fiber-diameter and spacing-size of PGA nonwoven fabrics

PGA nonwoven fabrics	Fiber-diameter in μm	Spacing-size in μm
	Mean±SD (n=30)	Mean±SD (n=30)
Fabric-0.7	0.69±0.28	1.80± 0.70
Fabric-0.9	0.90±0.65	10.80± 8.10
Fabric-3	3.00±1.86	9.00±10.00
Fabric-7	7.00±4.26	15.10± 9.40
Fabric-20	20.00±3.40	31.60±16.60

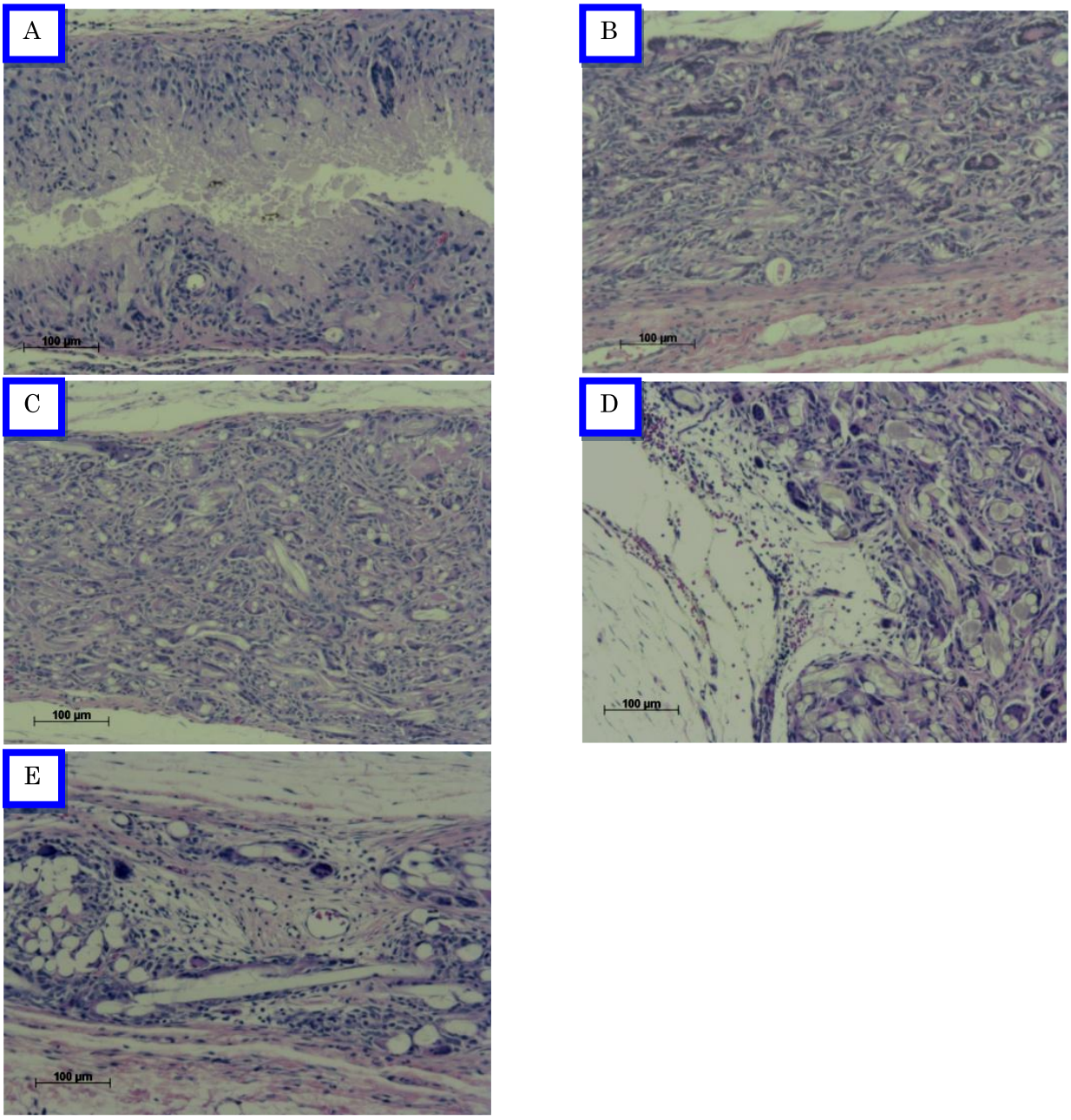

Fig. 3. Infiltrating cells on each 5 kind of square sheets of PGA nonwoven fabrics at 14 days.
A: fabric-0.7. B: fabric-0.9. C: fabric-3. D: fabric-7. E: fabric-20.

Table 3. The quantity of the four kinds of infiltrating cells, as assessed in the 6 specimens for each kind of the square sheet

Nonwoven fabrics	Lymphocytes	Fibroblasts	Macrophage	Multinucleated giant cells
Fabric-0.7	1	3	1	2
Fabric-0.9	0	4	0	4
Fabric-3	0	4	0	3
Fabric-7	2	3	1	3
Fabric-20	2	2	1	2

Table 4.
Classification of granulation-formation, evaluated in 6 specimens for each kind of square sheet

Nonwoven fabrics	Classification
Fabric-0.7	fair
Fabric-0.9	excellent
Fabric-3	good
Fabric-7	fair
Fabric-20	poor

ECM as much as possible. The structures of artificial scaffolds have two important characteristics, the fiber-diameter and the spacing-size. As fiber-diameter increased, the average spacing-size increased because of mechanical problems. [12] The present study evaluated 5 kinds of PGA nonwoven fabrics with different fiber-diameters and spacing-sizes. The study investigated the differences in the infiltrating cells and granulation-formation induced by different fiber-diameters and spacing-sizes.

The spacing-size was related to cell-infiltration. The size should be similar to the size of the infiltrating cells, nearly 10 μm. In the fabric-0.7 with a spacing-size of 1.8 μm, granulation tissue only formed in the superficial and shallow layer of the square sheets and no cells were observed to infiltrate into the central region of the square sheets. The cells could not infiltrate deeply into the thickness of the square sheets of fabric-0.7. In addition, the cells also could not infiltrate into the square sheets of fabric-7 and fabric-20. The cells were only observed to exist around the fiber bundles. On the other hand, granulation tissue was formed in the full-thickness of the square sheets in fabric-0.9 and fabric-3, which have a spacing-size of near 10 μm. Therefore, the cells were able to infiltrate easily and thus migrate into the deep layers of the scaffolds when the spacing-size is nearly 10 μm. This suggests that a spacing-size of near 10 μm is necessary for the cells to infiltrate deeply into the square sheet to form granulation tissues with the full-thickness of the scaffolds.

Many studies have investigated the cell response to nano- and micro-fiber nonwoven fabrics. Nanofiber nonwoven fabrics have larger relative surface areas onto which many kinds of internal proteins are adsorbed, and the internal proteins act to increase cell attachment and proliferation. [13] These studies reveal that nanofiber nonwoven fabrics are better than microfiber nonwoven fabrics in terms of their potential use as scaffolds. The fiber-diameter was therefore related to the density of the granulation tissues formed by the migrating and settled cells. Dense granulation-formation was seen with fabric-0.7, fabric-0.9 and fabric-3 which have fiber sizes of 0.7, 0.9 and 3 μm, respectively, whereas a sparse granulation was observed in the square sheets of fabric-7 and fabric-20 with fiber diameters of 7 and 20 μm, respectively. This result suggests that a fiber-diameter of less than 3 μm is suitable for migrating cells to settle and form dense granulation tightly rather than 7 μm larger diameter fibers.

5. Conclusion

The above described experiments suggest that a fiber-diameter of smaller than 3 μm and a spacing-size near 10 μm are suitable for the production of artificial scaffolds made with PGA fibers to induce the regeneration of dense granulation tissues in the full-thickness of the scaffold.

This work was supported, in part, by a grant from Science and Engineering Research Institute of Doshisha University.

References

1) C. P. Barnesa, S. A. Sella, E. D. Bolanda, D. G. Simpsonb and G. L. Bowlin, “Nanofiber technology: Designing the next generation of tissue engineering scaffolds”, Advanced Drug Delivery Reviews, 59, 1413–1433 (2007)
2) Z. Ma, M. Kotaki, R. Inai and S. Ramakrishna,

"Potential of nanofiber matrix as tissue-engineering scaffolds", Tissue Engineering, 11, 101-109 (2005)

3) Y. Dong, S. Liao, M. Ngiam, C. K. Chan and S. Ramakrishna, "Degradation behaviors of electrospun resorbable polyester nanofibers", Tissue Engineering Part B, Reviews, 15, 333-351 (2009)

4) D. Mooney and R. Langer, "Engineering biomaterials for tissue engineering: the 10–100 micron size scale", J. Bronzino (Ed.), The Biomedical Engineering Handbook, CRC Press, Boca Raton, pp, 1609–1618 (1995)

5) E. D. Hay, "Cell biology of extracellular matrix" 2nd edition, Plenum Press, New York (1991)

6) T. Elsdale and J. Bard, "Collagen substrata for studies on cell behavior", J Cell Biology, 54, 626–637 (1972)

7) E. Karl, F. David and A. John, "Collagen fibril formation", Biochem. J, 316, 1-11 (1996)

8) S. Ramakrishna, K. Fujihara, W. E. Teo, T. K. Lim and Z. Ma, "An Introduction to electrospinning and nanofibers.", World Scientific Pub, Singapore (2005)

9) S. R. Malkan, "An overview of spunbonding and meltblowing technologies", Tappi Journal, 78, 185-190 (1995)

10) JP 2005-3664367 (June 22, 2005)

11) J. E. Sanders, S. E. Lamont, S. B. Mitchell and S. G. Malcolm, "Small fiber diameter fibro-porous meshes: Tissue response sensitivity to fiber spacing", Journal of Biomedical Materials Research Part A, 72A, 335–342 (2005)

12) Q. P. Pham, U. Sharma and A. G. Mikos, "Electrospun poly(ε-caprolactone) microfiber and multilayer nanofiber/microfiber scaffolds: characterization of scaffolds and measurement of cellular infiltration", Biomacromolecules, 7, pp 2796–2805 (2006)

13) M. M. Stevens and J. H. George, "Exploring and engineering the cell surface interface", Science, 310, 1135-1138 (2005)