Micro and nano-scale *in vitro* 3D culture system for cardiac stem cells

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Abstract: Despite the success to prevent or limit cardiovascular diseases, the restoration of the function of a damaged heart remains a formidable challenge. Cardiac stem cells (CSCs), with the capacity to differentiate into cardiomyocytes, hold great potential as a source of cells for regenerative medicine. A major challenge facing the clinical application of differentiated CSCs, however, is the ability to generate sufficient numbers of cells with the desired phenotype. We previously established cell lines of CSCs using a c-kit antibody from adult rat hearts for use in regenerative medicine. C-kit -positive cardiac cells are well recognized as CSCs and have the potential to differentiate into cardiomyocytes. Here, before implant these cells *in vivo*, we first developed three-dimensional culture system (3D) using micro- and nano-scaled material. Sheets of poly(glycolic acid) (PGA) were fabricated by electrospinning. Composites of collagen-PGA were prepared that contained 0, 1.5, 3 or 6 mg of electrospun PGA nanofibers. The nanofibers were added as a sheet that formed a layer within the collagen sponge. The sponges were freeze-dried and then dehydrothermally crosslinked. A scanning electron microscopy (SEM)-based analysis of the surface of the sponges demonstrated a uniform collagenous structure regardless of the amount of PGA nanofibres included. The PGA nanofibers significantly enhanced the compressive strength of the collagen sponge. More CSCs attached to the collagen sponge incorporating 6 mg of PGA nanofibers than the sponge without PGA nanofibers. The attachment and proliferation of CSCs in the 3D culture was enhanced by incubation in a bioreactor perfusion system compared with 3D static and two-dimensional (2D; i.e. tissue culture plates) culture systems. The use of micro- and nano-scale materials in the fabrication of composites together with a 3D culture system is a very promising way to promote the culture of stem cells. © 2009 Wiley Periodicals, Inc. J Biomed Mater Res 94A: 1–8, 2010

Key words: miro- and nano-scale; *In vitro* 3D culture; composite; cardiac stem cells; nanofibers

INTRODUCTION

Many diseases including cardiac ischemia cannot be treated effectively with current methods because a loss of cells is the underlying cause. Replacing these lost cells using stem cell-based therapies offers the possibility of exciting alternative treatments for disease sufferers. Considering their proliferative activity and potential to differentiate, stem cells are promising for regenerative medicine. One recent attractive method is the usage of stem cells which upon transplantation into the infarcted myocardium repair and regenerate damaged tissue, and improves cardiac function.[1,2] Since c-kit positive cardiac stem cells (CSCs) are the only adult-derived cardiac stem/progenitor cells exhibiting all the characteristics of *bona fide* stem cells including clonogenic, self-renewing, multipotent and regenerative potential in animal models of myocardial infarction, their use in cardiac regenerative medicine strategies is considered promising.[1,2] However, a number of key hurdles have to be overcome before the clinical use of CSCs can be realized. In particular, large numbers of cells are required to treat a patient and so, in comparison to current strategies, the expansion of CSC populations has to be scaled up considerably in order to generate a sufficient quantity of specialized cells for treating cardiac ischemia. The overall objective of the present study is to develop systems that can be used to expand numbers of CSCs.

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The design of scaffolds for cell proliferation and differentiation is a key area of tissue engineering. Porous materials with a three-dimensional (3D) structure have been investigated for use as cell scaffolds because they have a larger surface for cell attachment and proliferation than two-dimensional (2D) materials and are preferable to assist the formation of 3D cell constructs which may better resemble the structure and function of tissues in the body.[3–5] The 3D scaffold is also important as a substrate for cell culture to increase numbers of cells.

In addition to designing scaffolds, it is necessary to develop a culture method suitable for the proliferation of cells and control of their phenotype and biological functions. It is important to create a local environment as similar as possible to natural ones. The conventional method of static culture does not always satisfy this requirement. The static medium cannot sufficiently supply oxygen and nutrients to cells in the 3D scaffold and remove cell waste. Our recent studies indicate that bioreactor perfusion culture facilitates the continuous exchange of medium and the constant removal of metabolic waste.[6–9] The results suggest that the bioreactor perfusion system and 3D scaffold provided better physiological conditions, resulting in an enhanced proliferative ability.

This study was undertaken to examine the behavior of CSCs in composites of collagen-PGA nanofibers as part of a 3D culture system. Collagen sponge was strengthened with 0, 1.5, 3 or 6 mg of electrospun PGA nanofibers not only to improve its poor mechanical properties and overcome shrinkage, but also to increase cell attachment. The attachment of CSCs to the composite was investigated, and the effect of different culture methods (static culture and perfusion bioreactor culture) on the proliferation of CSCs was evaluated.

MATERIALS AND METHODS

An aqueous solution of type I collagen, prepared from porcine skin by pepsin treatment, in HCl (3 mg/mL, pH 3.0) was kindly supplied by Nippi, Iabaraki, Japan. Poly (glycolic acid) (PGA) was purchased from Polysciences. Dulbecco's modified Eagle's minimal essential medium (DMEM; Sigma, St. Louis, MO) and fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) were purchased. Other chemicals were purchased from Wako Pure Chemical Industries, Japan.

Preparation and culture of CSCs

CSCs were obtained as described previously.[1,10] Briefly, rat hearts were digested by perfusion with a calcium-free Krebs-Henseleit buffer containing collagenase (Worthington Biochemical Corporation, Lakewood, NJ), hyaluronidase (Sigma, St. Louis, MO), trypsin (Sigma, St. Louis, MO), and DNAse (Sigma, St. Louis, MO). The whole cell population was then divided into myocytes and small cells by centrifugation. The small cells were treated with rabbit anti-c-kit primary antibody (Santa Cruz, CA). After being rinsed, the cells were treated with goat anti-rabbit secondary antibody conjugated with magnetic beads (Miltenyi'Germany). c-kit-positive cells were isolated by magnetic cell sorting (MACS) (Miltenyi'Germany), and cultured with complete medium (Comp M), DMEM (Sigma, St. Louis, MO) supplemented with 10% embryonic stem cell grade fetal bovine serum (Invitrogen, Carlsbad, CA), 5% horse serum (Sigma, St. Louis, MO), 10 ng/mL leukemia inhibitory factor (LIF, Millipore, Billerica, MA), 5 U/L Erythropoietin (EPO, Sigma, St. Louis, MO), penicillin-streptomycin (Wako, Japan), fungizone (Wako, Japan), and gentamycin (Invitrogen, Carlsbad, CA). Bulk cultures of c-kit positive cells (CSCs-BC) were passaged more than 40 times. And then, cloned cells were isolated from these BCs by the dilution method as reported previously.[10] Sphere-like balls (cardiospheres) were induced to form by the modified neurobasal medium, so called cardiosphere-forming medium.[11] The cardiosphere medium was composed of Dulbecco's MEM and Ham's F12 (ratio 1:1, Sigma, St. Louis, MO), bFGF (10 ng/mL, Wako, Japan), EGF (20 ng/mL, Wako, Japan), LIF (10 ng/mL, Millipore, Billerica, MA), insulin-transferrin-selenite (Invitrogen, Carlsbad, CA or Sigma, St. Louis, MO), B27 (Invitrogen, Carlsbad, CA), N2 (Invitrogen, Carlsbad, CA), penicillin-streptomycin (Wako, Japan), fungizone (Wako, Japan) and gentamyocin (Invitrogen, Carlsbad, CA). The CSCs-21E were cultured on bacterial dishes for 2 days to form spheroids, and then transferred to gelatin-coated dishes. Embryonic stem (ES) cells (ES-D3) were obtained from ATCC. The cells were grown and cultured according to the manufacturer's instructions. For comparisons of cell proliferation among the 2D static, 3D statistic, and 3D perfusion culture, DMEM/F12 (Sigma, St. Louis, MO) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) was used. In all cases, the medium was renewed at 3 or 4-day intervals.

Fabrication of nanofiber sheets

Solutions for electrospinning were prepared by dissolving 67 mg of PGA in 100 mL of 1,1,1,3.3.3 hexafluroro-2-2 propanol with stirring at 80°C for 48 h. The polymer solution was then delivered by a programmable pump (Harvard Apparatus, MA) to the exit hole of the electrode (Spinneret with a hole 0.7 mm diameter). The grounded platform for collecting the nanofibers was a silicon sheet. The flow rate was set to 10 mL/h. A high-voltage power supply was used to provide the necessary voltage for the fabrication of nanofibers. The voltage of the power supply and distance from the spinneret to the collector were set at 26 kV and 23 cm, respectively. The electrospinning was continued until a thick white layer of nanofibers (~5 mm) was fabricated. The sheet of PGA nanofibers was removed from the silicon sheet and processed for the fabrication of composite sponges.

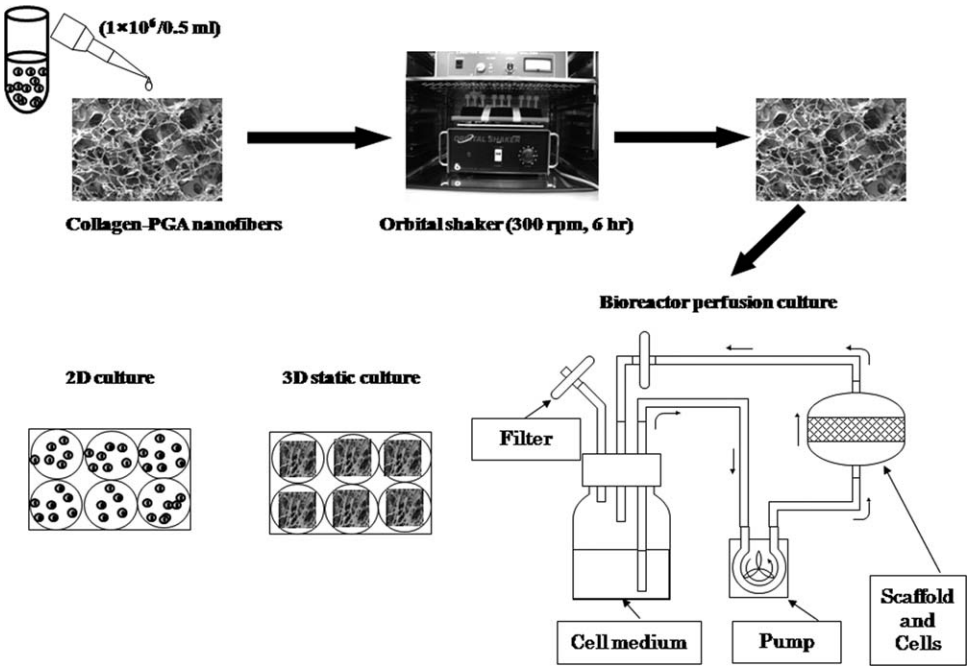

Figure 1. A schematic of *in vitro* culture systems.

Fabrication of composites of collagen-PGA nanofibers

Electrospun PGA nanofibers at weights of 1.5, 3, and 6 mg were placed into polystyrene moulds containing 0.75 mL of collagen solution. A further 0.4 mL of collagen solution was poured on top and the resulting mixture frozen at −20°C and freeze-dried to obtain a collagen sponge incorporating PGA nanofibers. The freeze-dried sponge was dehydrothermally crosslinked at 140°C for 16 h in a 0.1-Torr vacuum and then sterilized with ethylene oxide gas at 40°C. Samples of each sponge were coated with gold in an ion sputter machine and assessed morphologically by scanning electron microscopy (SEM) at a voltage of 10 kV.

Characterization of composites of collagen-PGA nanofibers

A DMA 242C (NETZSCH, Germany) was used to measure the mechanical properties of the composites. The versatility of the DMA 242C is particularly clear from the variety of sample holders that investigate the visco-elastic properties accurately over a wide modulus range. A cylindrical fixture was chosen to test specimens and evaluate their behavior as a whole along the compression axis, in the Z direction. Compression modulus was calculated from the slope of the initial linear portion of the strain–stress curve.

To evaluate shrinkage, the diameter of composites was measured before and after immersing the collagen sponge and composites of collagen-PGA nanofibers in cell culture medium. The Formula for shrinkage was calculated using the following formula:

Shrinkage (%) = (Initial diameter of the sample − Diameter of the sample after immersing in cell culture medium)/(Initial diameter of the sample).

In vitro 3D cell culture on composites of collagen-PGA nanofibers

An agitated seeding method effective in homogenously seeding cells into 3D porous scaffolds was used.[9] Briefly, 0.5 mL of cell suspension (1×10^6 cells/0.5 mL) and each composite were placed in a 50-mL test tube, and agitated at 300 rpm on an orbital shaker (Bellco Glass, Vineland, NJ) for 6 h at 37°C. The composites seeded with CSCs of collagen-PGA nanofibers were thoroughly washed with PBS (-) to exclude nonadherent cells.

Static and bioreactor perfusion systems were used to culture CSCs for proliferation. For the static culture, the cell-seeded composites were placed into six-well tissue culture plates (Asahi Techno Glass Corp., Chiba, Japan) and incubated in normal medium for 1–3 weeks at 37°C in an atmosphere containing 5% CO_2. The same procedure was used to culture CSCs in six-well plates without the composites, as 2D cultures.

The bioreactor perfusion system was designed to give a constant laminar flow of medium into the composites of collagen-PGA nanofibers. The cell-seeded composites were set in the sample holder, and perfused with normal medium (200 mL) delivered by a peristalic pump (Type Pst-100N, Iwaki brand, Scitech Div. Asahi Techno Glass Corp., Chiba, Japan) at a flow rate of 0.2 to 1.0 mL/min. The perfusion system was operated continuously in a 95% air-5% CO_2 atmosphere for 1–3 weeks. Half of the medium was exchanged every other day. A schematic design of the *in vitro* culture system is shown in Figure 1.

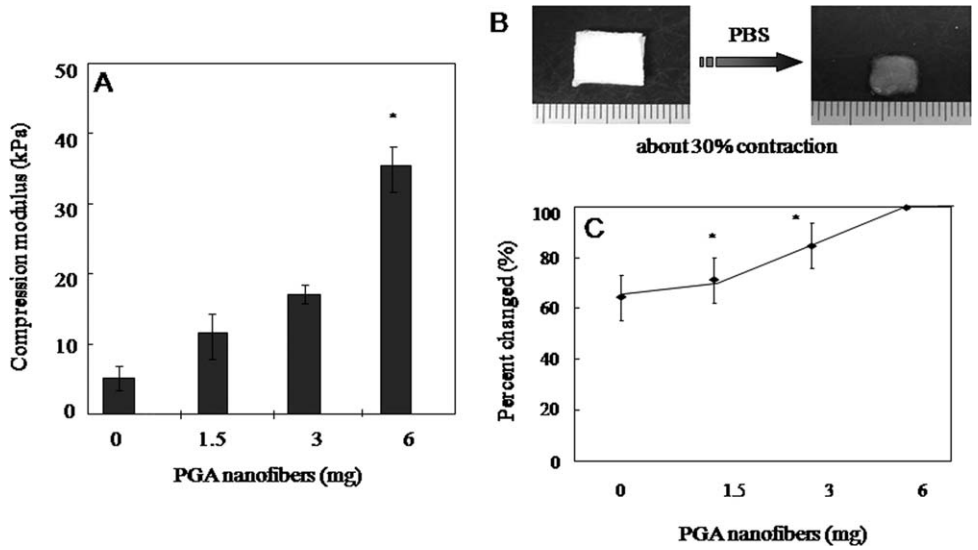

Figure 2. (A) Compression testing of composites of collagen-PGA nanofibers. (B) Shrinkage of collagen sponge. (C) Shrinkage assay of composites of collagen with different amounts of PGA nanofibers. *$p < 0.05$; significant relative to the sponge without PGA nanofibers (0 mg of PGA nanofibers).

DNA assay

The number of cells attached to the collagen-PGA composites was determined from the optical density of DNA. Briefly, the cell-seeded composites were washed with PBS and stored at −30°C until the assay. After thawing, the cells present in the composites were lyzed in a buffer solution (pH 7.4) containing 0.5 mg/mL proteinase K, 0.2 mg/mL sodium dodecylsulfate, and 30 m*M* saline-sodium citrate (SSC) at 55°C for 12 h with occasional mixing. The cell lysate (100 μL) was mixed with SSC buffer (400 μL). DNA concentrations were determined based on optical density (NanoDrop, ND-1000, Thermo Fisher Scientific, Rockford, IL). A calibration curve was prepared using cell suspensions with different cell densities. The DNA assay was performed three times independently for every experimental sample unless mentioned otherwise.

Statistical analysis

All the data were analyzed statistically to express the mean ± the standard deviation (SD) of the mean. Student's t test was performed and $p < 0.05$ was accepted as statistically significant.

RESULTS

Mechanical properties and morphology of composites of collagen-PGA nanofibers

Mixing in PGA nanofibers increased the compression modulus of the collagen sponge as shown in Figure 2. The collagen sponge showed about 30% contraction when kept in the PBS (-) solution. The shrinkage was reduced by adding the PGA nanofibers.

Figure 3 shows micrographs of cross-sections of composites containing 0, 1.5, 3 or 6 mg of electrospun PGA nanofibres. SEM of the surface of the composites demonstrated a uniform collagenous structure regardless of the amount of PGA nanofibers included.

In vitro cell culture

We isolated and characterized CSCs as described previously.[10] We used the cell line CSCs-21E established from CSC-BC21 and categorized as being of the cardiac lineage but multipotent (Machida et al., Submitted). Because CSCs-21E cells proliferated well and formed a distinct spheroid structure as observed in embryonic stem (ES) cells (ES-D3 cells) [Fig. 4(A,B)] when cultured in a cardiosphere-forming medium, we used them as a cell source for this study. Several line of evidences reported that cardiospheres could be a marker for harvesting CSCs, which showed differentiation into cardiac myocytes in infarcted hearts.[12,13] Cardiospheres were recently questioned to have cardiomyocyte potential.[14] However, we originally isolated CSCs using c-kit antibody, and the fraction of the cells could be different from their cells, since the population of c-kit positive cells was reported to be very small.[15]

SEM indicated cells present on the surface of each of composites of the collagen-PGA nanofibers. CSCc-21E were predominantly located within crevices rather than on flat surfaces and appeared greatest in number when the collagen sponge had been rein-

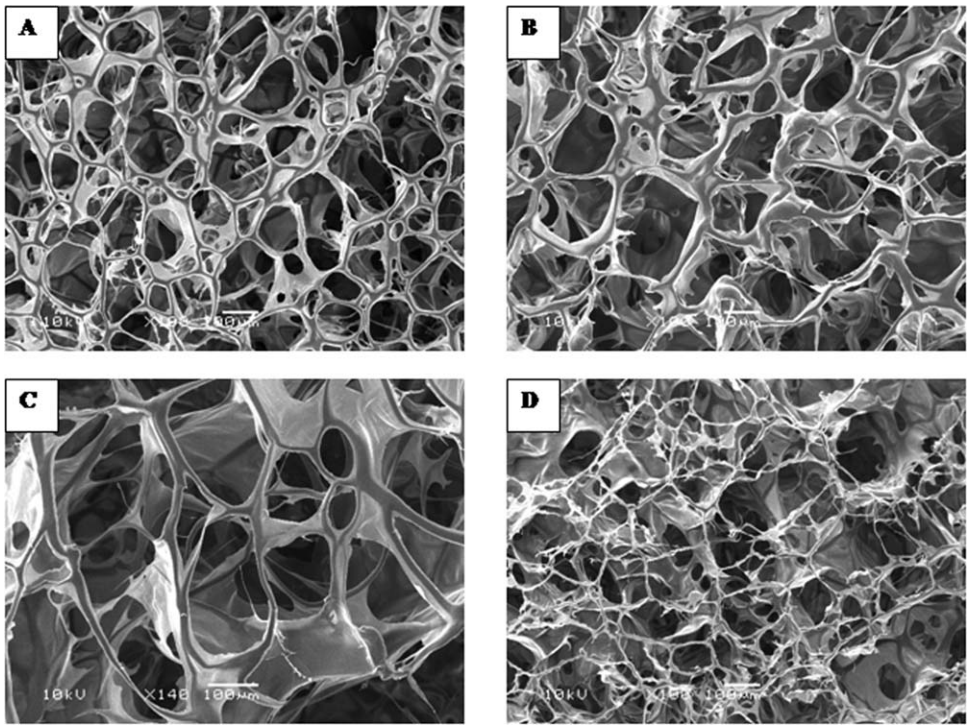

Figure 3. SEM of the surface of collagen sponges containing 0 (A), 1.5 (B), 3 (C), and 6 mg of PGA nanofibers (D). Scale bar = 100 μm.

forced with 6 mg of PGA nanofiber. Cells attached to both the collagen surface and, where visible, PGA nanofibers indicating that neither component was cytotoxic.

Proliferation of CSCs in the composites of collagen-PGA nanofibers in 3D culture systems

Figure 5 shows the number of CSCs-21E attached to the composites after the agitation seeding. There was a significant difference in cell number between the original collagen sponge and the collagen-PGA composites.

Figure 6 shows the proliferation of CSCs-21E cultured in the composites with the static and bioreactor perfusion systems. Irrespective of the method employed, the cell number tended to increase with incubation time. Of the three methods, the initial rate of cell proliferation was highest for the bioreactor perfusion method.

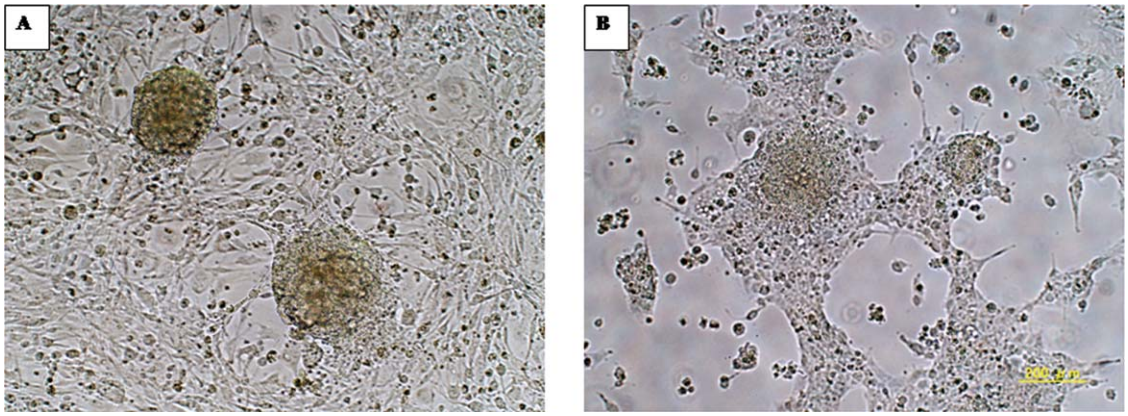

Figure 4. Light micrograph of ES cells; ES-D3 (A) and cardiac stem cells; CSCs-21E (B). The ES-D3 cells were cultured as described in the "Materials and Methods" section. Spheroid-like aggregates of CSCs-21E formed after the culturing in cardiosphere-forming medium on a gelatin-coated dish. The photograph was taken after 6 days of culture. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

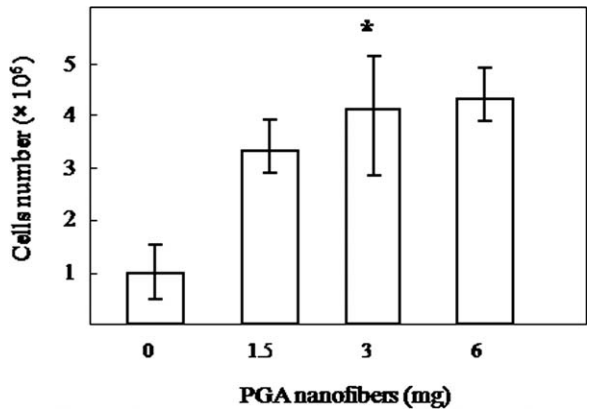

Figure 5. Initial cell attachment test for the collagen sponges with different amounts of PGA nanofibers 6 h after cell culture. $^{*}p < 0.05$; significant relative to the sponge without PGA nanofibers (0 mg of PGA nanofibers).

DISCUSSION

Among the many materials currently used as cell scaffolds, collagen has excellent biological properties and capacity to be processed (process ability). A collagen sponge is highly porous with interconnecting openings, good for cell infiltration and for supplying oxygen and nutrients to cells. However, its usage has been limited by poor mechanical properties. To overcome this problem, we attempted to fabricate composites of collagen with electrospun PGA nanofibers. The scaffold materials should mimic the native extracellular matrix (ECM) in terms of chemical composition and physical structure as reported by Ma et al.[16] Artificial nanoscaled fibers have great potential in the field of biomaterials and tissue engineering. There are three approaches to the formation of nanofibrous materials; phase separation, electrospinning, and self-assembly.[17–23] Phase separation and self-assembly can generate nanofibers in the same range as natural ECM collagen and allow for the design of macropores, while electrospinning only generates large-diameter nanofibers toward the upper end of the range possible with natural ECM collagen. Electrospinning is commonly used to fabricate scaffold for tissue engineering.[24] It is an easy technique and extremely cheap technique applicable to many different polymers. Our recent study demonstrated that PGA/collagen nanofibers fabricated through electrospinning significantly enhanced cell adhesion compared with PGA/collagen microfibers.[25] Based on these results, the present study aimed to fabricate an organ-like three-dimensional cell construct for use as a therapeutic cell-based system or as an *in vitro* model for the testing of cells to understand the effects of different methods on tissue development. The mixing of PGA nanofibers with collagen significantly increased cell attachment as demonstrated by SEM analysis and the initial cell attachment test (Fig. 5). Because the nanofibers also suppressed shrinkage of the collagen sponge [Fig. 2(C)], it is possible that the volume available for cell attachment was larger, resulting in a greater number of attached cells as shown by SEM and subsequent cell attachment test (Fig. 5). The experiments *in vitro* revealed that the number of attached CSCs increased with the incorporation of PGA fibers to a significant extent compared with that for the original collagen sponge (Fig. 5). A collagen sponge mechanically reinforced by PGA nanofibers is a promising scaffold for tissue regeneration.

In 2D cultures (i.e., tissue culture plates), the initial rate of cell growth is high, but the proliferation stops once a state of confluence is reached.[8,26] 3D cultures provide a larger surface area for attachment and spreading and can affect cell adhesion, spreading, and proliferation. The cell density in the 3D scaffold is high and the degradation of resorbable materials may release harmful substrates around cells, so that the physiological system cannot be sufficiently mimicked by the stagnant environment in conventional culture dishes (2D static culture method). Conventional methods of culturing with static medium may provide insufficient nutrition to support the survival and differentiation of cells in scaffolds in which the cell density is high and more active viability is expected. A bioreactor perfusion culture system allows for the continuous exchange of medium and constant removal of metabolic waste, and has been used for a variety of cells and tissues. The cells therefore receive a constant supply of nutrition and oxygen, while harmful metabolic products are removed from inside of the 3D scaffold. In our previous study, the osteogenic activity of mesenchymal stem cells was increased by the bioreactor perfusion method. This result can be explained by these features of perfusion culture. The enhanced

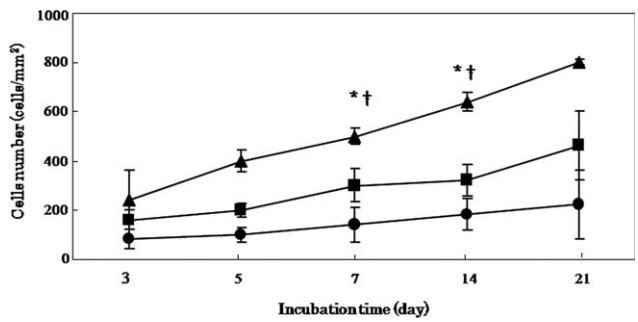

Figure 6. Proliferation of cells on composites of collagen with different amounts of PGA nanofibers in the 2D culture (●), 3D static culture (■) and bioreactor perfusion culture (▲). $^{*,\dagger}p < 0.05$; significant relative to the 2D and 3D static group.

viability and biosynthetic activity of cells are consistent with our previous research results.[8]

Figure 6 indicates that the bioreactor perfusion culture resulted in greater cell proliferation of CSCs than the 3D static culture. The supply of medium into the interior of the scaffold in the static culture is insufficient. This has been somewhat improved by perfusing the culture medium directly through the pores of the cell-seeded 3D scaffold, thereby reducing mass transfer limitations both at the scaffold is periphery and within its internal pores. The proposed technique of cell culture in 3D cell-scaffold constructs is based on the use of a 3D fibrous scaffold to guide cell organization. In comparison with a conventional culture, a 3D culture more closely resembles the situation *in vivo* with regard to cell shape and cellular environment that can influence the behavior of cells.[27–30] Therefore, the 3D culture system described here should be useful for *in vivo* transplantation in combination with CSCs, or other stem cells.

CONCLUSION

The present study aimed to combine micro- and nano-scale technologies to fabricate a 3D cell-substrate that mimics both mechanically and biologically the natural ECM and to enhance cell attachment. We demonstrated that bioreactor perfusion culture is a promising system that significantly enhanced the proliferation of CSCs *in vitro* compared with 3D static and 2D culture methods. Further characterization and optimization of the bioreactor perfusion method may help to overcome the limitations associated with 3D cell culturing, while also providing information about flow-mediated mechanical stimuli known to be major regulators of cellular functions.

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