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The Effect of a Long-Term Cyclic Strain on Human Dermal Fibroblasts Cultured in a Bioreactor on Chitosan-Based Scaffolds for the Development of Tissue Engineered Artificial Dermis

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Abstract: Mechanical stimulation is known to activate several cellular signal transduction pathways, leading to the induction of signaling molecules and extracellular matrix (ECM) proteins, thereby modulating cellular activities, such as proliferation and survival. In this study, primary human dermal fibroblasts (HDFs) were seeded onto chitosan-based scaffolds, and then cultured for 3 weeks in a bioreactor under a cyclic strain of 1 Hz frequency. Compared to control samples cultured under static conditions, the application of a cyclic strain stimulated the proliferation of HDFs in 1 week, and by week 3 the thickness of the cell/scaffold composites increased 1.56 fold. Moreover, immunohistochemical staining of the culture media obtained from the cell/scaffold samples subjected to the cyclic strain, revealed increases in the expression and secretion of ECM proteins, such as fibronectin and collagen. These results suggest that the preconditioning of cell/scaffold composites with a cyclic strain may enhance the proliferation of HDFs, and even facilitate integration of the engineered artificial dermal tissue into the host graft site.

Keywords: skin, chitosan, fibroblast, strain, artificial dermis, extracellular matrix.

Introduction

Tissue engineering is an emerging technology developed for the therapeutic reconstruction of damaged tissue. It involves deliberate and controlled stimulation of selected target cells through a systematic application of molecular and mechanical signals.[1] In the tissue engineering approach, a small number of cells are isolated from target organs and expanded via *in vitro* culture onto biocompatible and biodegradable scaffolds. These are then either allowed to develop into new tissue *in vitro*, or transplanted into a patient to replace functions of the lost or damaged tissue. There are numerous tissue-engineered products currently on the market including artificial skin and cartilage. Despite their usefulness, several clinical reports have suggested that current tissue-engineered products still have a much lower host integration rate than desired. Low integration rates are believed to result from failure of these engineered constructs to appropriately recapitulate the biological and mechanical functions of the native tissue.

In order for the engineered tissue to be clinically successful, specific cellular functions of the cell/scaffold composites must be regulated by providing appropriate signals in a temporally and spatially controlled manner.[2] In addition to growth factors and extracellular matrix (ECM) proteins that can stimulate cellular activities, mechanical strain has recently been shown to be a key stimulant of function in many cell types. The application of mechanical strain mimics the constant dynamic environment to which the viscous 3-dimensional networks of cells are exposed *in vivo*.

Previous research has demonstrated that application of dynamic stress to rat osteoblast-like cells, cultured on contractile gels, results in mechanical conditioning-dependent expression of several proteins and genes associated with the osteoblastic phenotype.[3] The application of cyclic strain was also shown to up-regulate elastin and collagen gene expression as well as increase tissue reorganization of engi-

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neered smooth muscle tissue. For example, applying a cyclic strain to the smooth muscle tissue of the rat aorta resulted in an up-regulation of elastin and collagen gene expression[4] whereas mechanical stimulation of the vascular smooth muscle cells of the human aorta increased PAR-1 mRNA expression.[5] Of particular interest is the finding that application of cyclic strain to human mesenchymal stem cells results in increased proliferation but that differentiation is not induced.[6] Such an outcome may prove useful for *in vitro* expansion of stem cells without altering their stem cell characteristics.[6]

Under normal physiological conditions, skin cells are also constantly exposed to the mechanical stimuli of tension and compression. The skin cells of certain sites, such as those of the sole and hip, are exposed to maximal compressive forces resulting in a more compact ECM structure and deeper rete ridges. Recently, Park *et al.* reported that application of an equibiaxial cyclic stretch to dermal fibroblasts, at a 10% maximal stretch, stimulated both fibroblast cell proliferation and fibronectin production without causing any transdifferentiation of fibroblasts into myofibroblasts.[7] But, the transdifferentiation of fibroblasts was commonly observed with growth factors treatment, which may lead to scar formation in damaged tissue.[7]

In the current study we attempted to create biologically functional dermal grafts by culturing human dermal fibroblasts (HDFs) onto chitosan-based scaffolds. Chitosan was selected as a tissue engineering scaffold due to the many desirable characteristics it possesses. For example, chitosan is a biopolymer consisting primarily of β-1,4-D-glucosamine repeating units and is formed through the *N*-deacetylation of chitin, an abundant polysaccharide obtained from crustacean shells. Chitosan has excellent biocompatibility since it has similar water attracting properties to those of hyaluronic acid and also resembles the structure of naturally occurring glycosaminoglycans.[8,9] In addition to this, chitosan is enzymatically degraded[10] to absorbable oligosaccharides, and can form insoluble complexes with common connective tissue components such as collagen and glycosaminoglycans,[11,12] making it especially suitable as a scaffold in tissue-engineering. However, some features of chitosan can be problematic as it can strongly adhere to fibroblasts and this often leads to an inhibition of cell proliferation.[12,13] To address this problem, we previously developed a biocompatible chitosan scaffold by applying a rigorous heat treatment, and demonstrated that HDFs attachment and growth were significantly increased.[14]

In this study, we combined the previously developed chitosan-based scaffold with a custom-designed bioreactor that can provide a constant cyclic strain to cell/scaffold composites. Specifically, we characterized the morphology of the engineered HDF tissue cultured onto the chitosan-based three-dimensional scaffolds following exposure to long-term cyclic strain conditions *in vitro*. The expression and secretion of ECM proteins from the artificial HDF remodeling process were also investigated.

Experimental

Materials. Chitosan from crab shell, whose the degree of deacetylation is 85% based on the amino content, was purchased from Korea Chitosan Ltd. (Seoul, Korea), and purified as follows; chitosan was dissolved in water with the stoichiometric equivalent of acetic acid. The solution was then filtered, and chitosan was reprecipitated by addition of sodium hydroxide up to pH 13. Pure chitosan was obtained by washing with distilled water, followed by drying in an oven. Bovine type I atelocollagen acidic solution was purchased from Nitta collagen (Japan). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (St Louis, MI, USA). Dulbecco's modified Eagle's minimal essential medium (DMEM), ethylenediaminetetraacetic acid (EDTA), and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Deionized water was obtained with a Milli-Q water filter system from Millipore Corporation (Bedford, MA, USA).

Preparation of Chitosan-Based Scaffolds. Chitosan was dissolved in 1% acetic acid to generate a 1.5% w/v solution. A 0.32% collagen solution, in 0.1 N HCl, was added to the chitosan solution in a ratio of 9:1 (chitosan:collagen). The mixed solution was subjected to mechanical stirring for 1 h at 4 °C, and then incubated in a dust-free environment, overnight, at room temperature to remove entrapped air bubbles. In order to prepare chitosonium acetate scaffolds, 35 g of the previously mixed chitosan:collagen solution was poured onto flat-bottomed pre-cooled molds (80 cm^2). These were then quickly frozen at -80 °C for 24 h and lyophilized at -55 °C under a vacuum of 0.2 torr for 24 h. The crude collagen-blended chitosonium acetate scaffolds were then heated in an oven at 110 °C, under vacuum, for 1 day. After the heat treatment, the scaffolds were washed in a series of solutions with decreasing ethanol concentrations for 1 h per wash. These were 90, 80, 70, 60, and 50% ethanol concentrations, respectively. Following this, the scaffolds were washed with deionized water until the filtrate reached neutral pH and the resulting scaffolds were freeze-dried for 24 h before being sterilized with ethylene oxide gas.

Porosity. The density and porosity of the scaffolds were measured using a liquid displacement method as previously published.[15] Briefly, the sample of known weight (W) was immersed in a graduated cylinder containing a known volume ($V1$) of ethanol for 5 min, and then a series of brief evacuation-repressurization cycles were conducted to force the ethanol into the pores of the foam. Cycling was continued until no air bubbles were observed to emerge from the foam. The total volume of ethanol and the ethanol-soaked foam was then recorded as $V2$. The volume difference ($V2$ -

$V1$) was the volume of pores within the scaffold. The ethanol-soaked foam was removed from the cylinder and the residual ethanol volume was recorded as $V3$. The volume of the ethanol held in the foam ($V1$ - $V3$) was determined as the void volume of the foam. Thus the total volume of the foam was: $V = (V2 - V1) + (V1 - V3) = V2 - V3$. The density of the foam (d) was calculated as;

$$d = W/(V2 - V3)$$

and the porosity of the foam (ε) was obtained using the following equation;

$$\varepsilon = (V1 - V3)/(V2 - V3)$$

Mechanical Testing. The dry scaffolds were cut into rectangular strips (20 × 10 mm) and the thickness of each strip was measured using common Vernier calipers. The samples were strained to breakage on an INSTRON universal testing machine (model 5567, Micrometritics, GA, USA) employing a gauge length of 10 mm and a 5-N maximum load cell with a crosshead speed of 1 mm/min. All specimens were drawn at ambient temperature with a relative humidity of 50%. To prevent slippage and damage of the specimen at the grips, a rectangular piece of overhead projector film that had been cut to size prior to testing, was glued to each end of the specimen. The ultimate tensile strength and breaking elongation of the scaffolds were directly obtained from tensile tests. Young's modulus was calculated from the slope of the initial section of the stress-strain curve. Replicate measurements (4 times) were made and the results were quoted as mean values. The hydrated samples were prepared by immersing the dry samples with the same dimension (20 × 10 mm) in phosphate-buffered saline (PBS) solution at room temperature for 3 h.

Isolation of HDFs and Cell Culture. HDFs were isolated from normal adult foreskins, removed by circumcision as previously described.[16] Circumcised foreskin samples were incubated in F-medium (1 part of Ham's F-12 medium and 3 parts of DMEM, 10% FBS) containing 1% penicillin/streptomycin and 250 ng/mL of fungizone (Gibco-Invitrogen, San Diego, CA, USA) at 4 °C. Cells were isolated within 24 h post-surgery. The foreskin samples were washed at least 8 times in PBS solution containing 5% penicillin/streptomycin (Gibco-Invitrogen, San Diego, CA, USA). The subcutaneous tissue was removed from the dermis with sterile surgical scissors, and the remaining skin was cut into fine pieces of less than 1-2 mm, before being treated with dispase II solution (2.4 U/mL, Roche, Mannheim, Germany) at 37 °C for 4 h. The dermal layer was then separated from the epidermal layer, washed, and then incubated in 10 mL of 0.35% collagenase (Roche, Mannheim, Germany) at 37 °C for 2 h. Isolated HDFs were dissociated into a single cell suspension by gentle pipetting, washed twice and then seeded onto culture plates in F-medium at a density of 2×10^4 cells/cm^2. The culture medium was changed two or three times per week.

***In vitro* Culture of Cells Under Mechanical Stimulation.** The chitosan-based scaffolds were cut into rectangular strips (40 × 15 mm) and immersed in PBS solution prior to use. On the day of cell seeding, the chitosan-based strips were clamped in the tissue culture chamber. Passage four primary cultured HDFs were seeded onto the scaffold at a density of 1.5×10^6 cells/scaffold. After 1, 2, and 5 h, 0.5, 1, and 30 mL of culture medium was added to each scaffold, respectively. The HDF-seeded scaffolds were maintained at 37 °C in 5% CO_2 for 4 days with the medium changed every day. After 1 week under the statically culture, the HDFs in the scaffolds were then subjected to cyclic stretching for 3 weeks using a bioreactor at a frequency of 1 Hz (1 cycle/sec) and an amplitude of 7% of the initial scaffold length. As a control, HDF-seeded scaffolds were fixed at only one end of the clamp and moved back and forth at the same frequency and amplitude as the mechanical stretching conditions described. The culture media was collected at each time point and stored at -80 °C until analysis for the measurement of fibronectin expression and procollagen metabolism was conducted.

MTT Assay. HDFs cultured as scaffold-cell hybrid constructs were subjected to a modified colorimetric MTT assay at 1 and 3 weeks to determine the proliferation rate of HDFs. Briefly, 1 mL of MTT solution was added to each well and incubated for 4 h at 37 °C. Cells were then washed with PBS solution, lysed with a 1:1 ethanol:dimethylsulfoxide (DMSO) buffer and agitated for 30 min in order to release cells from the scaffolds. For colorimetric analysis, 100 μL of supernatant solution was aliquoted into a 96 well plate and the optical density (OD) was measured at 540 nm on a Bio-Rad model 450 microplate reader (Richmond, CA, USA). All data are presented as mean values of three independent experiments.

Scanning Electron Microscopy (SEM). The cultured cell/scaffold composite constructs were washed with PBS solution, and fixed with 1% glutaraldehyde for 1 h at room temperature and incubated at 4 °C for 24 h in order to visualize cells in the scaffolds. The scaffolds were dehydrated for 15 min by immersing into a series of aqueous solutions with increasing alcohol content and freeze-dried under the same condition as described earlier. The samples were critical point-dried and coated with an ultra-thin gold layer (100 Å). A scanning electron microscope (Jeol, Model JSM-35CF) was used to image the samples.

Biological Assay. For histological analysis, the cultured cell/scaffold constructs were fixed with 10% (v/v) formalin in PBS and dehydrated by washing in a series of solutions with increasing alcohol content for 15 min. The samples were then washed with xylene and embedded in paraffin overnight. The embedded scaffolds were sectioned at a thickness of 4 μm using a rotary microtome. Hematoxylin and Eosin (H&E) staining as well as immunohistochemical

staining for vimentin and type I collagen were conducted using general immunohistochemical protocols.

The amount of fibronectin in the culture media was quantified using Western blotting analysis. Briefly, Triton X-100 and phenylmethylsulfonylfluoride (PMSF) were added to 1 mL of the thawed culture medium at final concentrations of 0.5% and 2 mM, respectively, and the resulting mixture was centrifuged at 1,500 rpm for 5 min. The supernatant was heat-denatured in 2X sample buffer for 5 min at 95 °C, and resolved on a 7.5% SDS-PAGE gel. The proteins were electrophoretically transferred onto a nitrocellulose membrane in a constant voltage of 100 mV overnight. The membrane was then immersed in 3% ponceau for 10 min, washed 3 times with deionized water, blocked with 5% FBS supplemented with 0.2% Triton X-100, and incubated in a skim milk:PBS solution at room temperature for 1 h. The membrane was then incubated with a primary antibody (human FN hybridoma culture supernatant) for 1 h at room temperature, washed with blocking water, and detected with a horseradish ALP conjugated secondary antibody. Immunoreactivity was determined by with the enhanced chemiluminescence reagent (ECL) (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Concentrations of procollagen, secreted in the cell culture supernatant, were determined using a solid phase ELISA kit (Procollagen type I C-peptide EIA Kit, Takara Bio Inc., Korea). After normalization according to a Bradford assay, 100 μL of antibody-POD conjugate solution was added to each well and 20 μL of each sample and standard solutions were then added to each well before incubating them at 37 °C for 3 h. Excess solution was aspirated and washed four times with 400 μL of PBS solution. Substrate solution (100 μL) was added to each well and incubated for 15 min at room temperature. The reaction was terminated by adding 100 μL of stop solution to each well. Concentrations were determined following measurement of absorbance at 540 nm on a microplate reader. The entire procedure was protected from the light.

Results and Discussion

Preparation of Chitosan-Based Scaffolds. The mechanical properties of skin layers enable the skin to withstand the complex mechanical stresses of the environment, including compression and stretching and therefore determine the physiological function of the skin and play an effective role in protecting internal organs.[17] Currently, tissue regeneration methods are increasingly being used for the treatment of extensive tissue damage and improvement of these methods is now relying on the development of enhanced tissue support such as chitosan-based scaffolds. When constructing a biologically relevant skin graft, it is of utmost importance to understand the effects of mechanical stimulation on cellular responses as well as to take the mechanical properties of supporting scaffolds into careful consideration. In the current study, we engineered artificial dermis and examined the effects of long-term cyclic strain on HDFs cultured on chitosan-based scaffolds in a bioreactor.

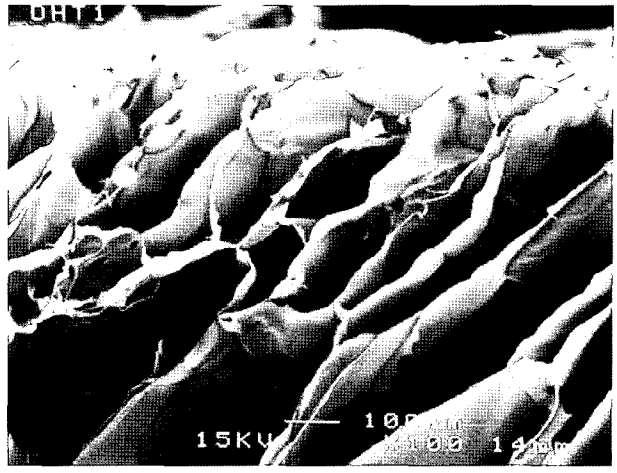

Figure 1. SEM micrographs of chitosan-based scaffold. Bar = 100 μm.

We first manufactured the heat-treated chitosan-based scaffolds that had previously developed as three-dimensional cell culture substrates for tissue engineering of the dermal layer, using the freezing-drying method previously described.[14] These scaffolds exhibit an interconnected porous structure, as displayed in Figure 1. A quantitative analysis of the pore structure of the scaffolds using mercury porosimetry and the ethanol displacement method was performed. The median pore diameter of the scaffold was approximately 81.38 mm, the total pore area was 517.6 m^2/g, and the porosity of the scaffolds was 90%.

Our next aim was to determine the elastic features of the chitosan-based scaffolds under various conditions. Table I shows that the tensile strength and elongation-at-break of the scaffolds differed significantly depending on the nature of the outer environment. For instance, in the dry state, the scaffold exhibited a tensile strength and the elongation-at-break of 833 kPa and 4.2%, respectively, while the tensile strength in the wet state decreased to 10.6 kPa and the elongation-at-break increased significantly to 30.1%. In addition, the modulus of the hydrated scaffold was reduced compared to the corresponding dry scaffold. The finding that in the

Table I. Mechanical Properties of Chitosan-based Scaffolds. The Values in the Table were the Average Values from 4 Specimens for Each Scaffold

Chitosan-Based Scaffolds	Young's Modulus (kPa)	Breaking Elongation (%)
Dried state	832.9 ± 18.7	4.2 ± 0.9
Hydrated state	10.6 ± 1.3	30.1 ± 2.4

wet state the scaffold exhibited greater elongation-at-break suggests that the chitosan-based scaffold is able to sustain a cyclic stress with minimal deformation or breakage. Thus our preliminary data revealed that the scaffolds constructed displayed no permanent deformation under the conditions applied during the culture period.

***In vitro* Culture of HDFs under Mechanical Stimulation.** The effect of mechanical stimulation on HDFs cultured on chitosan-based scaffolds in a bioreactor was examined. A cyclic stretch was applied to cell-seeded chitosan-based scaffolds, where the bioreactor shown in Figure 2 was utilized as previously described.[2] Briefly, the scaffolds cultured with HDFs were subjected to the cyclic strain, at frequency of 1 Hz, and amplitude of 7% of the initial scaffold length, that was generated by the movement of the crank back and forth as the eccentric disk connected to the crank rotates. It was reported that application of an equibiaxial cyclic stretch to dermal fibroblasts, at a 10% maximal stretch and frequency of 1 Hz with FlexerCell Stretch Unit, stimulated both fibroblast cell proliferation and fibronectin production.[7] But in this study, we chose the reduced amplitude of 7% of the initial scaffold length in order to escape from probable detachment of HDFs from scaffold during mechanical stimulation.

HDFs were seeded onto the chitosan-based scaffold and cultured for 1 week under static conditions and then a cyclic strain was applied for an additional 1 or 3 weeks. As shown in Figure 3, HDFs were distributed over the entire surface and cross-section of the scaffold by 1 week of static culture.

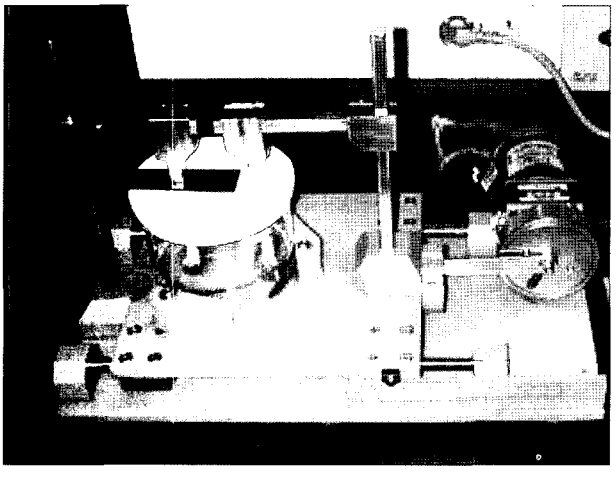

Figure 2. The *in vitro* bioreactor system utilized as previously described[2] for cyclic stretching to cell-seeded chitosan-based scaffolds. Scaffolds cultured with HDFs were subjected to cyclic stretch for 3 weeks at a frequency of 1 Hz and amplitude of 7% of the initial scaffold length by the movement of the crank back and forth as the eccentric disk connected to the crank rotates. As a control, seeded scaffolds were fixed at only one end of the clamps and moved back and forth at the same frequency and amplitude as the mechanical stretching conditions.

These results indicate that the distribution of HDFs within the scaffold is not the limiting factor and the chitosan-based scaffold is suitable as a tissue-engineered material. After 1 and 3 weeks of culture in the bioreactor, scaffolds retained their shape and showed no visible signs of physical deformation, suggesting that their original structures were preserved.

In addition, although similar levels of cell proliferation seemed to occur within the scaffolds irrespective of culture method, SEM imaging revealed an increase in HDF proliferation in the scaffolds that had been subjected to mechanical simulation (Figure 3). This led to complete coverage of the entire surface area of the scaffold by 1 week. In contrast, in control samples where HDFs were cultured under static conditions, cells seemed to grow at a slower rate and the cells did not completely cover the surface of the scaffold at 1 week. Furthermore, these control scaffolds displayed partial cell coverage at 3 weeks.

Biological Assays of *In vitro* Cultured Cell/Scaffold Composites.

MTT Assay: The proliferation rate of HDFs on the scaffold was examined quantitatively using a MTT assay. As shown in Figure 4, the results were consistent with the SEM images presented in Figure 3. It was evident that viable cells were attached to the surface of the scaffold for at least 4 weeks (1 week under static conditions and 3 weeks under mechanical stimulation). The cell number in the scaffold increased consistently with increasing culture time in both the static and the mechanically stimulated group. Interestingly, a 1.6 fold increase in cell number was observed in the sample that had been exposed to cyclic strain for 1 week. These results show that the proliferation rate of HDFs under stimulation appeared to be slower between 1 and 3 weeks despite demonstrating higher MTT activity. We speculate that HDFs cultured under cyclic mechanical loading may have quickly reached the plateau value of proliferation by 1 week, compared to cells in the static condition, and this may explain why a further increase in the proliferation rate was not observed at later time points.

Histological Analysis of Cell/Scaffold Composites. In order to examine the distribution of HDFs within the chitosan-based scaffold and the localization of secreted ECM proteins, immunohistochemical staining was carried out on the cell/scaffold composites. Figure 5 demonstrates that the HDFs exhibit distinct distributions depending on the culture conditions. It should be noted that under static conditions, cells are localized to the periphery of the scaffold whereas cells cultured under mechanical stimulation are distributed more homogeneously throughout the scaffold. In addition to the enhanced distribution of HDFs on the scaffold when exposed to mechanical stimulation, there was a significant increase in the scaffold thickness (1.56 fold) at 3 weeks. This may have occurred as a result of stimulated HDF metabolism in scaffolds exposed to mechanical strain thereby

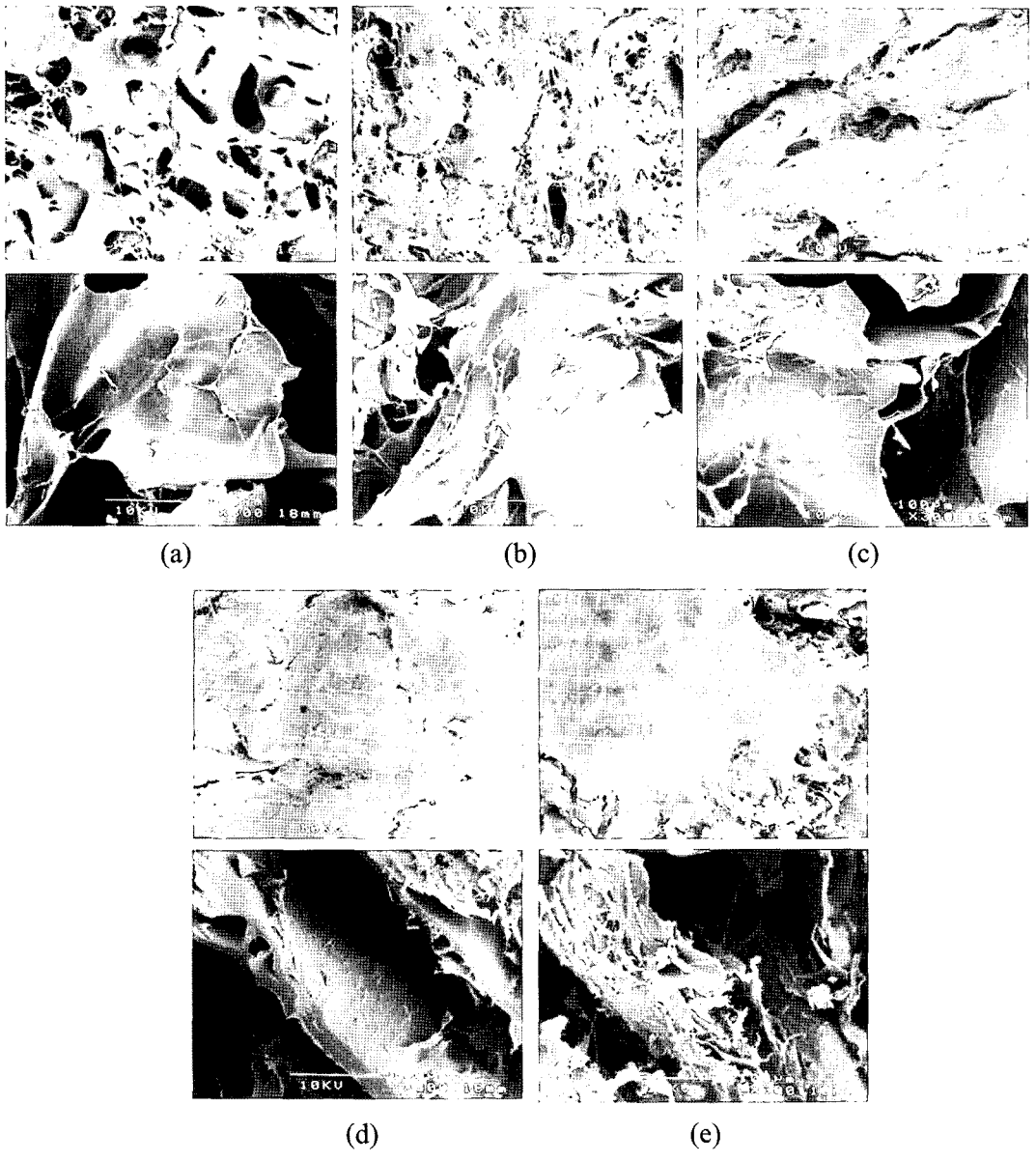

Figure 3. SEM of chitosan-based scaffolds at various culture periods (a) 1 week under the statically cultured, (b) additional designed time point of 1 week under the statically cultured, (c) additional designed time point of 1 week under the cyclic dynamically cultured, (d) additional designed time point of 3 weeks under the statically cultured, and (e) additional designed time point of 3 weeks under the cyclic dynamically cultured system (Magnification: Top × 100, and Bottom × 300).

increasing the secretion of ECM proteins and contributing to the volumetric expansion of the cell/scaffold composites. Histological and immunohistochemical analysis could not be conducted on the static culture at 1 week because we were unable to section the samples. The samples shattered when attempting to section them, possibly as a result of limited cellularity within the construct. Cellularity consists of cells and secreted molecules forming the ECM and usually contributes to the formation of a better framework of the histological section. Sections from other samples were obtained without difficulty.

We next examined the expression of various ECM proteins since increased production of ECM proteins may be the mechanism by which cell attachment and proliferation is promoted. ECM proteins such as vimentin and fibronectin are two major adhesive proteins in plasma and serum.[18] Fibronectin binds to integrin transmembrane receptors and also possesses multiple binding sites for other ECM components such as collagen, fibrin, and heparin.[19,20] The binding of fibronectin to cells and other ECM proteins mechanically links the intercellular structure to extracellular elements through a receptor-ligand mediated binding, which usually

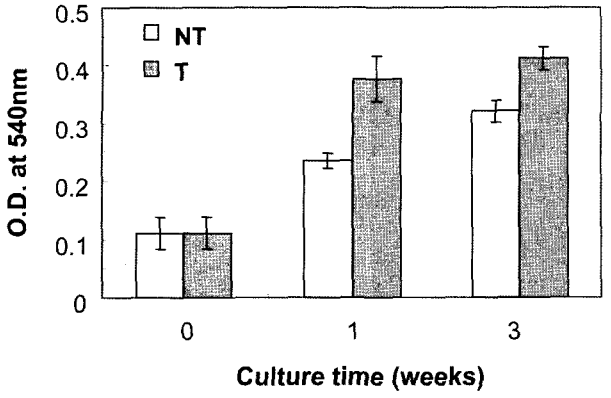

Figure 4. The effect of cyclic stretching on chitosan-based scaffolds on culture periods by using MTT assay of static culture conditions (NT, blank bar) vs. cyclic stretched culture (T, gray bar). Error bars indicate standard error of the mean, n = 4.

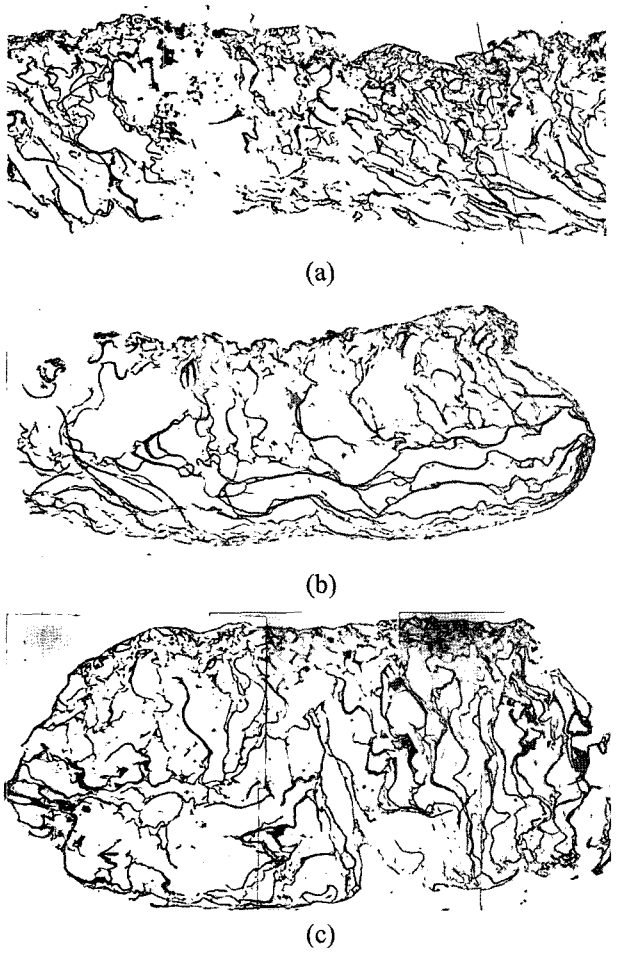

Figure 5. Pictures of cross-sectional H&E staining of chitosan-based scaffolds at various additional designed culture periods after 1 week under the static culture, (a) 3 weeks under the statically cultured, (b) 1 week under the cyclic dynamically cultured, (c) 3 weeks under the cyclic dynamically cultured system. Magnification: × 40.

triggers numerous signaling events and mediates critical cellular activities such as proliferation, differentiation, and migration.[21-23] Figure 6 shows that the expression of several ECM proteins including vimentin, fibronectin and collagen type I was up-regulated by mechanical stimulation at 3 weeks after the onset of cyclic strain in the cell/scaffold composites. This increase in the production of ECM proteins by cells cultured under mechanical stretch may be the mechanism by which cell attachment and proliferation is promoted.

Elevated expression of fibronectin was further corroborated by Western blotting of culture media collected at 1 and 3 weeks after the onset of the stretching. Although, the overall amount of secreted fibronectin reduced with increased culture time in both control and mechanically stimulated groups, fibronectin expression was up-regulated by the mechanical stretch at 1 and 3 weeks, compared to the static culture condition (Figure 7). This result was consistent with the immunohistochemical staining against fibronectin.

In addition the application of cyclic strain increased the expression and secretion of procollagen as demonstrated by ELISA analysis. Figure 8 shows that the amount of secreted procollagen was upregulated to approximately 28 and 68% at 1 and 3 weeks, respectively, after the onset of stretch, when compared to samples maintained under static culture conditions. Interestingly, the synthesis of procollagen was also reduced to 35 and 16% at 1 and 3 weeks, respectively, as culture time increased, irrespective of culture conditions. This observation may be consistent with the rapid increase in proliferation during the first week and the subsequent reduction of proliferation of HDFs cultured under mechanical stimulation. This is not unusual since expression levels of cell cycle-associated proteins are known to be induced rapidly in response to certain stimuli, then be down-regulated quickly and sometimes increase again. As such, it is important to note that an optimal culture time may be required to stimulate a cell cycle-associated protein after the onset of stretching. Further in-depth studies are required to investigate the mechanism by which stretch-induced up- or down-regulation of other factors occurs.

The gene expression of cells in engineered tissues may be regulated by various signals provided by scaffolds, including cell adhesion molecules, growth factors, and mechanical signals.[2] Among these are mechanical forces, which play a critical role in the regulation of various cell functions such as cell survival, proliferation, differentiation and synthesis of ECM.[24,25] Previously, we reported that the application of a mechanical stretch to HDFs induced mitogenic stimulation and consequently mechanical stretching could replace the mitogenic stimuli provided by serum or growth factors in HDFs.[7] This current study demonstrated and reconfirmed the importance of mechanical culture conditioning to mediate proliferation of fibroblasts and expression of several ECM proteins such as fibronectin, vimentin, and collagen.

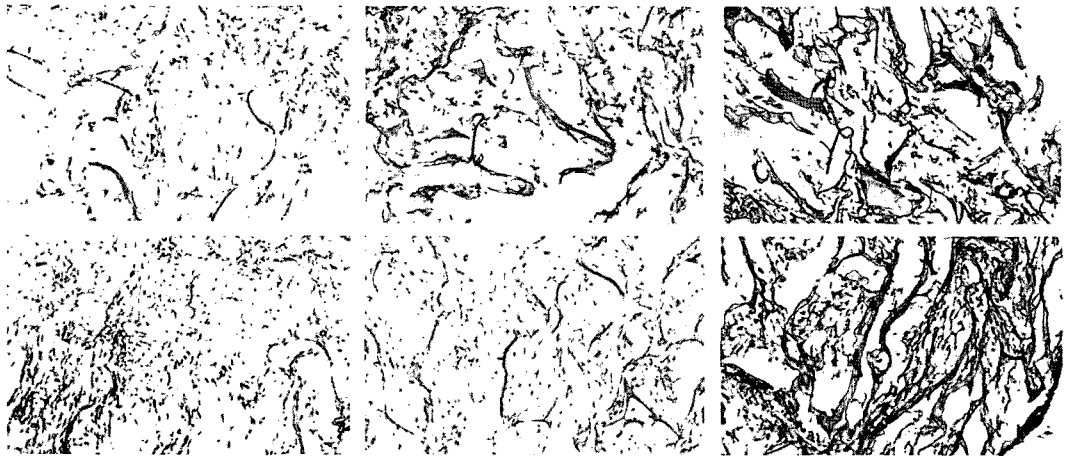

Figure 6. Pictures of cross-sectional immunohistochemical staining of chitosan-based scaffolds at various additional designed culture periods after 1 week under the static culture, (a) vimentin, (b) fibronectin, and (c) collagen type I. Top; 3 weeks under the statically cultured system, Bottom; 3 weeks under the cyclic dynamically cultured system. Magnification: × 100.

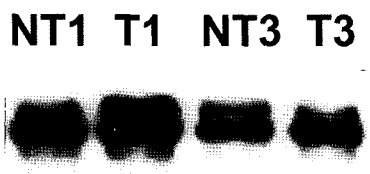

Figure 7. Western blots of fibronectin secreted in culture media under additional static (NT) or cyclic dynamic (T) condition for 1 and 3 weeks, after 1 week under the static culture of chitosan-based scaffolds.

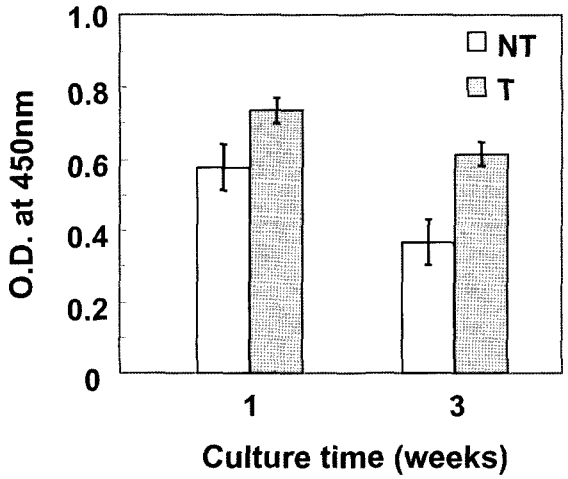

Figure 8. ELISA of procollagen secreted in the cell culture supernatant of chitosan-based scaffolds under additional static condition (NT, blank bar) or additional cyclic dynamic condition (T, gray bar). Error bars indicate standard error of the mean, n = 4.

Conclusions

In this study, we utilized the chitosan-based scaffold as a model system to investigate the effect of cyclic strain on the responses of HDFs. The MTT assay demonstrated that the cell number in the engineered scaffold continuously increased with culture time in both the static and mechanically stimulated groups with a rapid increase in the proliferation rate of HDFs cultured under the mechanical loading. The distribution of HDFs, cultured under the cyclic strain, was homogeneous throughout the scaffold while the cells cultured under static conditions were observed in larger numbers at the periphery of the scaffold. The localizations of ECM proteins, such as, vimentin, fibronectin, and collagen type I, were consistent with the distribution of cellularity. The increased expression of ECM proteins was also indirectly confirmed by an increase in scaffold thickness under mechanical loading by 1.56 fold at 3 weeks compared to that of cell/scaffolds in the static condition. The cyclic strain also stimulated the secretion of procollagen and fibronectin compared to the non-stretched control group. These results suggest that the application of a cyclic strain to cell/scaffold composites may increase cell survival and integration of the artificially engineered dermis at the graft site. In conclusion, understanding the relationship between mechanical stimuli and cellular function within biologically reinforced hybrid scaffolds may have significant implications for tissue and cell engineering.

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