Response of Human Skin Fibroblasts to Mechanical Stretch in Wound Healing Process Analyzed Using a Three-Dimensional Culture Model

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Abstract Hypertrophic scars are frequently observed at wound sites that had been subjected to cyclical stretch stimuli, such as skin of the anterior chest wall and lower abdomen. Previous studies found that cyclic stretch modulated fibroblast infiltration and collagen fiber remodeling compared with static culture conditions. However, these studies used homogeneous cultures that poorly replicated the physiological organization at the wound site. Similar to early studies, we hypothesized that cyclic stretch modulated fibroblast infiltration and collagen fiber remodeling compared with static culture conditions. However, we replaced the homogeneous culture condition used in previous studies with a novel two-gel wound model consisting of an inner decellularized collagen gel mimicking the wound site and an outer fibroblast gel simulating the epidermis. These models were then subjected to either 1-Hz uniaxial cyclical stretch for 3 h each day or were placed under static culture conditions. After day 4 in culture, we found two significant differences between specimens under the cyclic stretch conditions and those under static conditions. First, there were fewer fibroblast infiltrates in the inner wound-mimicking gel in cyclically stretched specimens than in statically stretched specimens. Second, the microstructure and orientation of collagen fibers in cyclically stretched specimens differed histologically from those in static culture. These results add to the growing evidence that cyclical stretch modulates the wound-healing process.

Keywords: skin fibroblast, collagen gel, wound healing, cell infiltration, cyclical stretch.


1. Introduction

A hypertrophic scar is a thick red epidermal tissue thought to result from aberrations of fibroblast behavior during the proliferative and remodeling phases of the wound healing process. Aberrations in the proliferative phase are characterized by insufficient fibroblast proliferation leading to reduced neoangiogenesis, granulation tissue formation at the wound site, and fibroblast apoptosis. This reduced fibroblast apoptosis leads to excessive collagen production, constriction of the wound surface, and development of hypertrophic scars during the remodeling phase of wound healing [1–5].

Although the process of hypertrophic scar formation is well understood, little is known about the cause of the pathogenic aberrations during the proliferative phase of wound healing. There are several reasons for this lack of knowledge. First, hypertrophic scars are unique to humans, making animal models difficult to develop. Second, many hypertrophic scar studies are treatment-focused clinical trials rather than investigations examining the cause of hypertrophic scar [6]. However, a few studies examining the impact of mechanical stress on hypertrophic scar formation have been published. Liu et al. [7] evaluated the effect of stretch on collagen fiber orientation. Akaishi et al. [8] used finite element methods to determine the effect of stretching on hypertrophic scar development. Only a few studies have evaluated the impact of mechanical stretch on an in vitro wound healing model. Takada et al. [9] reported a two-dimensional wound healing model using keratinocytes and acceleration of wound closing by transient stretch.

In this study, we developed a three-dimensional two-gel wound model consisting of an inner decellularized collagen gel mimicking the wound site and an outer fibroblast gel simulating the epidermis. Specimens under cyclic stretch condition were compared against specimens under static condition by evaluating fibroblast cell infiltration and collagen network remodeling as outcome measures.

2. Materials and Methods

2.1 Fibroblast-populated collagen gel preparation

Human neonatal skin fibroblasts (NB1RGB, Riken Cell Bank, Ibaraki, Japan) were cultured in Alpha Medium (Life Technologies, Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum and antibiotics/antimycotics, and passed every 3–4 days. Confluent cells between passages 6 and 8 were detached by 0.25% trypsin and resuspended in collagen solution at a cell density of 1.6 × 10⁶ cells/mL for collagen gel incorporation. Collagen solution (final concentration 2.4 mg/mL) was prepared by mixing reconstruction buffer (Nitta Gelatin Inc., Osaka, Japan), 10x-Alpha MEM, and 3 mg/mL type I acid-soluble collagen (AteloCell; Koken Co. Ltd, Tokyo, Japan) at a ratio of 1:1:8, and adjusting to neutral pH. The wound model was constructed by incorporating a cell-free collagen gel (mimicking wound) in the center, surrounded by a fibroblast-seeded collagen gel (Fig. 1a). Firstly, the fibroblasts/collagen mixture was poured
into a non-treated cell culture dish (#12-0712-3; Corning, NY) that contained a custom-made PDMS mold. The PDMS mold was a 5-mm thick disk with a 20 × 20 mm square cut out in the center. To incorporate an inner wound-mimicking gel, a cylindrical stainless steel piece (diameter, 5 mm) was placed in the center of the mold. Before pouring the collagen-cell mixture, non-woven sterilized mesh was positioned at the four corners of the square to be used later as anchors for handling and stretching. After 20-min incubation to solidify the gel, the stainless steel piece was removed, and fibroblast-free collagen solution was delivered into the well to mimic a wound. The cell-seeded collagen gel in the PDMS mold was cultured in 5 mL of culture media in a CO₂ incubator for an additional 24 h to confirm gel formation and cell adhesion to collagen fibers. The mold was removed before setting up the stretching device (Fig. 1b). The embedded non-woven mesh anchors and the additional 24-h culture allowed handling of the specimen without damage during the experiment. Ten wound model specimens (20 × 20 mm, 3 mm in thickness) were prepared. Five specimens were used in the cyclic stretch group and the remaining five in the static condition group (control group).

2.2 Uniaxial Stretching chamber and culture conditions
A stainless steel uniaxial stretching chamber was used to apply cyclical stretch to the wound model specimen. Fibroblast-seeded collagen gels were clamped within the chamber via the embedded non-woven mesh, and each chamber was used to apply a cyclical stretch stimulus simultaneously to three specimens (Fig. 2a). Before each experiment, the stretching chambers were autoclaved.

The cyclical stretching chamber was connected to a stepper motor driven stage (SGSP26-50X; Sigma Koki, Saitama, Japan) and a stage controller (PAT-001; Sigma Koki) controlled by a custom made program (LabVIEW 2012; National Instruments Corp., Texas, US). During cyclic stretch application, the chamber was maintained at a constant temperature of 37°C by a glass heater (ThermoPlate; Tokai Hit, Shizuoka, Japan). Desiccation was prevented by adding an acrylic cover to the device, and pH level was maintained using CO₂-independent culture medium (Life Technologies, Thermo Fisher Scientific, Waltham, MA).

Cyclic stretch frequency was set at 1 Hz to approximate the human heart rate. Tensile strain was set at 2–2.5% of specimen length to approximate human skin elongation rate in daily activities [10]. As shown in Fig. 2b, cyclic stretch was applied for 3 h/day outside the CO₂ incubator. For the remaining 21 h/day, the specimens were detached from the stretching chamber and cultured in free floating condition inside the CO₂ incubator. This procedure was repeated for 4 days. To monitor the size of the gel specimen, the length of the cyclically stretched gel specimen and

![Fig. 1](image) (a) Preparation and (b) gross appearance of the wound healing model using three-dimensional collagen gel culture.

![Fig. 2](image) (a) Schematic presentation of the cyclical stretching device and (b) time course of stretch application.
the long axis of the wound-mimicking circle under free floating condition were measured by stereo-microscope before the stretching experiment. In the process of clamping each specimen, the position of the stepper motor driven stage was adjusted to maintain the length of the gel equal to that under free floating condition to prevent excessive stretching. To confirm that the appropriate strain (2–2.5%) was imposed on the wound-mimicking gel, changes in diameter of the wound-mimicking circle were measured by stereo-microscope in the first and final 5 cycles of stretching. Control specimens were set in the same chamber without clamping and maintained in a CO₂-dependent medium outside the incubator during this period. Each specimen was cultured for 4 days.

2.3 Fluorescent microscopy and SEM imaging

Each specimen was stained with calcein-AM and the wound-mimicking region was imaged by fluorescent microscopy on day 4 of culture. The calcein-AM solution was prepared by mixing calcein-AM and Alpha MEM to a final concentration of $2.0 \times 10^{-3}$ mg/ml. Specimens were soaked in calcein-AM solution for 10–15 min and visualized by fluorescence microscopy. After visualization, the specimens were fixed for 24 h in 4% paraformaldehyde and then dehydrated in a graded ethanol solution series (50–99.5%). Then, the surfaces of the specimens were removed by a diamond knife and the specimens were immersed in t-butyl alcohol and freeze-dried with a t-butyl alcohol freeze dryer to prevent specimen shrinkage. Specimens were then osmium-coated using an osmium coater. Finally, collagen fiber structures were observed by a scanning electron microscope (SEM; JSM-7600F; JEOL Ltd., Tokyo, Japan).

2.4 Quantification of fibroblast infiltration ratio

The degree of fibroblast infiltration into the wound-mimicking region was determined by dividing the area (pixels) infiltrated by fibroblasts in the wound-mimicking site by the total area (pixels) of the wound-mimicking site (fibroblast infiltration ratio), using ImageJ (National Institutes of Health, USA). Wound-mimicking area was measured using the calcein-AM-stained fluorescent images. Fibroblast infiltration area (pixels) was acquired by thresholding the fluorescent images. This threshold was determined individually using the average of the maximum and minimum intensities in each calcein-AM-stained image.

Each data point represents the mean and standard deviation of five specimens. Experimental data were analyzed by Student’s $t$-tests to evaluate significant differences between the specimens with and without cyclic stretch.

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Fig. 3 (a) Four different positions of the wound area for SEM observation and (b) analysis of the flow of collagen fibers on SEM images.
2.5 Quantification of collagen fibers and orientation

The collagen fiber microstructure was evaluated using SEM images. Four different positions were imaged: the central part of the wound-simulating region (“center”), the middle of the wound-simulating region (named “middle”), the boundary between the wound-simulating and fibroblast-seeded regions (“boundary”), and the outer fibroblast-populated region (“exterior”) (Fig. 3a). To evaluate the effect of stretching direction, “boundary” was imaged at the intersection point between the wound-mimicking circle and axis parallel and perpendicular to the stretching direction (“boundary/parallel” and “boundary/perpendicular”). Fiber microstructure was compared among specimens and the aforementioned positions by ImageJ to trace the fibers in each SEM image. Briefly, the fibers were traced by straight lines and the length and angle of each line were measured (Fig. 3b). The effect of stretching on collagen fiber orientation was evaluated by the angular distribution of traced lines. The angles were measured from −90° to 90° relative to the stretching direction (0°). In a preliminary study, cell-free collagen gels with or without cyclic stretch were evaluated by this SEM image-based procedure. The result showed no difference in density of collagen fibers (data not shown). Therefore, the SEM image-based procedure is suitable for assessing the effect of cyclic stretch on fibroblast-induced collagen reorganization.

3. Results

3.1 Behavior of fibroblasts infiltrating into the wound-simulating region

Living cells were stained by calcein-AM to evaluate fibroblast infiltration into the wound-mimicking region. In both control and experimental conditions, fibroblasts invaded the wound-mimicking region and moved toward its center. However, significant differences existed between the two conditions. First, control specimens contracted by 50% compared to the initial gel area, while cyclical stretch specimens contracted only by 33%. Second, greater fibroblast infiltration was observed in control specimens (29% fibroblast infiltration) than in cyclically stretched specimens (16% fibroblast infiltration) (Figs. 4 and 5). Moreover, the fluorescent images indicated that the number of cells around the wound-mimicking region decreased and that many cells were round shaped in cyclically stretched specimens (Fig. 4a and c).

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Fig. 4 Calcein-AM-stained images and binary images of wound-simulating region. (a) and (b) Control specimens, (c) and (d) cyclically stretched specimens. Scale bar: 1 mm.
3.2 Collagen production and degradation by infiltrating fibroblasts

SEM images of the four different regions (center, middle, boundary, and external) in a control specimen and a cyclically stretched specimen are shown in Figs. 6 and 7, respectively.

Because the specimens were made of collagen gel, the collagen fibers derived from the gel were relatively longer and thicker than those secreted by fibroblasts. From this point of view, it would appear that two kinds of fibers coexisted in the fibroblast-seeded or -migrated area. The collagen fibers in cyclically-stretched specimens were thin and the surface of each fiber was smooth, while those in control specimens were thick and showed irregular surfaces.

To evaluate the effect of cyclic stretch on collagen fiber orientation, the fiber angles were compared between two regions of interest: center and boundary. Figure 8 shows the distribution of the angles of collagen fibers measured by the traced lines on SEM images. Variations in both length and angles were observed in control specimens, while longer fibers parallel to the stretching direction were found in cyclically stretched specimens. Therefore, the polar graphs showed that collagen fibers tended to align along the direction of cyclical stretch.

4. Discussion

In this study, we developed an in vitro three-dimensional wound healing model using a fibroblast-seeded collagen gel. Using this model, the proliferation phase of the wound-healing process
could be partially simulated. Although other researchers have developed wound or wound-like models utilizing three-dimensional matrices to study cell infiltrations from cell-dense areas to cell-free areas, their models could not be subjected to cyclic stimuli to replicate the physiological condition of the wound [11, 12]. The advantages of our model are that cyclic stretch can be applied to fibroblasts three-dimensionally and cell infiltration into the wound-mimicking area can be evaluated. In this study, we focused on one physiological condition: wound in the breast region subjected to cyclical stretch at the heart rate (nearly 1 Hz).

In both specimens (with and without cyclic stretching), fibroblast infiltration toward the cell-free area was observed. This infiltration could be explained by multiple factors. Dense populations of fibroblasts may generate chemotactic gradients via growth factor internalizing and degradation [13–15]. Also, contact inhibition of locomotion could explain directional infiltration of fibroblasts from densely cell-populated regions to sparsely populated regions.

On the other hand, the rate of fibroblast infiltration in cyclically stretched specimens was lower than that in control specimens. In addition, from the fluorescent images of cyclically stretched specimens, the fibroblasts became round shaped and the

![Collagen fiber microstructure in a cyclical stretched specimen.](image)
number decreased compared with static specimens. These results suggest that cyclical stretch retards the proliferation and migration of dermal-fibroblasts. These findings are in partial agreement with earlier work reporting that equi-biaxial stretch slowed epithelial cell migration in stretch wound assays [16, 17]. However, Takada et al. [9] created a two-dimensional wound-closing model using keratinocytes and reported that migration of cells was promoted by transient stretch. This disagreement might be derived from multiple differences in cell type (keratinocyte vs. dermal fibroblast), culture condition (two- vs. three-dimensional culture), and amplitude of stretch ratio (20% vs 2–2.5% in our study) between their research and the present study. However, additional studies by changing the amplitude and frequency of cyclic stretch are still required to further investigate the difference in cell infiltration.

The fibroblast infiltration behavior observed in this study could possibly be a result of integrin-mediated mechanosensing. Integrins is a cell adhesion molecule and is connected to stress fibers. On activation of integrins by cell-membrane deformation, stress fibers also activate the mechanosensitive channel of cells.

Fig. 8  Polar graphs of orientation angles and lengths of collagen fibers. (a) center of control specimens, (b) boundary of control specimens, (c) center of cyclical stretched specimens, (d) boundary/parallel of stretched specimens, (e) boundary/perpendicular of stretched specimens.
This sequence of events leads to cell transformation and migration to the wound area, resulting in cell infiltration. When cells are stretched during the wound healing process, a large amount of ATP is released, which in turn leads to an increase in Ca\(^{2+}\) concentration in the cell, and the Ca\(^{2+}\) reaction promotes cell infiltration [18, 19]. From this point of view, our results disagree with mechanosensing process. It was suggested that multi-cycle stretch in three-dimensional condition might inhibit normal Ca\(^{2+}\) reaction, promoting fibroblast migration. This disagreement in three-dimensional culture condition may be related to in vivo inhibition of normal wound healing process. In future studies, this hypothesis should be examined by analysis involving observation of stress fibers and quantification of Ca\(^{2+}\).

Collagen fiber microstructures in cyclically stretched specimens also differed from those in control specimens. In all regions of the wound-mimicking gel, the densities of collagen fibers in control specimens were greater than those in cyclically stretched specimens. In cyclically stretched specimens, the density of collagen in the central region was lower than in other regions. These results suggest that infiltration of fibroblasts in control specimens caused collagen contraction homogeneously, while fibroblast infiltration in cyclically stretched specimens was not sufficient to cause collagen gel contraction in the central region. Moreover, the cyclical stretch also affected collagen fiber orientation. The change in fiber orientation may be derived from collagen reconstruction by fibroblasts or anisotropic gel contraction under cyclic stretching. However, further studies are required to assess these speculations by distinguishing between collagen fibers that are produced by fibroblasts and those that are the component of the collagen gel; for example, enzyme-linked immunosorbent assay can be used to detect collagen fibers produced by fibroblasts.

5. Conclusion

We developed an in vitro wound healing model using a fibroblast-seeded collagen gel subjected to cyclic stretch. In this model, a central disc of collagen gel without cells to mimic skin wound was surrounded by fibroblast-populated collagen gel. Using uniaxial cyclic stretching in this model, we investigated the effect of stretching on the wound healing process by examining fibroblast infiltration ratio and collagen microstructure. The results suggest that 1-Hz cyclic stretch stimulus inhibits fibroblast infiltration into the wound area and may affect collagen orientation.

Conflict of interest

We have no conflicts of interest relationship with any companies or commercial organizations based on the definition of Japanese Society of Medical and Biological Engineering.

References

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