A novel FPCL model producing directional contraction through induction of fibroblast alignment by biphasic pulse direct current electric field

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ABSTRACT

Although parallel alignment of fibroblasts to the tension lines of scar has been evidenced in vivo, how scar contracture generates directional contraction remains largely unclear due to the lack of effective in vitro model. Fibroblast populated collagen lattice (FPCL), a widely used in vitro model, fails to mimic scar contracture since it produces concentric contraction with the random orientation of fibroblast. We hypothesized that a novel FPCL model with fibroblast alignment might produce directional contraction and then simulate scar contracture better. Here, we showed that although direct current electric fields (DCEFs) enabled fibroblasts aligned perpendicularly to the field vector, it also promoted electrotactic migration of fibroblast in FPCL. By contrast, biphasic pulse direct current electric fields (BPDCEFs), featured by reversal of the EF direction periodically, abolished the electrotactic migration, but induced fibroblast alignment in a pulse frequency dependent manner. Specifically, BPDCEF at a pulse frequency of 0.0002 Hz induced fibroblast alignment comparable to that induced by DCEF under the same field strength (300 mV/mm), leading to an enhanced contraction of FPCL along the direction of cell alignment. FPCL pretreated by BPDCEF showed an elliptical contraction whereas it was concentric in control FPCL. Further study revealed that F-actin redistributions acted as a key mechanism for the induction of fibroblasts alignment by BPDCEF. Cytochalasin D, an inhibitor of actin dynamics, abolished F-actins redistribution, and significantly suppressed the fibroblasts alignment and the directional contraction of FPCL. Importantly, BPDCEF significantly increased RhoA activity in fibroblasts, while this response was attenuated by C3 transferase pre-treatment, a potent inhibitor of RhoA, caused F-actin depolymerization and actin filament bundle randomly distributed. Taken together, our study suggests a crucial role for fibroblast orientation in scar contracture, and provides a novel FPCL model that may be feasible and effective for investigating scar contracture in vitro.

1. Introduction

Contractures following hypertrophic scar remain a big challenge for patients after traumatic or burn injuries, leading to poor cosmetic outcome, impaired joint range of motion, and even permanent loss of function [1,2]. The most important feature for contracture is the development of scar contraction along the direction of tension lines. Parallel alignment of fibroblasts as well as collagen fibers to the tension lines have been evidenced previously with electron microscopy, which raised an assumption that the aligned fibroblast coupling to the extracellular collagen fibers might be the key basis for scar contracture [3]. However, without an effective in vitro model to simulate the dynamics of contracture, whether or how fibroblast alignment contributes to the directional contraction of scars remains largely unclear.

Fibroblast populated collagen lattice (FPCL), as established in 1979 by Bell E., is a commonly used model for scar contraction in vitro [4]. Using this model, numerous studies have been conducted as an attempt to increase our understanding on scar contracture, but unfortunately have not resulted in notable therapeutic advances. This might be, at least partially, due to the fact that fibroblasts in FPCL are oriented randomly, but not aligned parallelly as that observed in scar contracture. As a consequence, FPCL produces concentric contraction, but not directional contraction [3,5]. We hypothesized that a modified FPCL model with fibroblast alignment might produce directional contraction and then simulate the scar contracture better in vitro.

Endogenous direct current electric fields (DCEFs), generated

instantaneously after skin injury due to disappearance of the transepithelial potentials, play an important role in cell migration and orientation [6,7]. Interestingly, exogenous application of DCEFs at physiologic strength was shown to induce fibroblasts alignment perpendicular to the field vector in either 2D or 3D culture although the mechanisms are largely unclear [8,9]. Nevertheless, DCEFs also promote directional migration of cells (electrotactic migration), which may lead to a unsymmetrical distribution of the cells and then should not be acceptable when considering constructing a new FPCL model with fibroblasts alignment by DCEFs stimulation. However, the onset of electrotactic migration normally takes tens of minutes depending on cell type and field strength [10]. This allows us to consider that a biphasic pulse direct current electric fields (BPDCEFs), featured by the reversal of EF direction periodically, might abolish the electrotactic migration, but still induce cell alignment.

In this study, we applied BPDCEF to FPCL with the aim to develop a novel FPCL with fibroblast alignment. We found that: 1) in comparison to DCEF, BPDCEF at a specified frequency enabled fibroblasts aligned perpendicularly to the field vector as DCEF did in FPCL, but abolished the electrotactic migration completely; 2) induction of fibroblasts alignment led to a directional contraction of FPCL along the cell alignment or the Y-axis of FPCL; 3) F-actin redistribution was found to be the key mechanism for fibroblast alignment induced by BPDCEF; 4) Cytochalasin D, an inhibitor of actin dynamics, abolished F-actins redistribution, and significantly suppressed fibroblasts alignment and the directional contraction of FPCL. 5) Rho signaling played an important role in BPDCEF-induced F-actin redistribution and cell alignment. Our study suggests a crucial role for fibroblast orientation in scar contracture, and provides a novel model that may be more effective for studying scar contracture in vitro.

2. Materials and methods

2.1. Ethics statement

BALB/c mice (male and female, 1–1.5 g) and SD rat (male, 200–250 g) were obtained from the Experimental Animal Department of the Third Military Medical University (Army Medical University). All of the animal procedures were approved by the Animal Experiment Ethics Committee of the Third Military Medical University. All methods were performed in accordance with the guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Pub. No. 85–23, revised 1996).

2.2. Cell culture

Fibroblasts were obtained from skin of newborn BALB/c mice (postnatal day 1–3) as described previously [11]. Briefly, fibroblasts were isolated from dermal tissue by 0.25% trypsin/0.04% EDTA solution (Invitrogen, USA) at 4 °C overnight. The fibroblasts were then plated into dishes and cultured in DMEM/F12 containing 10% fetal bovine serum (Gibco, USA) and 100 U/ml penicillin (Invitrogen, USA), and 100 mg/ml streptomycin (Invitrogen, USA). The cells were incubated at 37 °C in 5% CO2 and 95% humidity for 24 h and washed gently with warm phosphate-buffered saline (PBS) to remove non-adherent cells. The media were then refreshed. Passage 2 to Passage 5 cells were used in our experiments.

2.3. 3D FPCL culture

FPCL were generated by mixing rat tail collagen and fibroblasts together according to the method described previously [12]. The isolation of native collagen from rat tails was performed using the salt purification and then resuspended the pellet in 100 ml of ice-cold 0.1 M acetic acid [13]. Medium containing fibroblasts and serum, acetic acid containing collagen at a volume ratio of 1:1:8 mixed completely with

agitation at 4 °C. Once FPCL was formed, 1.0–2.0 ml medium was added into each dish and then incubated at 37 °C in 5% CO$_2$ for 4 days. The initial density of the collagen fibrils was 2 mg/ml. FPCLs were created at low (0.3×10^5; LD), moderate(1.5×10^5; MD) or high(15×10^5; HD) cell density.

2.4. EF stimulation and the imaging of cell migration or orientation and FPCL contraction

For observe fibroblasts orientation under EFs in 2D or 3D model. A previous galvanotaxis chamber was improved to stimulate cells with electric fields [6,14,15]. EFs were applied through two silver electrodes immersed in Steinberg's solution-filled reservoirs that were connected to wells at each end of the electrotaxis chamber (Height of 0.15 mm for 2D, Height of 0.45 mm for 3D) by two agar bridges (2% agar in Steinberg's solution). The culture media were supplemented with 15% fetal bovine serum and 20 mM HEPES buffer in the EF stimulation experiments. Direct current electric fields (DCEFs) was applied with strengths of 0, 100, 200 and 300 mV/mm for 4 h, which was the unidirectional flow of charged particles along the same direction (Supplemental Fig. S1A). Biphasic pulse direct current electric field (BPDCEF) was stimulated using biphasic square pulses at 300 mV/mm with frequencies of 100, 1, 0.01, 0.0002 Hz (duty ratio = 1, the proportion of power-on work time to total time was 100% in a pulse cycle) for 4 h, the pulse width during this experiment was 0.01 s, 1 s, 100 s, and 5000s corresponding to the above frequencies respectively, which was bidirectional and consists of two phases (Supplemental Fig. S1B). One phase left the isoelectric line, and after a finite time returned to baseline; another phase left the isoelectric line in the opposite direction, and after the equal time returned to baseline, which showed reversal of their direction periodically (Supplemental Fig. S1B). The time-lapse imaging of cell orientation or FPCL contraction was performed with a Zeiss imaging system (Carl Zeiss Meditec, Jena, Germany) or Nikon Eclipse imaging system. Time-lapse images were analyzed using NIH ImageJ software.

2.5. Inhibitor

Fibroblasts were treated with cytochalasin D (1 μmol/L), colchicine (5 μmol/L) or C3 transferase(20 μg/ml) for 10 min before EF stimulation. These pharmacological inhibitors are purchased from Sigma.

2.6. Immunostaining

Fibroblasts were seeded onto galvanotaxis chamber for 4 h under EFs or not. Then the cells were quickly washed twice with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature. After 3 washes with PBS, cells were blocked with 5% bovine serum albumin (BSA) (Sigma-Aldrich). Fibroblasts were stained with rhodamine phalloidin(proteintech, USA) at 1:35 dilution for the detection of F-actin or with Cyanine 3(proteintech, USA) at 1:100 dilution for the detection of α-tubulin.

2.7. Quantitative analysis of cell migration, cell orientation and FPCL contraction

Cell migration, cell orientation and FPCL contraction were analyzed using methods reported previously [15–17]. The directedness of cell migration was expressed as a function of cosθ, where θ was the angle between the EFs vector and a straight line connecting the start and end position of a cell. Values close to 0 represent random cell movement, and those close to 1 represent cells moving toward the cathode. Values close to −1 represent cells moving toward the anode. The values of cosθ range from −1 to +1 and quantify the direction of cell migration. The displacement speed was the straight-line distance between the start and end positions of a cell divided by time. Briefly, the orientation