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Interstitial fluid flow-induced growth potential and hyaluronan synthesis of fibroblasts in a fibroblast-populated stretched collagen gel culture

Natsumi Saito[a,1], Hiroaki Adachi[b], Hiroshi Tanaka[b], Satoru Nakata[b], Norifumi Kawada[a], Ken Oofusa[c], Katsutoshi Yoshizato[d,e,*]

[a] Department of Hepatology, Graduate School of Medicine, Osaka City University, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan
[b] Research Laboratories, Nippon Menard Cosmetic Co., Ltd., 2-7 Torimi-cho, Nishi-ku, Nagoya 451-0071, Japan
[c] IDEA Consultants, Inc., 1-24-22 Nanko-kita, Suminoe-ku, Osaka 559-8519, Japan
[d] Synthetic Biology Laboratory, Graduate School of Medicine, Osaka City University, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan
[e] PhoenixBio Co. Ltd., 3-4-1 Kagamiyama, Higashi-Hiroshima 739-0046, Japan

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ABSTRACT

Background: Tensioned collagen gels with dermal fibroblasts (DFs) as a dermis model are usually utilized in a static culture (SC) that lacks medium flowing. To make the model closer to its *in vivo* state, we created a device to perfuse the model with media flowing at a physiological velocity and examined the effects of medium flow (MF) on the cultures.

Methods: We constructed a medium perfusion device for human DF-embedded stretched collagen gels (human dermis model), exposed the model to media that flows upwardly at ~1 mL/day, and examined water retention of the gels, cells' growth ability, metabolic activity, expression profiles of nine extracellular matrix (ECM)-related genes. The obtained data were compared with those from the model in SC.

Results: MF increases the gels' water retention and cells' growth potential but had little effect on their metabolic activities. MF robustly enhanced hyaluronan synthase 2 (*HAS2*) and matrix metalloprotease 1 (*MMP1*) gene expressions but not of the other genes (*MMP2*, *HYAL1*, *HYAL2*, *HYAL3*, *COL1A1*, *COL3A1*, and *CD44*). MF significantly increased the amounts of cellular hyaluronan and adenosine triphosphate.

Conclusions: The MF at a physiological speed significantly influences the nature of ECMs and their resident fibroblasts and remodels ECMs by regulating hyaluronan metabolism.

General significance: Fibroblasts in tensioned collagen gels altered their phenotypes in a MF rate-dependent manner. Collagen gel culture with tension and MF could be utilized as an appropriate *in vitro* model of interstitial connective tissues to evaluate the pathophysiological significance of mechanosignals generated by fluid flow and cellular/extracellular tension.

1. Introduction

Researchers have been seeking a suitable cell culture method by which cells can be cultured under conditions as close as possible to the *in vivo* state. To accomplish this goal, various efforts have been made regarding dimensionality [planer [2-dimensional (2-D)] versus steric (3-D)], substrate compatibility (artificial versus native), medium supply method [static (not-flowing) versus flowing (perfusion)], and mechanical characteristics (relaxed versus stressed). So-called "collagen gel culture (CGC)" of dermal fibroblasts, originally described by Gey [1] and developed by Bell [2], was one of the trials to successfully recapitulate a living tissue (dermis in this case, "dermis equivalent") *in vitro*. Since then, the idea and technology of CGC have been applied to a wide variety of tissues, such as tendon [3], bone and cartilage [4], and liver [5]. CGC has thus become a common method for reproducing living tissues *in vitro*. Generally, the CGC cultivates cells in 3-D lattices of type 1 collagen fibril gels, and this culture is thought to better reflect the *in vivo* state than conventional 2-D cell culture, because it is 3-D

Abbreviations: CGC, collagen gel culture; (H)DFs, (human) dermal fibroblasts; ECM, extracellular matrix; SC, static culture; MF(C), medium flow (culture); 2-D, two-dimensional 3-D, three-dimensional; CF(s), collagen fibril(s); IF, interstitial fluid; ATP, adenosine triphosphate; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; DW, distilled water; DMEM/S, DMEM with supplements; PFA, perfluoroalkoxy; H & E, hematoxylin and eosin; MT, Masson's trichrome; PCNA, proliferating cell nuclear antigen; DAB, 3,3′-diaminobenzidine; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; HA, hyaluronan; ELISA, enzyme-linked immunosorbent assay

* Corresponding author at: Synthetic Biology Laboratory, Graduate School of Medicine, Osaka City University, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan.
E-mail address: katsutoshi.yoshizato@phoenixbio.co.jp (K. Yoshizato).
[1] Present address, Department of Plastic Surgery, Jichi Medical University 3311-1, Yakushiji, Shimotsuke, Tochigi 329-0498, Japan.

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(dimensionality), made of collagen fibrils (CFs) [substrate compatible, and receives tensile strength (mechanical qualification)]. Two types of fibroblast-populated collagen gels have been reported: gel that is floating in medium (detached, floating, or relaxed gel), and gel that is attached to a culture dish (attached, stretched, or stressed gel) [6].

CGCs have been well characterized using the detached collagen gel model, in which the gel floats in the standing medium. The contracted (shrunken) gel culture has been thought to be similar to a histologically connective tissue-like structure [2] and has been often utilized in studies on mechanisms of wound contraction [7]. Fibroblasts in the model robustly exert tractive force on CFs in an early phase of culture, which results in a decrease of the gel's original volume [2]. Then, if the culture dish surface is hydrophobic in its nature, the gel detaches itself from the dish surface and continues to contract until the cells' contractile force balances the gel's stiffness. The resulting gel, which floats in static medium and stably retains its volume for a long period ($>$ 1 week), has been utilized as an *in vitro* model of connective tissue [7].

It is thought that the living dermis context-dependently exhibits tensile strength intrinsically loaded by the collagen fiber-dominated ECMs [8], indicating that the dermis is in a mechanically elastic and tense state ("physiological tension"). Actually, several studies measured the tensile strength of living human dermis and reported variations of the values of Young's moduli, ranging from 8.3 kPa to 20 MPa, depending on the age of subjects, the tested part of body, anisotropy, and the measurement method [9–15]. It has been generally recognized that the tensile strength of ECM is vital in tissue homeostasis, morphogenesis of embryonic development [16–18], cell migration, matrix remodeling [19,20], and induction of differentiation [21]. These studies suggested to us that the CGC must be used under conditions in which both CFs and fibroblasts are continuously under physiological tension. As a matter of fact, there have been studies that characterized the stretched type of collagen gel cultures of fibroblasts mainly focusing on the expressions of ECM metabolism-associated genes in comparison with the relaxed type as reviewed by Wang *et al.* [22]. These studies generally agreed that the cells in the stressed CGC exhibit "synthetic" phenotypes, while those in the relaxed CGC exhibit "catabolic" one [23–26].

Generally, cells in living tissues are perfused with incessantly flowing interstitial fluid (IF), which not only provide cells with nutrients and O_2 but also with shear stress [27]. The order of magnitude of the interstitial fluid velocity in the mammalian tissues is considered to be 0.1 to 1 μm/s [28]. Researchers have well appreciated the biological and pathophysiological significance of IF flow as a basic regulator of the activities of the resident cells in tissues [29]. However, studies to verify such recognition under near-physiological conditions have been limited until recently largely due to the lack of appropriate experimental devices. Wang and Tarbell first developed a cell culture system as an *in vitro* model to examine the effect of interstitial fluid flow on physiological activities of vascular smooth muscle cells [30]. In the cited study the authors exposed the cells cultured in 3D collagen gels to the shear stress caused by fluid flowing at a velocity comparable to that of IF and showed that these cells produced prostaglandins in a unique manner responding to the stress as compared to those in the static collagen gel culture and those in a 2D culture. Importantly with respect to the present study, the CGC system was adopted in the cited study as a suitable model that faithfully reproduces the *in vivo* state of vascular connective tissues.

Since then, several studies have been reported that examined the roles of IF in regulating pathophysiology of connective tissue cell. For example, Shi *et al.* cultured vascular fibroblasts, myofibroblasts, and smooth muscle cells in collagen gels and loaded them with shear stress by flowing medium at a physiological velocity of ~0.5 μm/s; they then showed that these cells altered their gene expression profiles to respond to the shear stress [31,32]. These studies were mostly conducted in a short period of time (within a few hours) using vascular tissue-associated cells, the cells that are likely to be affected by stresses caused by fluid flowing. To our knowledge, there have been no studies that developed an in vitro 3-D model of dermis for investigating phenotypic alterations of caused by culture medium flowing over a long-time periods of culture against those in the non-flowing system.

In this study, we examined the effects of IF on human dermal fibroblasts (HDFs) in CGC, adopting the stretched type of fibroblast-populated collagen gel as a more faithful model of the living connective tissue based on the above-mentioned preceding studies. The present study was conducted with two purposes. Firstly, a culture system was developed in which HDFs were cultured in the tensioned collagen gel and bathed in medium continuously flowing at a velocity comparable to an *in vivo* IF velocity for a long period of time (up to at least 6 days). Secondly, utilizing this system, we determined the effects of medium flow (MF) on basic biochemical properties of collagen gels and HDF phenotypes. As a result, we demonstrated that the gels in MF culture (MFC) increase in dry weight and water retention compared with those in static culture (SC). Fibroblasts in MFC significant increase growth capacity, adenosine triphosphate (ATP) content, and expression levels of genes of ECM metabolism-associated enzymes, such as *MMP1* and *HAS2*. Notably, hyaluronan (HA) contents in MFC gels are prominently increased compared with those in SC gels.

2. Materials and methods

2.1. Preparation of collagen gel

Atelocollagen (KOKEN CO., LTD, Tokyo, Japan) was mixed on ice with 2-fold concentrated Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific Inc., Waltham, MA) with the following 2-fold concentrated supplements: 20% (v/v) heat-inactivated fetal bovine serum (FBS), 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma-Aldrich, St Louis, MO), 3.7 g/L of sodium bicarbonate (Sigma-Aldrich) and antibiotics (Sigma-Aldrich, 200 units/mL penicillin and 200 mg/mL streptomycin). When necessary, the final concentration of DMEM and supplements was correctly adjusted to 1-fold by adding distilled water (DW). HDFs were suspended in the obtained collagen sol for preparing HDF-populated collagen gels, as detailed below. The initial collagen concentration was optimized in its stiffness to improve the handling of the sol by changing its concentrations from 0.25 to 3 mg/mL. In this study, we usually used collagen gels with a final concentration of 2 mg/mL of collagen, considering the result of the optimization experiment.

2.2. Preparation of HDF-populated collagen gels

Neonatal HDFs (Thermo Fisher Scientific) were maintained in 75-cm^2 tissue culture-treated flasks (Greiner Bio One, Frickenhausen, Germany) at 37 °C with serial cultivation with a 1:1 split in DMEM with supplements (DMEM/S) as described above, in an incubator gassed with humidified air containing 5% CO_2. Cells at fewer than 12 passages were used in this study. The cell viability was determined by the trypan-blue (Wako Pure Chemical industries, Ltd. Osaka, Japan) exclusion test. HDFs in 75-cm^2 flasks were treated with 0.25% (w/v) trypsin-EDTA (Gibco) for 2 min at room temperature (RT), removed from the flasks by gently dispersing them twice with Dulbecco's phosphate-buffered Saline (D-PBS, Sigma-Aldrich), pelleted by centrifugation at 367*g* for 5 min at 22 °C and were homogenously suspended (usually at 2×10^5 cells/mL) in 2 mg/mL collagen sols in DMEM/S. The cell suspensions in collagen sols were dispensed into 35-mm Falcon™ cell culture dishes (Corning, NY), usually at 3 mL per dish, and were incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 2 h to gelate the sols. The gels were overlaid with 1 mL of DMEM/S and cultured.

2.3. Coating of culture dishes

We made 4 types of 35-mm culture dishes, whose surfaces had been