Collagen Matrix Stiffness Influences Fibroblast Contraction Force

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Abstract. Cell-embedded hydrogel has been widely used as engineered tissue equivalents in biomedical applications. In this study, contraction force in human aortic adventitial fibroblasts seeded within a 3D collagen matrix was quantified by a novel force sensing technique. We demonstrate that contraction forces in cells treated with histamine are regulated by the gel stiffness in a linear manner. These findings provide novel insights for the design of collagen-based biomaterials for tissue engineering and clinical applications.

1. Introduction

The culture of cells within 3D matrices has been used for several decades [1]. The behaviours of cells in tissue-like matrices, such as migration, contraction and proliferation, exhibit an ideal model of their *in vivo* phenotype. Several materials have been developed for the synthesis of cellular encapsulated hydrogels. Collagen, alginate, fibrin and agarose are extensively used as the natural polymer ingredients to construct engineered tissue equivalents [2]. Among these, collagen has been recognised as one of the major components in connective tissues, which forms stable fibrils and provides multiple cellular binding sites [3, 4]. Fibroblasts are the main cell type that influence remodelling and biosynthesis of collagen fibrils [1]. As the structure of collagen provides a mechanically stable framework,

the contractile properties of fibroblasts seeded within a collagen matrix provides a good model for investigating tissue physiology and pathology.

Currently, one of the most prevailing techniques to obtain cellularised collagen gels is modified from the method of Bell *et al* [5]. Measuring the contraction of cells embedded in 3D matrices is relatively straight forward using the conventional collagen gel contraction assay. When cells start to contract, the collagen matrix normally exhibits a noticeable reduction in size. However, previous studies using collagen gel-based assays to measure fibroblast contractility were limited to only examining the changes in overall gel radius and hence an accurate determination of cell contraction force was difficult to achieve. The concentrations of collagen used to form the gels reported in research studies are very different. Therefore even a same degree of gel radius shrinkage does not provide an accurate representation of the same magnitude of contraction force, because the quantity of matrix stiffness needs to be considered in the determination of cellular contraction forces [6]. To date, there are very few reports demonstrating that the stiffness of collagen matrices can influence cellular contraction forces.

At the site of vascular diseases such as atherosclerosis, arterial injury leads to enhanced adventitial fibroblast contraction and changes in the extra cellular matrix (ECM) contributing to vessel remodelling and restenosis [7]. Collagen fibrils form a tightly aligned structure due to the synthesis of connective tissues following vessel injury and inflammation. Fibroblasts can produce a large amount of collagen protein to increase the overall tissue tensile strength that contributes to vascular stiffening and impaired function [8]. The presence of differentiated contractile myofibroblast has been associated with the increase of ECM stiffness and they mediate the production of force for wound contraction [9]. The interaction between fibroblasts and various ECM components has been

investigated using a number of engineering tools. These methods include cell populated micropillar substrates [10] or Traction Force Microscopy (TFM) [11, 12]. For both methods, cell contraction force is determined indirectly based on the relative displacement of substrate materials, such as the deflections of micropillars or the movements of fluorescence microbeads embedded polymer substrate. However, both of these methods do not provide an accurate assessment of cell behaviour as they only measure the contraction of fibroblasts on a two-dimensional surface while the actual physiological environment *in vivo* for cell contraction within the extracellular matrix is three-dimensional.

To overcome these challenges in the accurate measurements of cell contraction force, we have recently developed a novel nano-indentation device to measure the gel elasticity, and in combination with mathematical modelling, cell contraction force can be determined accurately based on the measured thickness and area of a disk-shaped cell-embedded collagen matrix [13]. In this study, we have applied the new technique to investigate the effect of collagen gel stiffness on cell contraction force in a quantitative manner.

2. Materials and Method

Confluent cultures of human aortic adventitial fibroblasts (HAoAF, PromoCell, Germany) were detached from flasks using trypsin/EDTA and cells were re-suspended in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (FCS). Cells were then mixed with culture medium containing collagen I on ice, to achieve a final density of 2.16×10^5 cells/gel in DMEM containing 2mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 5% FCS and different collagen concentrations of 1.5, 2.0 and 2.5 mg/ml. The collagen matrix cell suspension was transferred into 35 mm Petri dishes (1.2 ml/dish) and incubated in a culture incubator (37°C, 5% CO_2) for 20 minutes to allow gel polymerisation before addition of a further 1.5 ml DMEM containing 5% FCS. After incubation overnight, the culture medium was replaced with fresh DMEM containing 5% FCS. Some dishes were then stimulated with the agonist histamine (100 µM) to elicit cell contraction. Collagen gels were then immediately dislodged from the dish using a sterile spatula with gel thickness and elasticity measurements conducted 5 hours later.

Histamine was employed in this study to elicit HAoAF contraction as it has been well characterised to act via G-protein coupled receptors. Upon activation, it triggers calcium release from the internal stores, leading to calmodulin-dependent myosin light chain (MLC) kinase activation resulting in MLC phosphorylation and subsequent actin-myosin crossbridge formation and cellular contractions [14-16]. We demonstrated in our previous study that addition of histamine (100 µM) increased single HAoAF cell contraction force by two-fold and contractions were attenuated by treatment with ML-7, an inhibitor of MLC kinase [13]. Therefore, we used histamine (100 µM) in this study to elicit short-term contraction events in HAoAF.

A depth-sensing indentation device (Fig 1A) was used to measure the thickness and Young's modulus of the cell-embedded collagen gel. The system has ultimate force and displacement resolutions of 10 nN and 100 nm, respectively. The gel indentation was performed at a controlled speed of 40 μm/sec to generate force-displacement curve. The first 30% of the curve was extracted to determine Young's modulus (*E*) by fitting a non-linear strain dependent elasticity model as shown in Fig 1B. The Young's modulus E_0 of collagen gel was determined from the measured Force-Displacement (*F-D*) curve fitting with the following non-linear mechanical equation [13], which was derived based on Hertz contact theory [17] in combination with non-linear strain dependent elasticity [18].

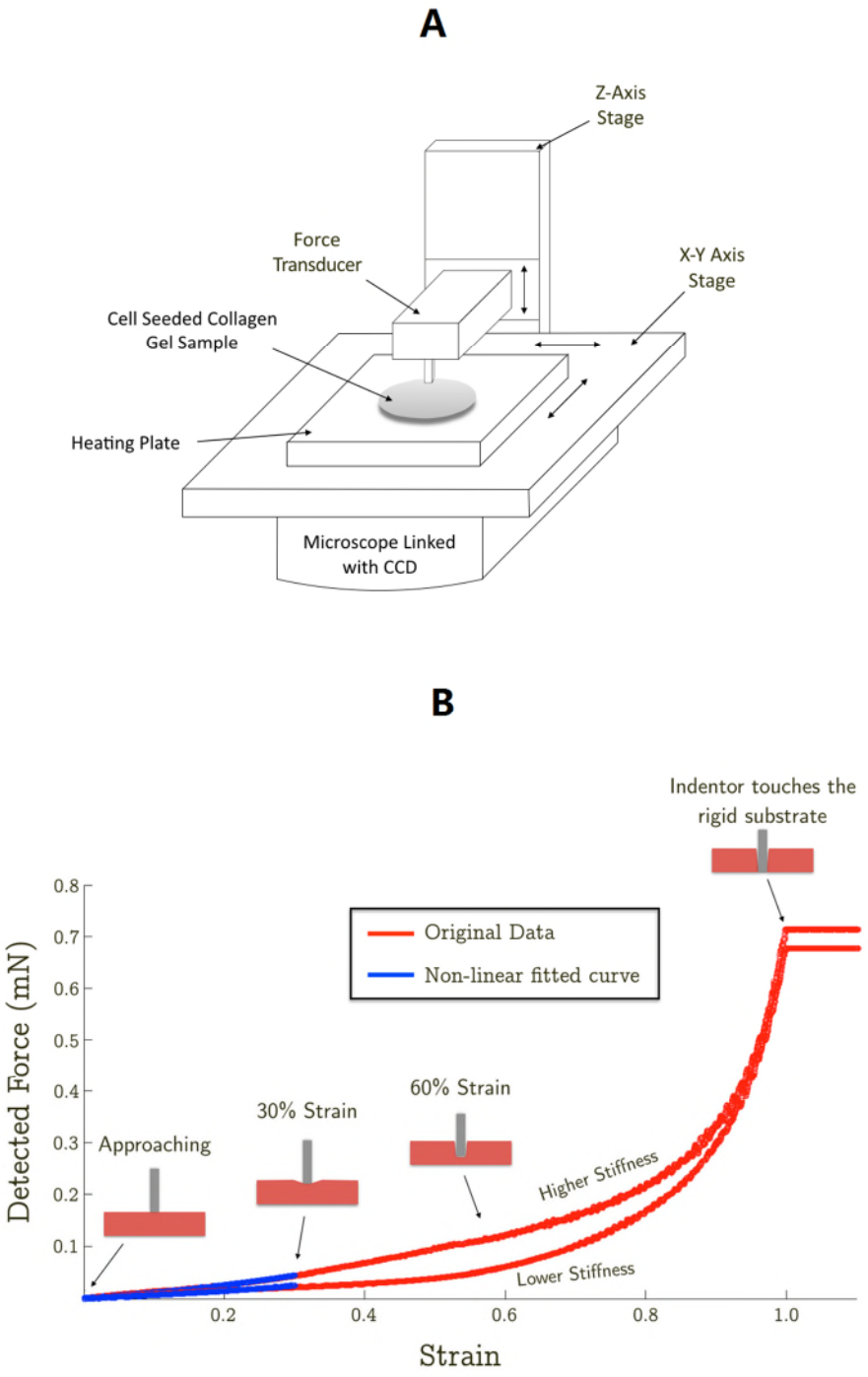

Fig 1. (A) The schematic setup of bio-nano-indentation tester. (B) Typical loading force-displacement curves of collagen gels with higher and lower stiffness indented by a flat punch.

$$F = E_0 \cdot \frac{2rh}{(1-\nu^2)} \cdot \frac{\bar{\epsilon}-\bar{\epsilon}^2+\frac{\bar{\epsilon}^3}{3}}{(1-\bar{\epsilon})^2} \quad (1)$$

Where E_0 and υ are Young's modulus and Poisson's ratio of collagen gel, respectively, F is the indentation force sensed by the force transducer, r is the radius of indenter, and $\bar{\epsilon}$ is applied strain which can be calculated as the ratio of the indentation displacement D normalized by the gel thickness h, *i.e.*, $\bar{\epsilon} = D/h$. The values of υ are in the range of 0.42-0.48, which were calculated based on the measured radii and thicknesses of the collagen gel disks before and after contraction. At equilibrium status, the cell contraction force (F^*) is balanced by the elastic restoration force of the deformed or shrunk gel and can be expressed by the following equation:

$$F^* = \pi(h_0 + h_1)(r_0 - r_1)\, E_0 \cdot \frac{1-\varepsilon^*+\frac{\varepsilon^{*2}}{3}}{(1-\varepsilon^*)^2} \quad (2)$$

where h and r with subscript *0* and *1* represent the thickness and radius of collagen gel at beginning and end of contraction, and ε^* is the overall strain generated due to gel shrinkage, i.e., $\varepsilon^*=(r_0-r_1)/r_0$ (see Fig 2).

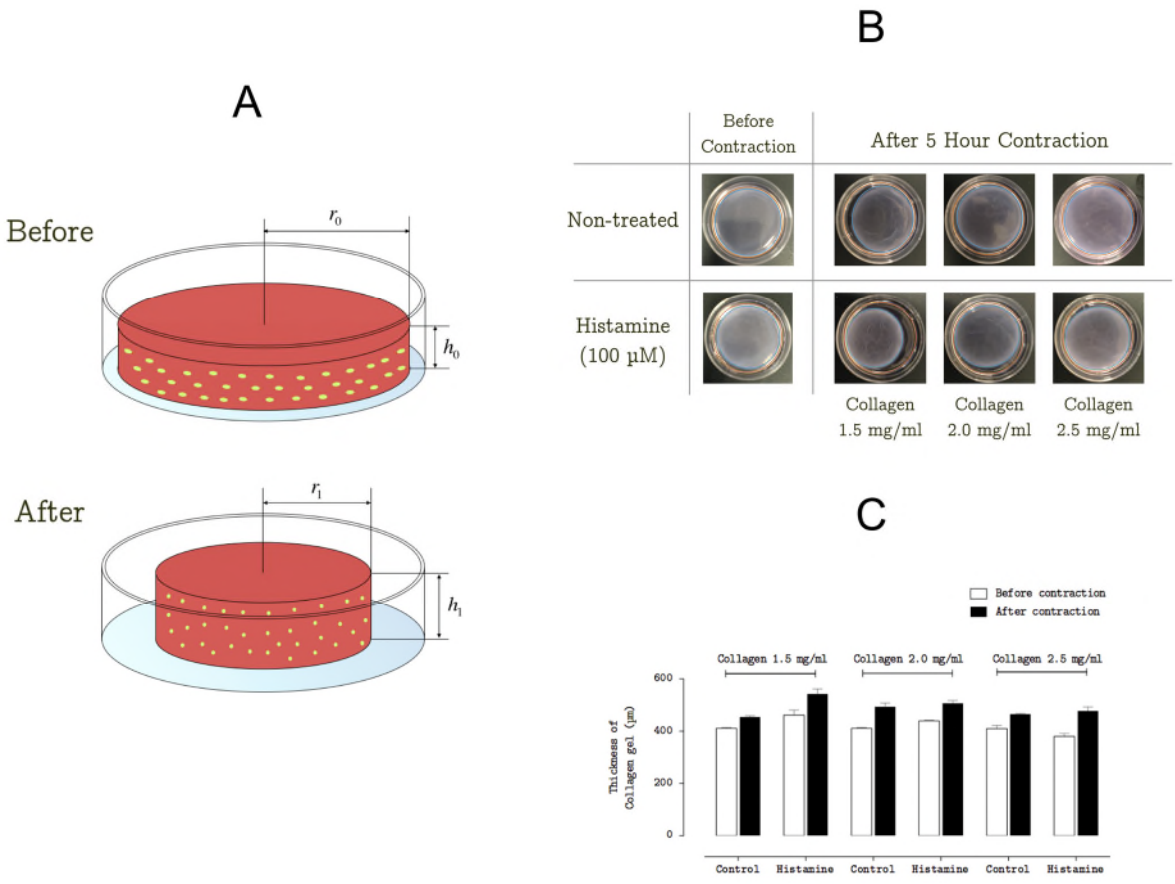

Fig 2. (A) Schematic of geometric parameters of collagen gel were measured before and after contraction for calculating contraction force. (B) Top view images of typical HAoAF-embedded collagen gel before and after 5 hours with/without histamine (100 μM) treatment. The areas circled with orange and blues lines denote the sizes of Petri dish (35 mm in diameter) and collagen gels respectively. (C) Measured thickness of collagen gel in the absence and presence of agonist histamine with different collagen concentration (1.5, 2.0 and 2.5 mg/ml).

3. Results

As illustrated in Fig 2, when fibroblast starts to contract, the contraction force will lead to gel radius shrinkage with a concomitant change in thickness of the disk-shaped gel. The thickness and radius of collagen gel were measured at the beginning and end of treatment to determine the overall fibroblast contraction force (see Eq. (2)). Ten random positions were measured to calculate average gel thickness (shown in Fig 2C). The depth sensing indentation device was used to measure displacement difference from the gel top surface to the Petri dish surface. The top view images of the disc-shaped collagen were captured and used to measure the gel radius before and after shrinkage by referencing the culture surface within the Petri dishes (illustrated as Fig 2B).

Fig 3A shows the results derived from measurement of Young's moduli of both the treated and untreated gels, demonstrating a dose-dependent increase with collagen concentrations. Interestingly, histamine treatment slightly reduces the Young's modulus of collagen gel compared with the untreated gels at the same collagen concentration. Fig 3B shows the overall contraction force of fibroblasts which was calculated based on the Eq. (2) using the measured geometric parameters and Young's modulus of the gel. The result shows the contraction force decrease as the collagen concentration increases. It was evident that histamine treatments doubled overall contraction cell force in every concentration group. Fig 3C demonstrates that cell contraction force decreases monotonically as the gel concentration increases. Histamine treated gels exhibited a significant linear regression model in the overall contraction force against collagen concentration ($P=0.0002$) compared with untreated cells ($P=0.16$).

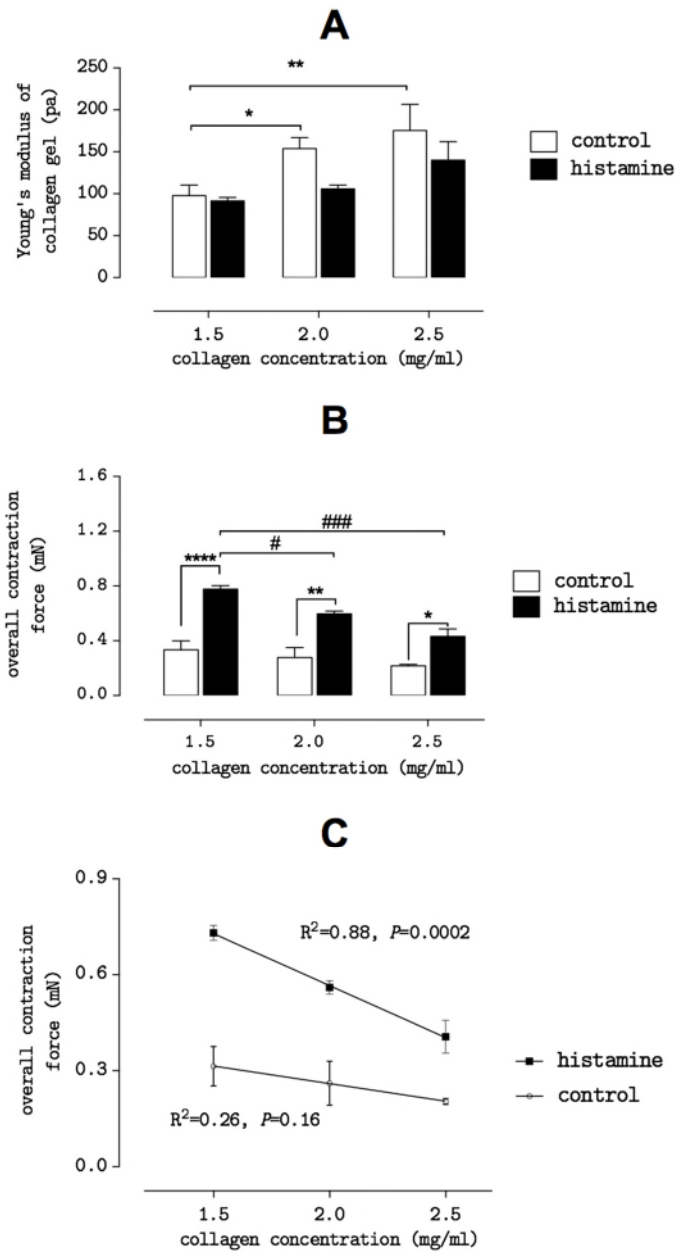

Fig 3. HAoAF were embedded into Type I collagen gels, formed at concentrations of 1.5, 2.0 and 2.5 mg/ml. (A) Young's modulus of each gel at different collagen concentration. (B) Overall contraction force per gel in the absence or presence of histamine treatments (100 μM, 5 hours). (C) Linear regression fitting of overall cell contraction force. Data denote mean ± s.e.m from three independent measurements (N=3). *P-V*alue was calculated using one-way ANOVA with Bonferroni post hoc test. **** $p<0.0005$; ** $p<0.05$; * $p<0.5$; ### $p<0.005$; # $p<0.5$.

4. Discussion

In this study, adventitial fibroblast contraction forces were measured based on cell-populated collagen gels to investigate the effect of gel stiffness on overall contraction force generated by the fibroblasts. The Young's modulus (or stiffness) of collagen gel, as calculated by the non-linear strain dependent mechanical model [18, 19], demonstrates a clear increase as the gel collagen concentration increases. The increase in the gel Young's modulus regulated fibroblast contraction force as the cell contractility was significantly reduced in the stiffer matrices. Moreover, the addition of the agonist histamine elicited significant fibroblast contraction forces. In all collagen concentration groups, the histamine treated fibroblasts exhibited a two-fold increase in their contraction forces (Fig 3B) despite the decrease in Young's modulus of the collagen gels (Fig 3A). This proves the Young's modulus of gel matrices should be considered as a key parameter to properly assess cellular contraction forces using gel-based assays.

The results clearly demonstrate that the mechanical properties of collagen matrices will influence the contractile responses of fibroblasts. Cells in stiffer (higher Young's modulus) materials exhibit a significant reduction in their contractility measured by our novel method, which is comparable to the results obtained by using other techniques, such as CFM [20]. For histamine-induced cell contraction assay, we showed a more significant decrease of overall contraction force as the matrix stiffness increases. With the activation of myosin light chain phosphorylation mediated by the agonist histamine, cell contractility increases, however, fibroblast contraction force will also be affected by the interaction between the cells and the collagen matrix. The detailed mechanisms of how the cells translate the sensing of substrate stiffness into the downstream

signals for regulating contraction requires further biomechanical studies. In the blood vessel wall, inflammatory disease processes would increase ECM collagen content thereby causing vascular dysfunction due to enhanced stiffness which impacts on the contractility of resident fibroblasts.

In the earlier reports, a loss of mass has been shown in fibroblast collagen gel assays during cell contraction process [5]. This finding also implies that there were a potential loss in cell numbers in the collagen-gel-based contraction assay for long-term (> 48 hours) measurements. Hence, a certain degree of inaccuracy is likely in the determination of fibroblast contraction force using previous techniques (e.g. culture force monitor (CFM) [21, 22], because the mass and cell loss may cause alterations in the mechanical properties of the collagen matrix. However, since the treatment time in our current study is only 5 hours, there was likely to be no significant loss of cells or gel mass.

5. Conclusion

We have demonstrated that in a 3D collagen matrix, the cells exhibit different levels of contractility depending on the collagen concentration and gel stiffness. The finding confirms that the mechanical properties of the collagen matrix should be considered in the cellular contraction events induced by physiological changes (e.g. effect of agonist treatments). Different cell types may have varying degrees of response depending on the mechanism of their sensing of the substrate stiffness. Overall, the study has shown the importance of matrix stiffness on cell contraction forces in the design of collagen-based biomaterials for clinical applications.

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