Endothelial-cell-mediated Displacement of Extracellular Matrix during Angiogenesis

Yue Du, Sahan C. B. Herath, Qing-Guo Wang, Harry Asada, and Peter C. Y. Chen

Abstract—Mechanical interaction between endothelial cells (ECs) and the extracellular matrix (or collagen gel) is known to influence the sprouting response of endothelial cells during angiogenesis. This influence is believed to impact on the capability of endothelial cells to sense soluble chemical cues. Quantitative analysis of endothelial-cell-mediated displacement of the collagen gel provides a means to explore this mechanical interaction. Existing analysis in this context is generally limited to 2D settings. In this paper, we investigate the mechanical interaction between endothelial cells and the extracellular matrix in terms of the endothelial-cell-mediated displacement of the collagen gel in both 2D and 3D. Digital image correlation and Digital volume correlation are applied on confocal reflectance image stacks to analyze cell-mediated displacement of the gel. The skeleton of the sprout is extracted from phase contrast images and superimposed on the displacement field to further investigate the link between the development of the sprout and the displacement of the gel.

Keywords—Angiogenesis, digital image correlation, digital volume correlation, interaction between ECs and ECM.

I. INTRODUCTION

ANGIOGENEIS is the process of forming capillary blood vessels from pre-existing blood vessels. Under proper conditions (such as the presence of a VEGF gradient), endothelial cells (ECs) form sprouts extending into the extracellular matrix (ECM). A sprout is known to consist of a tip cell that leads the extension and a collection of ECs (known as stalk cells) that follow the tip cell to eventually form the lumen. Since blood vessel is related to various biological processes, developing an understanding of the mechanisms involved in angiogenesis has important implication on medical treatment of various illnesses, such as cancer, tumor formation, stroke, Alzheimer's disease, etc. [1].

The process of sprouting during angiogenesis is influenced by soluble cues and the mechanical properties of the ECM. Moreover, the latter is known to affect the sensitivity of the sprouting process to the soluble cues [2]. For example, the mechanical stress imposed on extracellular matrix (ECM) can influence the formation of receptor of VEGF, which plays a role in triggering the migration of ECs and in increasing microvascular permeability [3,4]. Various studies have attempted to reveal the effect of change in certain ECM mechanical properties on the sprouting process; one such property is the stiffness of the ECM [5-9]. Findings from these studies indicate that ECs seeded on rigid gel become spread and form larger lumens, whereas these on soft gel become round and form dense interconnecting multi-cellular structure with smaller lumens. In additional, researches have been working on quantitative analysis of the interaction between cells and the ECM, mainly focusing on the cell-mediated displacement of ECM and surface traction force. Their results indicate that certain patterns of surface traction force are associated with the activities of cells, and that these activities are influenced by the ECM.

These studies are usually limited to a 2D setting, where cells are seeded on a substrate and then the contraction of the surface of the substrate is investigated. Results obtained from such a 2D setting do not reveal the full behavior of cells in a 3D ECM *in vivo*. Min-Cheol *et al.* have suggested that the migration behavior of cell in 3D is different from that in 2D [10]. A recent effort extends the traditional 2D analysis by investigating the *z*-direction traction force, but still with the cell seeded on a 2D substrate [11].

In this paper we investigate the interaction between ECs and the ECM in terms of EC-mediate ECM displacement in both 2D and 3D. We use a microfluidic device that enables ECs to migrate into collagen gel in 3D, and subsequently obtain image stacks using a confocal microscope. With the image stacks, cell-mediated displacement field of the gel in 2D and 3D can be generated upon the application of the digital image correlation and its extension - digital volume correlation method [11], respectively. In particular, we investigate the combined effect of multiple ECs on ECM displacement, which reflects more accurately the situation in sprouting, and complements preceding research works that focus on analyzing the effect of a single cell.

The remainder of the paper is organized as follows. Section II discusses materials and methods that we used in this study, including the preparation of the device and collagen gel, the culturing and seedling of the cells, and capturing and processing of images. Section III presents the results on the time-evolution of displacement of gel mediated by ECs during angiogenesis in both 2D and 3D, which enables the evaluation of consistency between 2D and 3D analyses. Section IV summarizes the results, discusses their implications, and outlines directions for further research.

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II. MATERIALS AND METHODS

A. Device and Gel Preparation

The microfluidic device designed by Farahat and Wood [12] is used in this study. It is made from polydimethylsiloxane (PDMS) and bonded onto a glass slide. The design of this device includes a cells-seeding and media-filling channel, gel-filling channel and media-filling channel (shown in Fig. 1). There are trapezoidal-shaped posts created inside the gel channel, which are used for forming a surface tension during the filling of the gel to avoid the leak of the gel into other channels. The gel to be filled in the channel contains 2.5 mg/ml Rat Tail Collagen Type I. The streptavidin-coated magnetic beads (diameter in ~1.5 µm, BM551 from Bangs Laboratory) are mixed with collagen uniformly before the polymerization of the gel. Then, the mixed collagen gel is filled in PDL-coated device and allowed to polymerize in the incubator for one hour. During the polymerization, the beads are binding onto the collagen and moves along with the collagen once there is a movement of the collagen due to the activities of the cells.
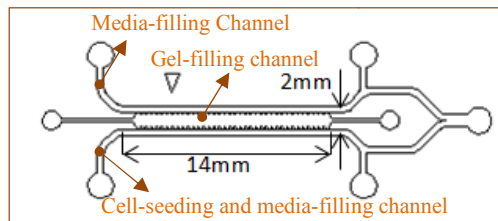

Fig. 1 The microfluidic device

B. Cell Culture and Confocal Microscopy

The media consisting of Basal media (EBM-2), FBS, Growth factor are added in the media channel before cell seeding. Then, human Micro Vascular Endothelial Cells (hMVECs) are seeded in the device with a concentration of 2.5 million/ml after the treatment of trypsin for 1 minute. The gradient of angiogenic factor is created in the gel after the confluent layer of cells forms through adding VEGF in cell-seeding channel and its opposite channel with different concentration. Low concentration VEGF is added in cell-seeding channel and high concentration VEGF is added in opposite channel, thus triggering the movement of sprouts in the gel towards the channel with high concentration. The media is replaced every 12 hours.

Three-dimension image stacks are captured using confocal system mounted on an inverted optical microscope. Two laser lines are used to obtain phase-contrast and reflectance images. Phase-contrast images are used to extract the boundary of the sprout and reflectance images showing the location of the beads are used to analyze cell-mediated displacement of collagen fibrils and thus the interaction between cells and ECM. One example of 3D confocal reflectance image stack is shown in Fig. 2. The bright dots inside the image are the beads and the white and thick line shows the scale of the image. One slice of this image stack is demonstrated in Fig. 3. Image stacks are taken every 30 minutes with step size equal to approximately 6 microns.
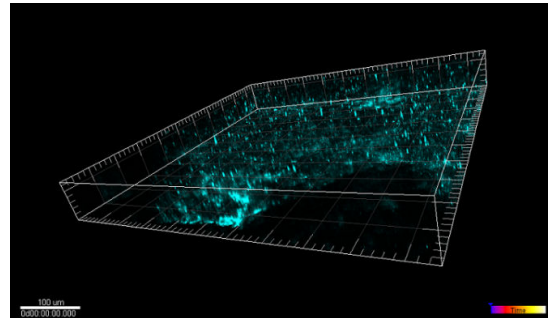

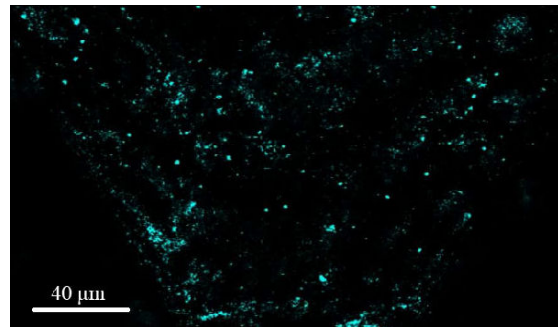Fig. 2 Three dimensional confocal reflectance image of beads

Fig. 3 One slice of confocal reflectance image of beads

C. Measurement of ECM Displacement

The method of digital image correlation that used for the analysis of the velocimetry of the particle in 2D is applied on the confocal reflectance images to generate cell-mediated displacement of the gel in 2D. The location of the beads can be extracted because of their high intensity compared with the intensity of the gel. The recognition of sub-images within a pair of images exposed with a certain time separation is done using the digital image correlation based on the distribution pattern of the particles. This is realized by calculation of the cross-correlation value of sub-images of a pair of images. The peak values of cross-correlation results are targeted. This peak gives the movement of each sub-image within the time separation. The details can be found from [13, 14]. The open source software MatPIV (http://folk.uio.no/jks/matpiv/) developed for the purpose of the digital image correlation is used in this study.

The extension of the digital image correlation – the digital volume correlation developed in [11, 15] is also applied to analyze cell-mediated displacement of the gel in 3D. Instead of cross-correlation of sub-images, the digital volume correlation conducts the cross-correlation of individual cubic subsets in a pair of 3D image stacks. The displacement vectors can then be calculated by determining the spatial location of the peak value of the correlation function. The code provided by Franck et al. on the website is used for this purpose (http://franck.engin. brown.edu/~christianfranck/FranckLab/Downloads.html).

III. RESULTS

A. Time-evolution of Cell-mediated Displacement of Gel in 2D

Fig. 4 shows phase contrast images of the gel and a sprout inside the gel, which grows continually after the sensation of the gradient angiogenic factor. The image is taken every 30 minutes for a total one and a half hours. The outline of the sprout is extracted manually using ImageJ (http://rsb.info.nih.gov/ij/). It is superimposed on the 2D displacement field of the gel to observe the connection between the change in the morphology of the sprout and the displacement of the gel.
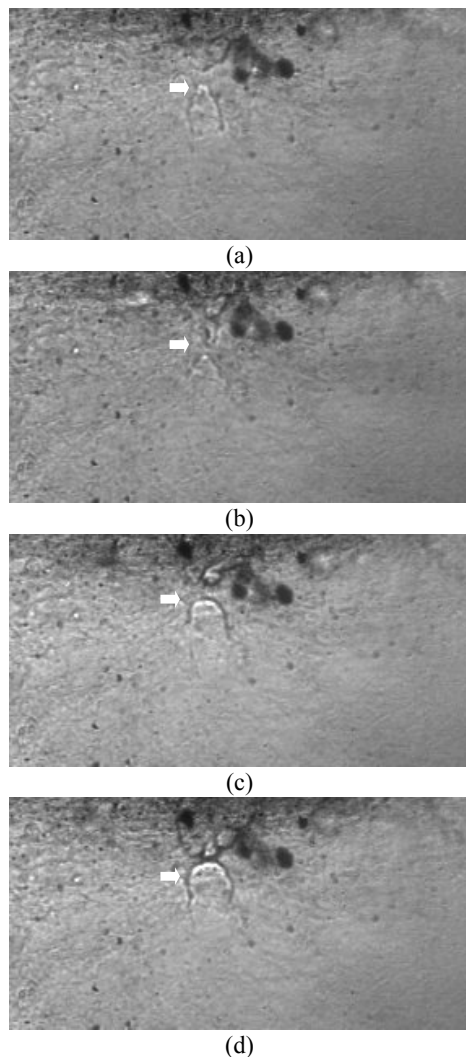

(a)

(b)

(c)

(d)

Fig. 4 Phase contrast images of the sprout and the gel. The image is captured every 30 minutes for a total one and a half hours. White arrow indicates the location of the sprout

Fig. 5 shows cell-mediated displacement field of the gel in 2D. The displacement vectors are generated using digital image correlation based on the pattern of distribution of beads in a pair of confocal reflectance images captured before and after 30 minutes. The superimposition of the skeleton of the sprout on the displacement field enables us to investigate the connection between the development of the sprout and the displacement of the gel.

Comparing the skeleton of the sprout at each time step in Fig. 4(a) - (d), the change in the lumen size or the elongation/ contraction of the sprout can be observed. Fig. 5(a) shows that during the first 30 minutes the sprout extends in radial direction. Our observation is that the direction of the displacement of the gel near the extending part of the sprout points toward this part. This may imply that the sprout seek for the energy to extend by pulling the gel. Additionally, the forward arrows can be observed in (a), indicating that the pushing force exists at the rear of the sprout. In (b) within the second 30 minutes, the contraction of both the sprout and the gel are clearly observed. This movement may prepare for the elongation of the sprout in the next 30 minutes. Fig. 5 (c) demonstrates the significant elongation of one branch of the sprout towards the media channel that has higher concentration of the angiogenic factor within the third 30 minutes. The arrows point to the tip of this branch can be clearly seen from this (c), through which cell may be sensing the stiffness of the ECM or contracting the gel for seeking for the energy for the further elongation in the next 30 minutes. The significant in-plane displacements within this one and a half hours range 1 to 2.5 microns.
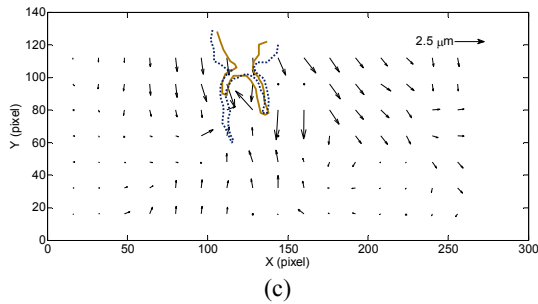

(a)

(b)

(c)
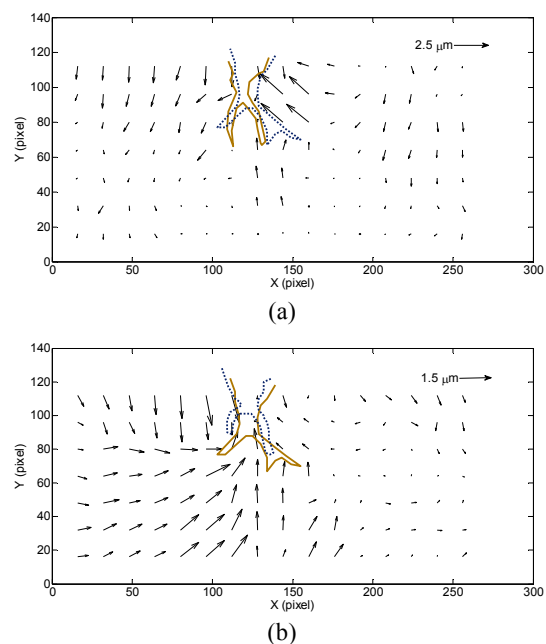
Fig. 5 Cell-mediated displacement of gel in 2D. Each sub-image is generated from a pair of confocal reflectance images with time separation equal to 30 minutes. Both the outlines of the sprout before and after 30 minutes are superimposed on the displacement field. The black arrows show the displacement of the gel with length in proportional to the magnitude of the displacement. The orange curve shows the outline of the sprout before 30 minutes and the dark blue curve shows the outline of the sprout after 30 minutes. The scale of the arrows is shown by arrow with text at the right corner of each sub-image

B. Cell-mediated Displacement of Gel in 3D

Fig. 6 shows phase contrast images of the sprout and the gel in 3D. To explore the general feature of the sprouts during interacting with the gel, the sprout that is different from the one used in previous sub-section is chosen here. White arrows indicate the sprout in 3D.
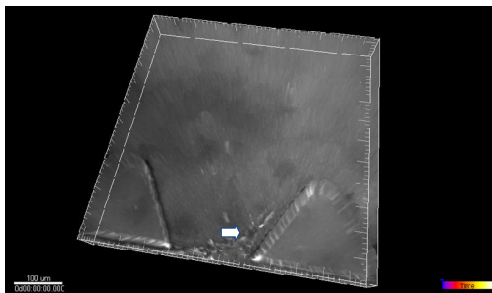

Fig. 6 Phase contrast images of the sprout and the gel (white arrow indicates the location of the sprout)

Fig. 7 shows cell-mediated 3D displacement vectors of the gel. The green displacement vectors in each sub-image are generated from a pair of 3D confocal reflectance image stacks captured before and after 30 minutes. It can be seen from the (a) that the magnitude of z- component of most of displacement vectors is larger than that of x- and y-components. Furthermore, most of vectors have negative z-component, resulting in downward vectors. There are a few of upward vectors surrounding the tip of the sprout. Those downwards and upwards movements of the gel may be generated separately by the stalk cell and the tip cell. It is also observed that a cluster of vectors shown by a black empty arrow closely surrounding the sprout points downward and outward relative to the sprout. Another cluster of vectors shown by a black solid arrow distributing slightly away from the sprout points downward and inward relative to the sprout.

There is a blank area shown by dashed circle between these two clusters. This may be the cleaved area of collagen due to the proteinases released by ECs. We assume that the outer cluster of vectors show the movement of the gel caused by the main motion of the sprout that is contracting the gel in order to move, whereas the inner cluster of vectors show the additional movement of the fraction of degraded collagen associated with the main motion of the sprout, for example, a squeeze from the sprout. The projection of the outer cluster of vectors is consistent with the pattern of the direction in 2d that is pointing towards the active parts of the sprout. Fig.7 (b) shows that the 3d displacement vectors of the gel at the second 30 minutes. The different pattern of the displacement of the gel from that in previous 30 minutes can be observed. The more active part of the sprout shifts to the frontier of the sprout shown by the solid empty arrow where the dense displacement vectors with significant magnitude are observed. Furthermore, the pull-down of the gel shown by the empty arrow at the rear of the sprout can also be observed from Fig. 7 (b).
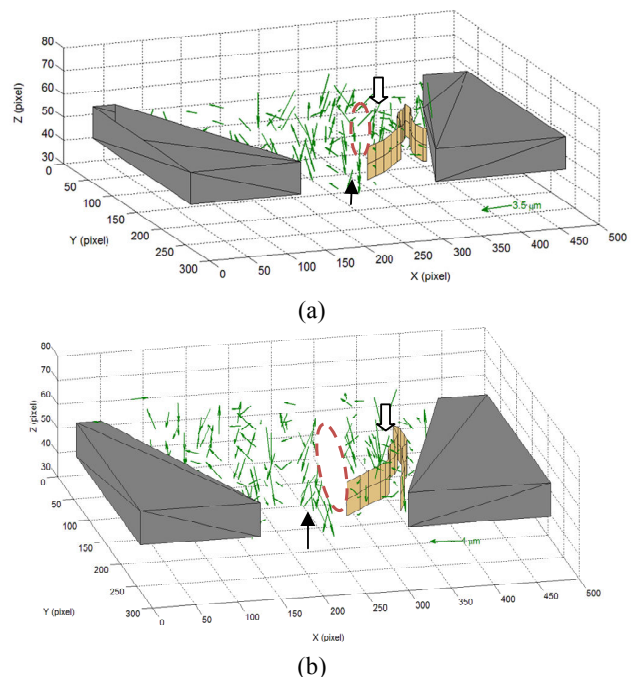

(a)

(b)

Fig. 7 Cell-mediated displacement of gel in 3D. Each sub-image is generated from a pair of 3D confocal reflectance image stacks with time separation equal to 30 minutes. The green vectors show the displacement of the gel in 3D with the length in proportional to the magnitude of the displacement. The orange surface shows the skeleton of the sprout after 30 minutes and the grey blocks represent the slots. The arrow with text in each sub-image shows the scale of the displacement vectors

IV. DISCUSSION

The work reported in this paper investigates the mechanical interaction between endothelial cells and the extracellular matrix by conducting quantitative analysis on cell-mediated displacement of the collagen gel. The method of digital image

correlation and its extension – digital volume correlation are applied on confocal reflectance image stacks to analyze cell-mediated displacement of the gel in 2D and 3D, respectively. The skeleton of the sprout is extracted from phase contrast images and superimposed on the displacement field to further study the link between the development of the sprout in term of morphology and the displacement of the gel.

The observations from cell-mediated gel displacement in 2D as well as the development of the sprout are:

(i) There is a link between the evolution of the morphology sprout and the displacement of the gel.
(ii) The extension of the sprout in either the radial or the axial direction associated with the pulling of the gel by the sprout towards itself.
(iii) Pushing forces are exerted on the gel by the sprout at the rear of the sprout.

The first observation can be explained by the fact that the evolution of the morphology of the sprout is mediated by the migration of the tip cell or the stalk cell, or both. The migration of cells depends on the traction forces exerted on the focal adhesion site, which results in the gel being displaced. Observation (ii) is consistent with the finding reported in [16] that the tip cell of a sprout pulls collagen fibrils towards itself during migration. Our finding further reveals that not only the tip cell but also the stalk cell tend to pull on the gel in order to migrate in the gel. The hypothesis is that this activity indicates that the tip cell or the stalk cell may be sensing the stiffness of the gel in order to facilitate its movement.

The observations from the quantitative analysis of cell-mediated displacement of the gel in 3D are:

(i) The magnitude of cell-mediated displacement of the gel in the z-direction is comparable to or even more significant than that in the other two dimensions.
(ii) There is a cleaved area without displacement vectors located slightly away from the sprout.
(iii) A dense displacement field pointing downward and inward relative to the sprout is found at the location just beyond the cleaved area or near the tip cell.

We found that the interaction between ECs and ECM during angiogenesis is in three dimensions. Therefore, quantitative analysis of the interaction in 3D would provide a more accurate description of the actual situation compared to the traditional method focusing on in-plane pattern of the interaction. This finding is supported by the results reported in a recent paper [15] that the magnitude of z-direction displacement of collagen gel caused by a fibroblast cell seeded on the substrate is comparable to that in the other two dimensions. Additionally, a cleaved area devoid of displacement vectors was found surrounding the sprout. This may be due to the fact that the tip cell is continually degrading the surrounding gel during migration by releasing the proteinases such as the series of MMPS (MT1-MMP, MMP2, MMPS9, MMP1, etc. [17-19]). It is further observed that two

clusters of displacement vectors, namely, the inner cluster and the outer cluster, are located at each side of the cleaved area. The inner cluster is defined as the one located at the side very close to the sprout. The outer cluster is defined as the one located slightly far away from the sprout. The outer cluster of displacement is found to be pointing towards the sprout, whereas the inner cluster is pointing away from the sprout. The hypothesis is that the outer cluster of vectors indicate the movement of the gel caused by the main motion of the sprout that is contracting the gel in order to move, whereas the inner cluster of vectors indicate the additional movement of the fraction of degraded collagen associated with the main motion of the sprout (for example, a squeeze from the sprout).

To extend the work reported in this paper, we plan to incorporate end-point staining of cells into our proposed method. With information provided by cell staining, the displacement of the gel can be related directly to the tip cell or the stalk cell and also to their locations inside the lumen. Moreover, the end-point staining of MMP would be helpful in locating the cleaved area of collagen that has been observed in this study. Another issue we will address is the quality of images. The analysis reported in this paper based on the particle velocimetry method, including digital image correlation and its extension-digital volume correlation. To obtain more accurate results, the quality of the images needs to be improved by de-noising to generate confocal image stacks with higher quality.

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