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INVESTIGATING THE EFFECT OF CYCLIC STRETCH ON MULTICELLULAR AGGREGATES

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INTRODUCTION

Many tissues in the body undergo cyclic stretch, and recapitulating this dynamic environment in cell-level studies is critical for understanding the mechanobiology of development and disease. Cells exist individually in connective tissues, in monolayers lining the organs, and also in aggregates when acting collectively in morphogenesis, healing, and in some disease states. Whereas the responses of single cells and monolayers to stretch have been studied extensively (e.g.,[1]), to date, there are a limited number of studies which examine the behavior of multicellular aggregates under cyclic stretch [2-4].

Microcontact-printed aggregates of cells (also referred to as cell islands) are a useful system for mechanobiology studies. They enable the study of collective cell behavior which often results in nonuniform mechanical environments (e.g., with higher stress in the exterior and lower in the interior) [2, 5]. Microcontact-printed aggregates exposed to cyclic stretch exhibit increased proliferation and contractile protein expression in their interior cells [2] and transiently increased traction forces with stretch and depressed forces with stretch release [3]. The effect of cyclic stretch on other important cell behaviors such as realignment [6] and apoptosis [7] have been studied in single cells and monolayers, but to our knowledge not in aggregates.

The goal of this work is to study the effect of cyclic stretch on valvular interstitial cell alignment and apoptosis in microcontact-printed aggregates to better understand multicellular-based mechanisms of valve disease.

METHODS

Preparation of Substrates

Polyacrylamide gel-coated PDMS substrates were prepared according to previously described protocols [8] . Briefly, approximately 500 µL benzophenone solution was pipetted onto each well. It was left covered from light for 1 minute. The solution was then removed and the wells were rinsed with methanol. The wells were covered from light and placed in a vacuum for 30 minutes. The vacuum chamber was then flooded with nitrogen gas. Polyacrylamide gels were prepared by mixing 29.9% acrylamide solution (40%, Bio-Rad), 5.9% bisacrylamide (2%, Bio-Rad), 53.9% HEPES, 0.3% TEMED, 10% ammonium persulfate. 50 µL droplets of gel solution were placed on each well, and a square coverslip micropatterned with 400 µm collagen islands was placed on top. The gels were then polymerized under UV light, and finally the coverslips were removed.

Cell Culture and Stretch

Porcine valvular interstitial cells (VICs) were seeded into each 28 mm square well (125,000/well). Media was changed every four days. Cells were given time to grow to confluence on the micropatterned protein circles before stretch. Cells were cyclically stretched uniaxially overnight in an incubator at 10% strain at 1 Hz on a custom stretch device described previously [9].

Staining and Imaging

Aggregates were stained for active Caspase to indicate apoptosis and with Hoechst for nuclear identification and imaged on a Zeiss microscope. Images were analyzed using ImageJ. The percent of the aggregate positive for apoptotic activity was determined as follows. The area of the aggregate was outlined manually. The background was subtracted, and a threshold for apoptosis was determined by the user. The images were converted to binary, and the area of positive pixels was divided by the total area to calculate the percent positive for apoptosis.

Cell alignment was determined as follows. Contrast was adjusted to minimize the background brightness for the Hoechst channel image.

The ImageJ "find edges" command was used to outline the nuclei. Contrast was increased to better delineate the nuclei. The image was converted to binary, nuclei were outlined and filled in, the watershed command was used to separate closely packed nuclei. The alignment angle was then found using the "analyze particles" command.

Statistical Testing

A two-sample t-test was used to determine difference between control and stretched groups with significance set to $p<0.05$.

RESULTS

Circular aggregates of cells with ~ 400 μm diameter were obtained. The aggregates were cultured for 2 days and then stretched overnight (n=9 to 11 aggregates; ~170 cells/aggregate). Many of the aggregates were partially confluent or had small gaps and thus not used in this study. Only fully confluent aggregates were selected for analysis. Caspase images indicated low levels of apoptosis in both static control and stretched aggregates.

Preliminary data did not demonstrate a statistically significant difference in apoptotic activity between unstretched controls and stretched aggregates, as shown in Figure 1. In both cases, the level of apoptosis is very low (<3% of the area of the aggregate).

Analyzing the angle of the cell nucleus, in the control group, the average of the angle was 91.4°, with a standard deviation of 5.24°. Similarly, for the stretched group, the angle was 88.0°, with a standard deviation of 3.93°. See Figure 2. The t-test showed no significant difference between control and stretched group, p value=0.31.

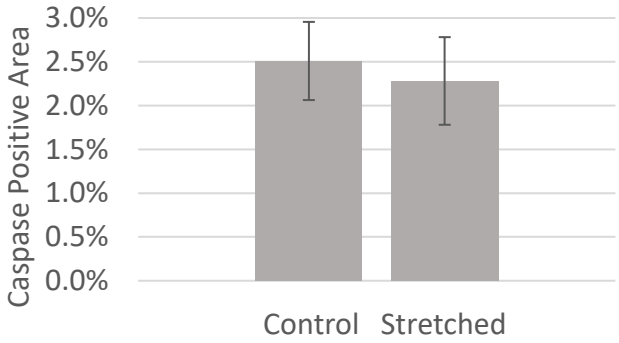

Figure 1: Apoptotic activity in stretched aggregates and static control aggregate. n=9 control aggregates, 11 stretched aggregates (all control aggregates from a single well and all stretched aggregates from a single well). Error bars indicate standard error of the mean. Difference is not statistically significant.

DISCUSSION

Here we demonstrate a method to study multicellular aggregates under cyclic stretch. Preliminary data did not show differences between stretched and unstretched aggregates in apoptosis or cell alignment.

In comparison, a previous study examining the effect of cyclic stretch in alveolar epithelial cell monolayers found that 20% stretch induced apoptosis but preconditioning with 5% stretch (normal for these cells) reduced this effect [7]. This supports the idea that normal physiological stretch can reduce apoptosis. Preliminary data in our system does not indicate a difference in apoptosis between stretched and unstretched aggregates. Longer time in culture before stretch may increase the levels of apoptosis and allow for better comparison. Also, by outlining the cell boundaries, the proportion of apoptotic cells may be quantified, rather than %area positive for caspase.

Although it has repeatedly been demonstrated that single cells [9] and monolayers [10] reorient away from the cyclic stretch direction, in this study we did not observe changes in the direction of the cells. We believe that this difference between single cells and aggregates are the results of the forces applied by nearby cells and from the different mechanical environment of the aggregate as a whole i.e., the collective cell behavior generated in response to the external constraint of being cultured on a protein island is a stronger mechanical signal than cyclic stretch.

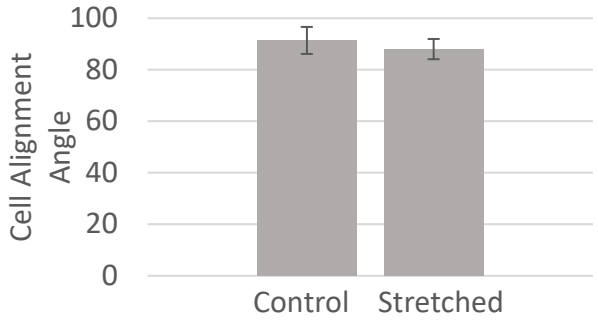

Figure 2: Cell alignment in stretched aggregates and static control aggregate. n=9 aggregates in both scenarios (all control aggregates from a single well and all stretched aggregates from a single well). Error bars indicate standard deviation. Difference is not statistically significant (p=0.31).

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