CELLS ON GELS: ADHESION VERSUS DIFFERENTIATION OF SKELETAL MUSCLE CELLS

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ABSTRACT

Substrate flexibility is emerging as an important determinant of cell adhesion, cell organization, membrane tension, and other key features that define the state of a cell. For skeletal myocytes, in particular, substrate compliance can be understood as key to cell contraction and, as described here, cell differentiation. Although differentiated myocyte cultures have been studied for many years, the syncytia they form are generally highly branched, interconnected, and complex. In order to grow isolated, well-defined myotubes and study their cell state as well as adhesive mechanisms, collagen was patterned as microstamped strips and crosslinked to polyacrylamide gels with a tunable elastic modulus. Cells were examined at 2 days, 2 weeks, and 4 weeks for cell patterning efficiency as well as marker of differentiation expression (eg. myosin and desmin). Efficient cell patterning was initially seen after just 2 days and was maintained over the life of the culture, independent of substrate flexibility. Cell differentiation on stiffer gels was very evident at 2 weeks with notable striation and multinucleation; cells on 10-fold softer gels did not express the differentiation markers nearly as strongly or as organized. After 4 weeks in culture, however, evidence of differentiation was noticeable in all cultures, with cells on stiff gels showing more organization than cells on either rigid glass or soft gel. Interestingly, adhesion strength as measured by micro-peeling of cells, showed that myocyte adhesion increases monotonically with substrate stiffness. Like the optimum seen years ago in cell motility, the results here imply that long-term myogenesis relies on an optimal balance of contractility and adhesiveness as influenced by substrate compliance.

INTRODUCTION

Myogenesis begins with the fusion of motile myoblasts to create well anchored myotubes. These cells differentiate further with increasing expression and assembly of many proteins, including cytoskeletal myosin and desmin as well as novel adhesion proteins. With increasing maturation, the cytoskeletal markers reorganize along the length of a myotube into the characteristic striations of muscle, serving as visible indicators of both differentiation and function [1].

Given the potential coupling between the development of contractile structures and contraction itself, one potential obstacle to in vitro differentiation is the rigid nature of common culture substrates, e.g. glass or polystyrene. Firm attachment to these would only allow isometric work to be done – if the adhesions hold – which may not be optimal compared to contraction allowed on a gel. Another potential complexity of myogenesis in vitro is the branched architecture of cultured muscle cells which will tend to limit long-range organization of myofibril-like striations seen with myotubes in vivo.

Patterned, flexible substrates permit isolated myotubes to contract without disrupting cell-substrate adhesions. While extremely soft surfaces inhibit cell spreading [2], moderately stiff, patterned surfaces will be shown to foster proper assembly of the actin-myosin contractile apparatus without the presence of branched syncytia. The tunable elastic modulus of polyacrylamide (PA) gels [3] has been exploited to investigate how a wide range of surface stiffnesses modulates both cell adhesion and myogenesis. Not only does the gel enable long-term cell cultures needed for differentiation, but it also provides a more biomechanically relevant surface than the substrates used today.

METHODS AND MATERIALS

Experiments were performed on C57 primary mouse and C_2C_{12} cells, cultured in Dulbeco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were plated on PAG substrates of variable stiffness and observed for up to four weeks. PAG samples were prepared on glass slides with a modified method established by Wang and Pelham [3]. Glass supports for PAG were prepared by attaching aminosilanes to glass. N,N' methylene-bis-acrylamide (0.03% to 0.3%) was added to solutions of 5% acrylamide (C_3H_5NO) in distilled water, which when gelled, produces the tunable elastic modulus. Polymerizing reagents were mixed with the acrylamide solution, and as the solution began to gel, 25µl was added to the silanized glass surface. The solution was covered by a coverslip to create a uniform surface. The gel thickness was approximately 70µm, which was confirmed optically.

Due to the anti-adhesive properties of PAG however, collagen was chemically crosslinked to the gel surface by a photoactivated heterobifunctional crosslinker, Sulfo-SANPAH. Collagen was attached to PAG in a pattern of long lines using a method of micropatterning and self-assembling monolayers. A 2.5 cm x 0.75 cm glass stamp, consisting of 20µm gaps spaced 100 µm apart, was designed and fabricated using common photolithographic and glass etching techniques. Collagen (1 mg/mL) was added to the grooves in the glass stamp, and the stamp was inverted and applied to the PAG surface in the presence of the UV-activated crosslinker. Samples were kept at pH 8.5 and placed in a 37°C incubator overnight. Controlling the collagen distribution during this binding process allowed crosslinks of collage to PAG in the grooved areas of the stamp only, promoting cell attachment only in the 20µm lines of ECM protein (Fig. 1).

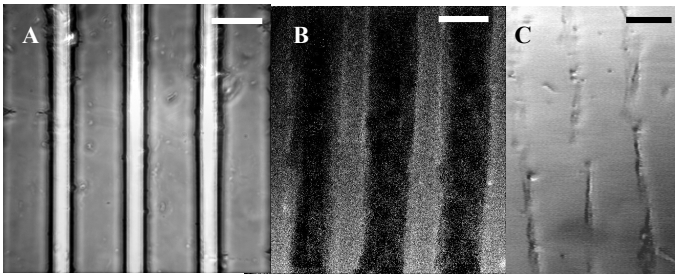

Figure 1. Micropatterning results of the (A) glass stamp, (B) fluorescent collagen type I, and (C) patterned C_2C_{12} cells on PAG indicating cell alignment. Scale bars are 100um.

Gel rigidity was determined from correlated measurements of the elastic modulus determined by both nano-scale and continuum-based techniques of applied Cauchy-Lagranian stresses and strains in an established technique [2]. Nano- and continuum measurements were done as a function of crosslinker concentration, indicated a tight correlation between the two methods, and produced a working compliance range of between 1kPa (soft) and 8kPa (stiff) gel.

Cells were observed on patterned surfaces of various stiffnesses after 2 days, 2 weeks, and 4 weeks to examine the efficiency of the pattern and the morphological development of myotubes.

RESULTS

After 2 days in culture, C57 primary and C_2C_{12} skeletal myoblasts had recognized the collagen pattern, aligned themselves to form myotubes, maintained the pattern width (20µm) and spacing (100µm), and begun to fuse into myotubes, regardless of elastic modulus. C_2C_{12} cells were observed at 10x on an 8kPa gel (Fig. 1C). While not shown, C57 and C_2C_{12} cells exhibit similar pattern recognition on both gel stiffnesses, and at high magnification, display membrane fusion.

After 2 and 4 weeks in culture, C57 primary and C_2C_{12} myoblasts remained non-motile, tended to maintain the collagen pattern initially presented to them, and continued to fuse together to form myotubes, independent of the elastic modulus. The combined patterning rate for C57 primary and C_2C_{12} myoblasts did decrease slightly over time and as a function of substrate elasticity, possibly be due to the inhibition of spreading on soft gels and detachment similar to cultures on glass substrates for stiff gels. Myotube patterning was not a concern however, especially due to an average 90% pattern efficiency, the ability of myotubes to remain isolated instead of forming crossbridges between adjacent isolated myotubes.

Additionally after 2 and 4 weeks in culture, C_2C_{12} cells were stained for myosin, desmin, and nuclei to examine their overall differentiated and multinucleated state on soft and stiff gels and glass (Fig. 2). Myotubes on soft gels did not show organization after 2 weeks in culture, with organization noted in a small subpopulation after 4 weeks. On stiff gels, myotubes displayed striations in a subpopulation, and after 4 weeks, organized striations were prevalent. On glass, organized myosin and desmin were noticeable in a smaller subpopulation. Other brightly fluorescent cells, showing partial assembly

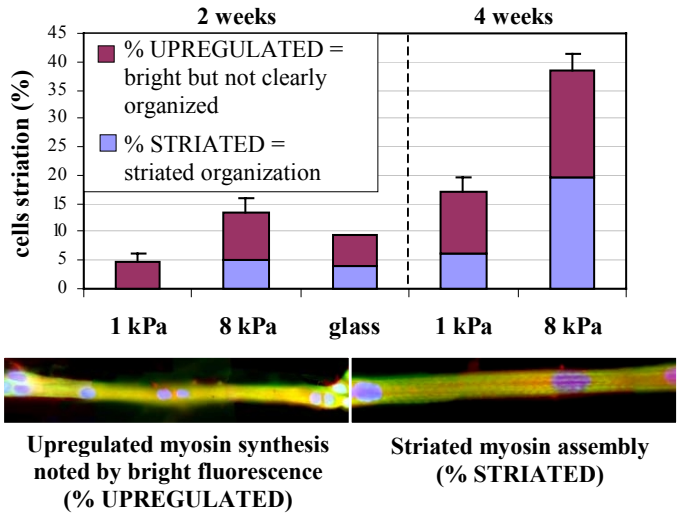

Figure 2. Quantified cell differentiation expressed in terms of the total cell population.

Overall, the results can be understood of in terms of competing functions of adhesion energy or membrane tension versus the contractile force, i.e.- the amount of myotube differentiation. Adhesion-dependent cells have membrane tensions that are governed by the rigidity of the substrate and limited by cell spreading. This tension, determined from cell peeling on different substrate compliances, limits the allowable contractile force, and thus the allowable differentiation before cell detachment occurs. Optimal attachment and viability for long-term study of cell mechanics will occur when these functions equilibrate (Fig. 3), which is elucidated by the bell-shaped myotube differentiation (Fig. 2) and increasing membrane tension (unpublished data).

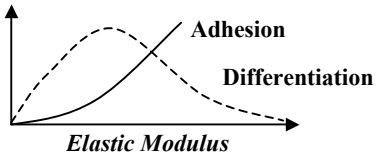

Figure 3. Schematic of the potential mechanism for cell differentiation on tunable elastic substrates.

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