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Substrate stiffness regulates apoptosis and the mRNA expression of extracellular matrix regulatory genes in the rat annular cells

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ABSTRACT

Cells are subjected to static tension of different magnitudes when cultured on substrates with different stiffnesses. It has long been recognized that mechanical stress is an important modulator of the intervertebral disc degeneration. Here we studied the influence of substrate stiffness on cell morphology, apoptosis and extracellular matrix (ECM) metabolism of the rat annulus fibrosus (AF) cells which are known to be mechanosensitive cells. Polyacrylamide gel substrates with three different stiffnesses were prepared by varying the concentration of acrylamide and bisacrylamide, and the elastic modulus of the different gel substrates were measured with atomic force microscopy (AFM). First-passage rat annular cells were cultured on soft, intermediate, rigid substrates or plastics for 24 or 48 h. The percentages of apoptotic cells were detected by flow cytometry and caspase-3 activity, and morphologic changes were visualized by Hoechst 33258 staining and F-actin staining. In addition, the expression of ECM genes (Col1α1, Col2α1, aggrecan, MMP-3, MMP-13 and ADAMTS-5) were analyzed by RT-PCR. The three different substrates had elastic moduli varying between 1 ± 0.23 kPa (soft, 5% gel with 0.06% bis), 32 ± 2.89 kPa (intermediate, 10% gel with 0.13% bis) and 63 ± 3.45 kPa (rigid, 10% gel with 0.26% bis) with a thickness about 60–70 μm. Most of the rat AF cells appeared small and rounded, and lost most of their stress fibers when cultured on soft substrate. There was a significant increase in the percentage of apoptotic cells in the rat AF cells cultured on soft and intermediate substrates relative to those on plastic surface, with a parallel decrease in the area of cell spreading and nucleus. The AF cells grown on intermediate or rigid substrate had reduced expression of Col1α1, Col2α1 and aggrecan and enhanced expression of MMP-3, MMP-13, and ADAMTS-5 at 24 h or 48 h, respectively, relative to those cultured on plastic surface. Conversely, we observed an up-regulation of Col2α1 and aggrecan and no change in the gene expression of MMP-3, MMP-13, and ADAMTS-5 in AF cells on soft substrates. Rat AF cells are sensitive to substrate stiffness which can regulate the morphology, growth, apoptosis and ECM metabolism of rat AF cells, thus indicating the importance of substrate choice for cell transplantation and regeneration for the treatment of disc degeneration using tissue-engineering technique.

1. Introduction

Intervertebral disc (IVD) degeneration causes neck or low back pain and disc herniation which are the most common disorders leading to morbidity or the deterioration in quality of adult life (Waddell, 1996). Excessive apoptosis of disc cells leads to a decrease in cell density (Ahsan et al., 2001; Gruber and Hanley, 1998) and catabolic metabolism of extracellular matrix (ECM) (Ahsan et al., 2001; Park et al., 2001), both of which play pivotal roles in IVD degeneration. As current surgical techniques may accelerate adjacent IVD degeneration while providing mobilization and fusion with instrumentated fixation (Ishihara et al., 2004), cell transplantation and regeneration by tissue-engineering techniques may be a therapeutic strategy choice for disc degeneration. The mechanical properties of the substrates used in the engineered tissues not only provide a physical support vehicle but have important effects on cell proliferation, extracellular matrix metabolism and remodelling, and the activation of cell signalling pathways (Hohaus et al., 2008; Leung et al., 2006; Sebastine and Williams, 2007). Therefore, it is requisite to clarify the response of disc cells to substrate stiffness.

Most cells in multicellular organisms are embedded in much softer tissues than the glass or plastic surfaces on which nearly all studies are done *in vitro*. Some authors have demonstrated that substrates with different stiffnesses can create static tension of different magnitudes to the cells cultured on them (Wang et al., 2000; Yeung et al., 2005). Moreover, static tension, the counteracting force of substrate to cells, is greater when cells are cultured on stiffer substrate (Beningo and Wang, 2002; Georges and Janmey, 2005). Various bioengineering materials, such as extracellular matrix gels, silicone membranes, polyacrylamide gels, have been used to investigate the effect of mechanical properties of substrates on cellular organization and function.

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A variety of factors can induce the excessive apoptosis of intervertebral disc cells. In addition to the biochemical factors, externally applied mechanical stimuli also lead to disc cell apoptosis and disc degeneration, as shown in both animal models of over loading-induced disc degeneration and cultured disc cells or tissues subjected to unphysiologically static compression or cyclic stretch (Ariga et al., 2003; Court et al., 2001; Lotz and Chin, 2000; Lotz et al., 1998; Rannou et al., 2004; Walsh and Lotz, 2004). To date, it has been demonstrated that the internal static tension created by cell tugging on the substrates with different stiffnesses activates the signal transduction and regulates cell functions (Beningo and Wang, 2002; Bischofs and Schwarz, 2003; Karamichos et al., 2008; Wang et al., 2000). Nevertheless, not all cell types display the same sensitivity to the tension from substrate, such as transformed cells (Wang et al., 2000) and neutrophils (Yeung et al., 2005) appearing not to respond to it. Most mechanosensitive cell types grown on stiffer substrates, which create stronger tension to cells assemble more actin stress fibers (Georges and Janmey, 2005), exhibit a more spreading phenotype (Pelham and Wang, 1997), adhere and survive better (Wang et al., 2000; Yeung et al., 2005). Unfortunately, it is not clear whether the disc cells, like the other mechanosensitive cell type, respond similarly to change caused by the substrates with different stiffnesses.

In the present study, we investigated the responses of rat AF cells to substrate stiffness, specifically on the cell morphology, cytoskeleton organization, apoptosis and ECM metabolism. To accomplish this task, we developed three polyacrylamide gel substrates with different stiffnesses by varying substrate stiffness using a well-established polyacrylamide gel method (Pelham and Wang, 1997), and then examined the elastic modulus (E) of the substrates with atomic force microscopy (AFM). Using morphological and apoptotic analysis, we have observed that AF cells not only change their morphology and cytoskeletal organization but also regulate apoptosis. Moreover, we have detected by real-time PCR analysis that substrate stiffness has an evident effect on the mRNA expression of extracellular matrix regulatory genes in rat AF cells. Taken together, our results may increase new understanding on disc cell apoptosis and facilitate development in the biomechanical properties of the substrates used in the engineered tissues for potential therapies for disc degenerative disease.

2. Results

2.1. Development of polyacrylamide gels with various stiffnesses

To study the effect of substrate stiffness on the cellular morphology and function, we developed three substrates with constant chemical property but different stiffnesses by varying the concentration of acrylamide and bisacrylamide. The substrates with three different stiffnesses used in this article have an elastic modulus (E) varying between 1 ± 0.23 kPa, 32 ± 2.89 kPa and 63 ± 3.45 kPa with a thickness of about 60–70 μm, as coincided with the results in other studies (Qin et al., 2007; Tracqui et al., 2008; Yeung et al., 2005). In the discussion hereafter, these substrates will be referred to as soft ($E = 1 \pm 0.23$ kPa), intermediate ($E = 32 \pm 2.89$ kPa), and rigid ($E = 63 \pm 3.45$ kPa) substrates, respectively.

2.2. Effects of substrate stiffness on cell morphology

Distinct cell morphology was observed in rat AF cells cultured on different polyacrylamide gel substrates under phase-contrast microscopy (Fig. 1A, B and C). Cells grown on the rigid substrate were not distinguishable from those on plastic surfaces in cell morphology and growth rate (data not shown). However, when rat AF cells were cultured on increasingly soft substrate, they became less well spread and rounded or irregularly shaped. In addition, rat AF cells on soft and intermediate substrates appeared small and rounded and lost most of their stress fibers revealed by FITC-phalloidin staining of actin filaments (Fig. 1D and E). On the rigid substrate, the predominant cell morphology (approximately 80%) was a large, flattened cell or spindle-shaped with stress fibers yet (Fig. 1F, 3–24 h).

2.3. Effects of substrate stiffness on cell grow, apoptosis and area of cell spreading and nucleus

We assessed the effect of substrate stiffness on the growth characteristic of rat AF cells cultured under four conditions using the cell counting method by haemacytometer. The proliferation rate of the AF cells cultured on soft and intermediate substrates for 48 h was significantly decreased compared to those on rigid substrate and plastic, and cell growth was completely arrested on soft substrate based on cell counting method (Fig. 2A).

The percentage of apoptotic cells in the AF cells cultured on soft and intermediate substrates for 24 h was $14.3 \pm 1.7\%$ and $8.1 \pm 1.3\%$, respectively. By 48 h, the percentage of apoptotic cells increased further to $20 \pm 2.2\%$ and $12 \pm 1.6\%$ in cells on soft or intermediate substrates (Fig. 2B). There were no obvious differences in the percentage of apoptotic cells, determined by flow cytometry (Fig. 2C), between cells cultured on rigid substrate and plastic surface ($3 \pm 0.85\%$ and $2.8 \pm 0.85\%$, respectively). For the detection of apoptosis by annexin V/PI staining *in situ*, early apoptotic cells are positive in annexin V staining but they exclude propidium iodide (PI-negative). In the late stages of apoptosis cells were costained by PI and annexin V due to permeabilization of the plasma membrane. We observed that apoptosis apparently was induced in AF cells cultured on intermediate substrates and the number of apoptosis in AF cells cultured on soft substrates increased markedly (Fig. 3).

An alteration in cell spreading was also observed in AF cells after prolonged incubation on substrates with different stiffnesses. The time courses of the spreading of rat AF cells after initial seeding to different substrates were shown in Fig. 1D, E and F. As shown in Fig. 4A, a reduction in substrate stiffness caused a decrease in the cell spreading area.

The area of the nucleus may reflect the degree of cell spreading and DNA synthesis (Folkman and Moscona, 1978). To investigate whether cell spreading is associated with rat AF cells survival/apoptosis, we stained the cells cultured on the substrates with different stiffnesses with Hoechst 33258 (Fig. 3) and assessed their nuclear area and the percentage of apoptotic cells. The nuclear area of the cells cultured on soft and intermediate substrates decreased significantly, as compared to that on rigid substrate or plastic surface (Fig. 4B). Moreover, the percentage of apoptotic cells in rat AF cells was inversely proportional to the nuclear area of cells and the substrate stiffness. Interestingly, those results would seem to suggest that there is a physiological limit of cell spreading area and nuclear area for AF cells to maintain survival. When the spreading area and nuclear area of the cell was below this limit, the percentage of apoptotic cells significantly increased (Fig. 4C and D).

These findings taken together, including the area of cell spreading and nucleus, and the percentage of apoptotic cells, strongly suggest that soft substrate-induced AF cell apoptosis might result from the inhibition of cell spreading, which indicated that cell spreading was required for the growth and survival of AF cells.

2.4. Effects of substrate stiffness on caspase-3 activity

The enhanced caspase-3 activity is one of the most important events in the process of apoptosis through the mitochondrial pathway. To determine whether the release of caspase-3 was involved in soft substrate-induced apoptosis, AF cells were cultured on the substrates with different stiffnesses or a plastic dish for 24 or 48 h. We surveyed the caspase-3 levels in cell lysates and found that the caspase-3 level increased significantly in AF cells cultured on soft and intermediate