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Short communication

Aging enhances the vulnerability of mesenchymal stromal cells to uniaxial tensile strain-induced apoptosis

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ABSTRACT

Mechanical priming can be employed in tissue engineering strategies to control the fate and differentiation pattern of mesenchymal stromal cells. This is relevant to regenerative medicine whereby mechanical cues can promote the regeneration of a specific tissue type from mesenchymal precursors. The ability of cells to respond to mechanical forces is dependent upon mechanotransduction pathways that involve membrane-associated proteins, such as integrins. During the aging process changes in the mechanotransduction machinery may influence how cells from aged individuals respond to mechanical priming.

In this study mesenchymal stromal cells were prepared from young adult and aged rats and exposed to uniaxial tensile strain at 5% and 10% for 3 days, or 2.5% for 7 days. Application of 5% tensile strain had no impact on cell viability. In contrast, application of 10% tensile strain evoked apoptosis and the strain-induced apoptosis was significantly higher in the mesenchymal stromal cells prepared from the aged rats. In parallel to the age-related difference in cellular responsiveness to strain, an age-related decrease in expression of α2 integrin and actin, and enhanced lipid peroxidation was observed.

This study demonstrates that mesenchymal stem cells from aged animals have an altered membrane environment, are more vulnerable to the pro-apoptotic effects of 10% tensile strain and less responsive to the pro-osteogenic effects of 2.5% tensile strain. Thus, it is essential to consider how aged cells respond to mechanical stimuli in order to identify optimal mechanical priming strategies that minimise cell loss, particularly if this approach is to be applied to an aged population.

1. Introduction

Mesenchymal stromal cells can be expanded in culture and mechanically primed towards specific lineages (Sonnaert et al., 2014). These cells are mechano-responsive and the application of mechanical stimuli has been demonstrated to control their differentiation (Kearney et al., 2010; Haugh et al., 2011).

Integrins are mechanosensitive proteins that activate downstream signalling to evoke a response to biophysical stimuli (Popov et al., 2011; Seong et al., 2013). In mesenchymal stem cells the α2 integrins anchor the cells to collagen I (Warstat et al., 2010) and regulate the osteogenic pathway (Kuo et al., 2012; Becerra-Bayona et al., 2012).

We have previously described how the application of cyclic uniaxial tensile strain can regulate the differentiation and viability of young adult rat mesenchymal stromal cells (Kearney et al., 2008, 2010; McMahon et al., 2008). With an increasingly aged population (Lutz et al., 2008) it is pertinent to examine how aged cells respond to biophysical stimuli. The aim of the current study was therefore to examine the effect of aging on the influence of tensile strain on the viability of aged mesenchymal stromal cells.

2. Materials and methods

2.1. Culture of mesenchymal stromal cells

Mesenchymal stromal sells were prepared from 3-month old (250–300 g) and 22-month old (700–750 g) Wistar rats (Charles River (UK)), as previously described (Dominici et al., 2006). Animals were sacrificed by CO_2 asphyxiation and cervical dislocation in accordance with European guidelines (86/609/EEC). The epiphyses of the femur and tibia were cut and the marrow was flushed out with Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Gibco BRL, Dublin), 100 U/ml penicillin/streptomycin (Gibco BRL, Dublin), 2 mM glutamax (Gibco BRL, Dublin), 1 mM L-glutamine (Gibco BRL, Dublin) and 1% non-essential amino acids (Gibco BRL, Dublin). Following centrifugation at 650g for 5 min at 20 °C the pellet was re-suspended in DMEM for trituration before passage through a 40 μm nylon cell strainer. The cells were then plated out at a density of 5×10^7 cells per T75 culture flask (Sarstedt, Leicester, England). Cells were passaged upon reaching 80–90% confluency (to a maximum of 5 passages) and culture medium was changed every 3–4

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days between passages. Fluorescence activated cell sorting (FACS) demonstrated that 96.8 ± 0.4% of the cells isolated from young rats were positive for CD90, a putative MSC marker, whereas 2.1 ± 0.8% of cells were positive for the haematopoietic stem cell marker, CD45. Similarly, in cells isolated from aged rats 96.4 ± 0.4% were positive for CD90 whereas 2.2 ± 0.4% of the cells were positive for CD45.

2.2. Lipid peroxidation

Lipid peroxidation was identified using the thiobarbituric acid reactive substances (TBARS) assay (Cell Biolabs, Inc., San Diego, USA) in accordance with the manufacturer's instructions using an MDA (malondialdehyde) standard curve. Samples and standards (100 μl) were lysed in SDS (sodium dodecyl sulphate) lysis solution at room temperature and incubated with the TBA reagent at 95 °C for 60 min. Samples were centrifuged at 3000*g* for 15 min and the supernatant (from MDA standards and samples) was removed for spectrophotometric analysis of absorbance at a wavelength of 532 nm. Data obtained from the MDA standard curve are expressed as nmol MDA produced per g of tissue.

2.3. Application of mechanical strain and culture conditions

Silicone strips 600 mm^2 (10 × 60 mm^2; Speciality Manufacturing, Saginaw, MI) were coated with rat-tail collagen type I 1% (Sigma-Aldrich, Dublin, Ireland) and seeded with 2.5×10^6 cells/ml. Cyclic uniaxial tensile strains of 2.5%, 5% and 10% were applied continuously at 0.17 Hz for 3 or 7 days using a custom-made stretching device, as previously described (Moretti et al., 2004). Non-strained (static) control cells were grown on silicone membranes placed in a tissue-culture dish.

2.4. Immunofluorescent staining for α2 integrin, actin and active-caspase-3

MSCs were fixed in 4% paraformaldehyde for 25 min at room temperature (RT), permeabilised with 0.1% Triton- × 100 for 10 min and washed. Non-reactive sites were blocked with 20% goat serum for 2 h at room temperature (Vector Laboratories, USA). α2 integrin was labelled with a rabbit anti-α2 integrin (1:500; Millipore, Ireland) overnight at 4 °C. Labelled protein was detected with goat anti-rabbit secondary antibody conjugated to biotin (1:500; Vector Laboratories, USA) for 1 h at RT. MSCs were then incubated with avidin-conjugated FITC (1:500; Sigma-Aldrich, England) for 1 h at RT. Actin immunoreactivity was labelled with a mouse anti-actin antibody (1:1000; Sigma Aldrich, England) overnight at 4 °C and detected with horse anti-mouse secondary antibody conjugated to biotin (1:500, 1 h RT; Vector Laboratories, USA) followed by incubation with avidin-conjugated FITC (1:500; Sigma-Aldrich, England) for 1 h at RT. Caspase-3 was labelled with a rabbit anti-active caspase-3 (1:1000 in 30% blocking buffer; Promega G7481, England) overnight at 4 °C. Labelled protein was detected with goat anti-rabbit secondary antibody conjugated to biotin (1:1500 in 30% blocking buffer; Vector Laboratories, USA) for 1 h at RT. MSCs were then incubated with avidin-conjugated FITC (1:500; Sigma-Aldrich, England) for 1 h at RT. Coverslips were then mounted with mounting medium (Vector Laboratories, USA) and incorporated fluorophores were examined with a confocal microscope (Carl Zeiss, Germany).

2.5. Polyacrylamide gel electrophoresis and Western immunoblot

α2 integrin and ß-actin expression was assessed by Western immunoblot. Cells were harvested by scraping gently into lysis buffer (25 mM HEPES, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml pepstatin A, 2 μg/ml leupeptin, 2 μg/ml aprotinin, pH 7.4, 4 °C), centrifuged (15,000*g* for 20 min at 4 °C) and the supernatant diluted with sample buffer (150 mM Tris–HCl pH 6.8, 10% v/v glycerol, 4% w/v SDS, 5% v/v β-mercaptoethanol, 0.002% w/v Bromophenol Blue). Proteins (10 μg per lane) were separated by electrophoresis on a 10% polyacrylamide minigel, transferred to nitrocellulose membrane and immunoblotted with an anti-α2 integrin (Millipore, Ireland) or anti-ß actin antibody (Sigma-Aldrich A2228, England). Immunoreactive bands were detected using horseradish peroxidase conjugated anti-goat IgG (for α2 integrin) or anti-mouse IgG (for actin) and enhanced chemiluminescence. Blots were stripped and re-probed for GAPDH using an anti-GAPDH antibody (1:500, Abcam, UK) and horseradish peroxidase conjugated anti-mouse IgG to normalise protein loading. Band widths were quantified by densitometry (D-Scan PC software).

2.6. TdT-mediated-UTP-end nick labelling

Apoptotic cell death was assessed using the DeadEnd™ colorimetric apoptosis detection system (Promega Corporation, Madison, WI) in accordance with the manufacturer's instructions. Cells were fixed with paraformaldehyde (4%, 30 min), permeabilized with Triton × 100 0.1% and nuclear DNA strand breaks were detected by enzymatically labelling free 3-OH termini with biotinylated nucleotide using the enzyme terminal deoxynucleotidyl transferase TdT. TdT-mediated- UTP-end nick labelling TUNEL stained nuclei were visualised by detecting the peroxidase using hydrogen peroxide and the chromagen, diaminobenzadine (DAB) and cells were counter stained with methylene green. Cells were viewed under light microscopy at 40x magnification.

2.7. Statistical analysis

Statistical analysis between two groups was performed using a Student's *t*-test. When comparing more than two groups, a one-way analysis of variance (ANOVA), followed by the Neuman–Keuls *Post-hoc* test was used. When comparing more than two groups or factors, statistical analysis was performed using a two-way ANOVA, followed by the Bonferroni *Post-hoc* test.

3. Results

3.1. Expression of α2 integrin in rat MSCs is decreased with age

A significant age-related reduction in expression of the α2 subunit of integrin was observed (Fig. 1(a)); ($p < 0.001$, Student's *t*-test, $n=5$). Distribution of α2 integrin in the plasma membrane was localised in dense regions around the membrane indicative of focal adhesion complex formations. Western immunoblot detected α2 integrin expression bands at 165 kDa and a significant 25% decrease in the expression of α2 integrin was observed in MSCs isolated from aged rats ($p < 0.05$, Student's *t*-test, $n=4$).

3.2. Expression of actin in MSCs is decreased with age

An age-related decrease in actin immunoreactivity and stress fibres was observed (Fig. 1(b); $p < 0.01$, Student's *t*-test, $n=5$). Western immunoblot detected actin expression bands at 42 kDa and confirmed a significant 40% decrease in the expression of actin in MSCs isolated from aged rats ($p < 0.05$, Student's *t*-test, $n=4$).

3.3. Membrane lipid peroxidation in MSCs is increased with age

Membrane lipid peroxidation can modify plasma membrane fluidity. Fig. 1(c) demonstrates a significant increase in lipid peroxidation in MSCs isolated from aged rats compared to MSCs isolated from young rats (4.79 ± 0.70 nmol MDA/g *vs* 2.15 ± 0.34 nmol MDA/g; $p < 0.01$, Student's *t*-test, $n=6$).

3.4. Caspase-3 activity is increased in MSCs exposed to 10% cyclic tensile strain and this is enhanced in MSCs isolated from aged rats

Cells were exposed to 10% tensile strain for 3 days and apoptosis was evaluated by measuring the expression of the active form of apoptotic caspase-3 (Fig. 2) and DNA fragmentation (Fig. 3). A significant increase in active caspase-3 immunoreactivity was observed in MSCs exposed to 10% strain, compared to static controls. There was a significant effect of strain ($F_{1,16}=145.1$, $p < 0.001$, two-way ANOVA) and age ($F_{1,16}=11.27$, $p < 0.01$, two-way ANOVA). *Post-hoc* analysis revealed that both young and aged MSCs exposed to 10% strain showed a significant increase caspase-3 activity compared to static control. Furthermore, a significant increase in caspase-3 activation was also observed between young and aged cells exposed to 10% strain (Young 10%: 328 ± 7.62 units, Aged 10%:412.40 ± 29.52 units, $p < 0.01$, $n=5$). These results indicate the induction of apoptosis in MSCs exposed to 10% strain, with an increased response occurring in MSCs isolated from aged compared to young rats. The application of 5% tensile strain for 3 days (Fig. 2(c)) did not evoke activation of apoptotic caspase-3 in MSCs prepared from either young or aged rats.