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Maturational alterations in gap junction expression and associated collagen synthesis in response to tendon function

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ABSTRACT

Energy-storing tendons including the equine superficial digital flexor tendon (SDFT) contribute to energetic efficiency of locomotion at high-speed gaits, but consequently operate close to their physiological strain limits. Significant evidence of exercise-induced microdamage has been found in the SDFT which appears not to exhibit functional adaptation; the degenerative changes have not been repaired by the tendon fibroblasts (tenocytes), and are proposed to accumulate and predispose the tendon to rupture during normal athletic activity. The anatomically opposing common digital extensor tendon (CDET) functions only to position the digit, experiencing significantly lower levels of strain and is rarely damaged by exercise. A number of studies have indicated that tenocytes in the adult SDFT are less active in collagen synthesis and turnover than those in the immature SDFT or the CDET. Gap junction intercellular communication (GJIC) is known to be necessary for strain-induced collagen synthesis by tenocytes. We postulate therefore that expression of GJ proteins connexin 43 and 32 (Cx43; Cx32), GJIC and associated collagen expression levels are high in the SDFT and CDET of immature horses, when the SDFT in particular grows significantly in cross-sectional area, but reduce significantly during maturation in the energy-storing tendon only. The hypothesis was tested using tissue from the SDFT and CDET of foetuses, foals, and young adult Thoroughbred horses. Cellularity and the total area of both Cx43 and Cx32 plaques/mm^2 of tissue reduced significantly with maturation in each tendon. However, the total Cx43 plaque area per tenocyte significantly increased in the adult CDET. Evidence of recent collagen synthesis in the form of levels of neutral salt-soluble collagen, and collagen type I mRNA was significantly less in the adult compared with the immature SDFT; procollagen type I amino-propeptide (PINP) and procollagen type III amino-propeptide (PIIINP) levels per mm^2 of tissue and PINP expression per tenocyte also decreased with maturation in the SDFT. In the CDET PINP and PIIINP expression per tenocyte increased in the adult, and exceeded those in the adult SDFT. The level of PINP per mm^2 was greater in the adult CDET than in the SDFT despite the higher cellularity of the latter tendon. In the adult SDFT, levels of PIIINP were greater than those of PINP, suggesting relatively greater synthesis of a weaker form of collagen previously associated with microdamage. Tenocytes in monolayers showed differences in Cx43 and Cx32 expression compared with those in tissue, however there were age- and tendon-specific phenotypic differences, with a longer time for 50% recovery of fluorescence after photobleaching in adult SDFT cells compared with those from the CDET and immature SDFT. As cellularity reduces following growth in the SDFT, a failure of the remaining tenocytes to show a compensatory increase in GJ expression and collagen synthesis may explain why cell populations are not able to respond to exercise and to repair microdamage in some adult athletes. Enhancing GJIC in mature energy-storing tendons could provide a strategy to increase the cellular synthetic and reparative capacity.

1. Introduction

Energy-storing tendons significantly improve locomotor efficiency by storing kinetic and potential energy as elastic energy during weight-bearing, and releasing it during recoil and the subsequent swing phase of the limb (Alexander, 1988). These "biological springs" including the human Achilles tendon and the functionally equivalent

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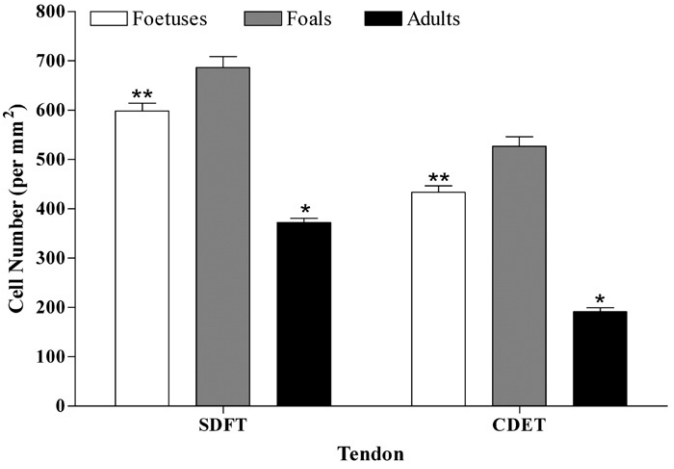

Fig. 1. Cellularity in the equine SDFT and CDET. Cellularity was quantified in cryosections from mid-metacarpal segments of the SDFT and CDET obtained from three age groups; foetuses (age <1 day, $n=5$), foals (age <1 month, $n=5$) and young adults (age 2–5 years, $n=10$). Propidium iodide was used to stain nuclei and these were counted. Each data set represents the mean ± SEM of 5 images per horse. *Indicates significantly lower than all other age groups ($p<0.001$) and **indicates significantly lower than the foal group ($p<0.05$). All CDET values were significantly lower than SDFT values for each age group ($p<0.001$).

equine superficial digital flexor tendon (SDFT) are subject to relatively high strain levels during normal activity, operating close to their physiological limits during maximal exercise, with narrow biomechanical safety margins (Riemersma and Schamhardt, 1985; Shadwick, 1990; Wilson and Goodship, 1991). Consequently, exercise-induced injury to energy-storing tendons is extremely common in both equine and human athletes (Gibbon et al., 1999; Williams et al., 2001). In the horse, the anatomically opposing common digital extensor tendon (CDET) is useful for comparison as it functions only to position the digit prior to weight-bearing, experiences significantly lower levels of strain and is rarely injured (Birch et al., 2008; Kear and Smith, 1975).

Tendon rupture during normal athletic activity is often preceded by a period of accumulation of age- and exercise-related microdamage in injury-prone sites, including degeneration of existing collagen fibrils and an increase in type III collagen (Birch et al., 1998; Goodship et al., 1994; Kannus and Jozsa, 1991; Miles et al., 1994; Patterson-Kane et al., 1997; Smith et al., 1999). It is proposed that resident tendon fibroblasts (tenocytes) constantly repair and synthesise matrix components, otherwise all tendons would eventually weaken and rupture as a result of normal use (Ker, 2002). However, excessive, repetitive and/or non-uniform loading followed by insufficient recovery times may overwhelm the cellular capacity for matrix repair in certain tendons and/or directly damage or kill cells (Leadbetter, 1992; Yuan et al., 2002).

Tenocytes are arranged in longitudinal rows separated by parallel bundles of collagen fibrils (Benjamin and Ralphs, 2000). At points of cell–cell contact within and between rows, the latter via cytoplasmic processes, they are linked by gap junctions (GJ) and adherens junctions (McNeilly et al., 1996; Ralphs et al., 2002). Gap junctions connect cells into networks by allowing diffusion of ions and small molecules (<1 kDa) between their cytoplasms, and facilitating electrical coupling (Willecke et al., 2002). In tendon this allows coordinated synthetic responses to mechanical stimuli (i.e. mechanotransduction), and is known to be a requirement for strain-induced collagen synthesis (Amiel et al., 1995; Banes et al., 1999; Ko and McCulloch, 2001). Gap junction intercellular communication (GJIC) may also facilitate provision of nutrients to tenocytes in this lowly vascular tissue (Tanji et al., 1995). Gap junctions are aqueous pores, comprised of two hemichannels (connexons), each of which is provided by one of two neighbouring cells. Gap junctions cluster together in the cell membranes to form plaques, typically comprised of hundreds to thousands of channels and measuring up to several μm in diameter (Forge et al., 2003; Segretain and Falk, 2004). Each connexon is comprised of six transmembrane proteins termed connexins (Cx). There are at least 21 connexins in the human genome (as classified by molecular weight), various combinations of which create channels with differing permeability (Bevans et al., 1998). Connexin 43 (Cx43) and 32 (Cx32) have been identified in situ in equine, human, avian and rat tendons, and may have differential effects on collagen synthesis (McNeilly et al., 1996; Ralphs et al., 1998; Stanley et al., 2007; Waggett et al., 2006).

Significant maturational and age-related reductions in tenocyte collagen synthesis occur in the SDFT but not the CDET (Batson et al., 2003; Birch et al., 2008; Goodman et al., 2004). Collagen turnover as assessed by measurement of collagen-linked fluorescence and levels of matrix metalloproteinase (MMP) gene expression and enzymatic activity, are significantly lower in the SDFT than in the CDET of the adult horse (Batson et al., 2003; Birch et al., 2008). The cross-sectional area of the SDFT increases more than two-fold between 50 and 365 days as foals rapidly increase in bodyweight, requiring a synthetically active cellular population (Kasashima et al., 2002). In the adult SDFT it has been proposed that the tenocytes "switch off" their synthetic machinery to maintain matrix stiffness and elasticity within narrow optimal limits for energy storage, however this restricts their ability to respond to matrix damage (Smith et al., 2002). In one previous study using a limited number of horses, significant reductions in cellularity and expression of Cx43 and Cx32 protein per tenocyte were measured within the first few months of life in both the SDFT and CDET (Stanley et al., 2007). However, a reduction in the amount of connexin protein does not necessarily indicate decreased efficiency of GJIC or of collagen expression (Cotrina et al., 2001; Sia et al., 1999). These investigations led to the hypothesis that there are high levels of GJ expression, communication efficiency, and associated collagen synthesis in the immature SDFT and CDET, with significant declines in all of these parameters during maturation in the SDFT only. Expression of Cx43, Cx32 and collagen mRNA and protein from SDFT and CDET tissue of skeletally immature and mature horses was analysed by quantitative real-time polymerase chain reactions (qRT-PCR), quantitative confocal laser scanning microscopy (qCLSM) and biochemical collagen analysis. Fluorescence recovery after photobleaching (FRAP) was used to assess functional dynamics of GJIC in monolayer cell culture.

2. Results

2.1. Cellularity in tendon tissue samples

There were maturational and tendon-related differences in cellularity (mean tenocyte number per mm^2 area) in all groups for both tendons

Table 1
Connexin 43 and 32 gene expression in SDFT and CDET of foetuses, foals and adult Thoroughbred horses.

Gene	SDFT			CDET		
	Foetuses	Foals	Adults	Foetuses	Foals	Adults
Cx43	0.633 ± 0.567*	2.423 ± 1.786	0.630 ± 0.713	0.556 ± 0.839*	2.423 ± 1.786	0.630 ± 0.713
Cx32	0.028 ± 0.055	0.168 ± 0.185	0.652 ± 1.648	0.004 ± 0.007	0.010 ± 0.007	0.027 ± 0.030

Copy number normalised to GAPDH. Data shown is mean ± S.D. *Indicates a significant difference to Cx32 ($p<0.05$).