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Formation of lamellar cross bridges in the annulus fibrosus of the intervertebral disc is a consequence of vascular regression

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Abstract

Cross bridges are radial structures within the highly organized lamellar structure of the annulus fibrosus of the intervertebral disc that connect two or more non-consecutive lamellae. Their origin and function are unknown. During fetal development, blood vessels penetrate deep within the AF and recede during postnatal growth. We hypothesized that cross bridges are the pathways left by these receding blood vessels.

Initially, the presence of cross bridges was confirmed in cadaveric human discs aged 25 and 53 years. Next, L1-L2 intervertebral discs (n=4) from sheep ranging in age from 75 days fetal gestation to adult were processed for paraffin histology. Mid-sagittal sections were immunostained for endothelial cell marker PECAM-1. The anterior and posterior AF were imaged using differential interference contrast microscopy, and the following parameters were quantified: total number of cross bridges; percentage of cross bridges staining positive for PECAM-1; cross bridge penetration depth (% total lamellae); and PECAM-1 positive cross bridge penetration depth.

Cross bridges were first observed at 100 days fetal gestation. The overall number peaked in neonates then remained relatively unchanged. The percentage of PECAM-1 positive cross bridges declined progressively from almost 100% at 100 days gestation to less than 10% in adults. Cross bridge penetration depth peaked in neonates then remained unchanged at subsequent ages. Depth of PECAM-1 positive cross bridges decreased progressively after birth. Findings were similar for both the anterior and posterior.

The AF lamellar architecture is established early in development. It later becomes disrupted as a consequence of vascularization. Blood vessels then recede, perhaps due to increasing mechanical stresses in the surrounding matrix. In this study we present evidence that the pathways left by receding blood vessels remain as lamellar cross bridges. It is unclear whether the presence of cross bridges in the aging and degenerating intervertebral disc would be advantages or detrimental, and this question should be addressed by future studies.

Keywords

Intervertebral disc; annulus fibrosus; blood vessel; cross bridge; development

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1. Introduction

The intervertebral disc is composed of three distinct anatomical regions: the central, proteoglycan-rich nucleus pulposus (NP), the surrounding, fibrocartilagenous annulus fibrosus (AF), and two hyaline cartilage end-plates that interface superiorly and inferiorly with the vertebral bodies. These structures act synergistically to transfer and distribute loads, and facilitate spinal mobility. The AF has a hierarchical microarchitecture comprised primarily of collagen I fiber bundles arranged in approximately concentric lamellae around the NP. The oblique angle of these fiber bundles alternates with each consecutive lamella (Marchand and Ahmed, 1990). This structure is established early in fetal development, with resident cells aligning to form the template for subsequent extracellular matrix deposition (Hayes et al., 1999; Rufai et al., 1995; Smith et al., 2011).

Within the AF lamellar architecture, a series of secondary structures have been identified and referred to collectively as 'radial bridging elements' or 'cross bridges' (Melrose et al., 2008; Pezowicz et al., 2006; Schollum et al., 2010; Schollum et al., 2008, 2009; Yu et al., 2007; Yu et al., 2002). These structures have been observed in the discs of sheep (Melrose et al., 2008; Schollum et al., 2010; Schollum et al., 2008, 2009) and bovine tails (Pezowicz et al., 2006; Yu et al., 2007; Yu et al., 2002). While the presence of cross bridges in the human AF is assumed, to date there has been no published evidence.

The origin and function of cross bridges are unknown. It has been suggested that cross bridges might form as an adaptive remodeling response to a changing micromechanical environment during aging or following injury, and are designed to reinforce the structural integrity of the lamellar structure (Melrose et al., 2008; Schollum et al., 2010; Schollum et al., 2008). The adult AF, however, has a limited capacity for remodeling and repair, as evidenced its response to trauma. For example, a stab injury to the AF results in scarring rather than regeneration (Hampton et al., 1989; Osti et al., 1990), and may initiate a degenerative cascade (Elliott et al., 2008; Sobajima et al., 2005). This limited remodeling and repair capacity can be attributed to the small number of resident cells (around 9000 cells/mm³, low compared to other skeletal tissues such as articular cartilage or knee meniscus (Maroudas et al., 1975)). Additionally, the metabolic capacity of these cells is limited due to the lack of a local blood supply: they must survive and function in a hypoxic microenvironment and rely exclusively on diffusion via the disc end-plates and the AF periphery for nutrition (Urban et al., 2004).

While the adult AF is predominantly avascular, during development this is not the case. In the human disc, blood vessels penetrate deep into the lamellar structure from as early as 35 weeks fetal gestation (Nerlich et al., 2007; Rudert and Tillmann, 1993). These blood vessels subsequently recede and by the second decade of life disappear almost completely, remaining only at the AF margins. It is possible that the microstructural disruption in the lamellar architecture never fully regenerates following vascular regression due to the limited remodeling capacity of the AF. We hypothesized the pathways left by receding blood vessels during postnatal growth are never fully integrated into the surrounding microstructure and remain into adulthood as lamellar cross bridges. Indeed, the highly interconnected lamellar cross bridge network present in the adult ovine disc resembles an interconnected vascular bed (Schollum et al., 2008). Also in support of this theory, some cross bridges in the AFs of ovine neonates stain positive for perlecan, a proteoglycan that is synthesized by vascular endothelial cells (Melrose et al., 2008).

The objectives of this study were as follows: firstly, to confirm the existence of a lamellar cross bridge network in the human intervertebral disc at skeletal maturity and following the onset of degenerative changes, and secondly, to undertake a quantitative investigation of

lamellar cross bridge formation during pre and postnatal development. Given the difficulties in obtaining human spines spanning pre and postnatal ages, an ovine model was selected for this second objective. The ovine lumbar disc has established similarities with the human lumbar disc with respect to mechanical function, geometry and composition (Beckstein et al., 2008; O'Connell et al., 2007; Wilke et al., 1997).

2. Results

2.1 Histological Observations

Morphological grades of 2 (mild signs of degeneration) and 3 (moderate signs of degeneration) were assigned to the 25 and 53 year old human discs respectively (Thompson et al., 1990). Cross bridges were present in the anterior and posterior AF of both these samples (Figure 1), and appeared morphologically similar to those previously described in large animal discs (Schollum et al., 2008, 2009). In ovine samples, cross bridges were observed at all ages except F75. At F100, a well established lamellar structure contained large number of PECAM-1 positive cross bridges in both the anterior and posterior regions (Figure 2). The majority of cross bridges at F100 appeared to bridge only a single lamella. In neonates combinations of both single and multi-lamella cross bridges were observed, with large numbers staining positively for PECAM-1 (Figure 3). At 3 months, vascular regression was observed, evidenced most clearly by the depth-dependant PECAM-1 staining of large, multi-lamellar cross bridges (Figure 4). At 7 months and in adults, an extensive network of cross bridges was present (Figure 5), similar to that described previously for sheep (Schollum et al., 2008, 2009); however very few appeared to stain positively for PECAM-1. The transition between PECAM-1 positive and PECAM-1 negative cross bridges in the anterior AF at each subsequent age while the overall distribution of cross bridges overall remains similar, is illustrated schematically in Figure 8.

2.2 Quantification

2.2.1 Number of Lamellae—In the anterior and posterior AF, the number of distinct lamellae did not change with age, with the exception of a small but significant decrease at 7 months and in adults relative to 3 months in the anterior, and at 7 months relative to F100 in the posterior (Figure 6A). The total number of distinct lamellae was significantly greater for the anterior AF than the posterior AF for all ages.

2.2.2 Number of Cross Bridges—In the anterior AF, the total number of cross bridges initially increased from 31 ± 18 (mean $\pm$ SD) at F100, to 76 ± 24 in neonates and 82 ± 24 at 3 months ($p < 0.05$, compared to F100), but was not statistically different at subsequent ages (Figure 6B). In the posterior, the total number of cross bridges did not change significantly from fetal day 100 through to adults (Figure 6C). As the total number of lamellae was found to be significantly greater in the anterior than the posterior, this parameter was normalized by dividing by the total number of lamellae at each age to facilitate an accurate comparison of the number of cross bridges between the anterior and posterior AF. The total number of cross bridges per lamella was found to be greater for the anterior AF than the posterior AF at all ages, but with statistical significance only for neonatal, 3 month, and adult samples (Figure 6D).

2.2.3 Percentage of Cross Bridges Staining Positive for PECAM-1—In the anterior, at F100 $99 \pm 3\%$ of cross bridges stained positively for PECAM-1. This percentage then decreased progressively at each subsequent age, to $87 \pm 4\%$ in neonates, $38 \pm 12\%$ at 3 months ($p < 0.05$ compared to F100 and neonates), $12 \pm 10\%$ at 7 months ($p < 0.05$, compared to F100, neonates and 3 months), and finally $1 \pm 3\%$ in adults ($p < 0.05$, compared to F100, neonates and 3 months) (Figure 6E). In the posterior, 100% of cross bridges stained positive

for PECAM-1 at F100, then decreased progressively to $91\pm11\%$ in neonates, to $60\pm17\%$ at 3 months ($p<0.05$, compared with F100 and neonates), to $17\pm11\%$ at 7 months ($p<0.05$, compared with F100, neonates and 3 months), to $8\pm15\%$ in adults ($p<0.05$ compared with F100, 3 months and neonates) (Figure 6E). The percentage of PECAM-1 positive cross bridges was not significantly different between the anterior and posterior for any age except 3 months, where it was lower for the anterior (Figure 6E).

2.2.4 Cross Bridge Penetration Depth—In the anterior, at F100, cross bridges were present inward from the AF periphery up to a peak depth of $67\pm14\%$ of the total number of lamellae. This peak depth remained unchanged at all subsequent ages (Figure 7A). In the posterior, at F100, cross bridges were present inward from the AF periphery up to a peak depth of $63\pm17\%$ of the total number of lamellae (Figure 7A). This peak depth remained statistically unchanged at all subsequent ages, except in the adult samples, where the peak depth was significantly lower than for 3 month and neonatal samples ($p<0.05$). The peak depth of cross bridges was not significantly different between anterior and posterior for any age except adult, where it was significantly lower for the posterior (Figure 7A).

In the anterior, the peak depth of cross bridges staining positive for PECAM-1 was not statistically different between F100 and neonates ($67\pm14\%$ and $71\pm8\%$ respectively), then decreased progressively at subsequent ages, to $43\pm14\%$ at 3 months ($p<0.05$, compared with F100 and neonatal), $15\pm13\%$ at 7 months ($p<0.05$, compared with F100, neonatal and 3 months), and $2\pm3.5\%$ in adults ($p<0.05$, compared with F100, neonatal and 3 months) (Figure 7B). In the posterior, the peak depth of cross bridges staining positive for PECAM-1 was not statistically different between F100 and neonates ($63\pm17\%$ and $79\pm10\%$ respectively), then decreased progressively at subsequent ages, to $41\pm19\%$ at 3 months ($p<0.05$, compared with neonatal), $19\pm14\%$ at 7 months ($p<0.05$, compared with F100 and neonatal), and $6\pm11\%$ in adults ($p<0.05$, compared with F100, neonatal and 3 months) (Figure 7B). The peak depth of PECAM-1 positive cross bridges was not significantly different between the anterior and posterior at any age (Figure 7B).

Progressive changes in the distributions of PECAM-1 positive and negative cross bridges in the anterior AF are illustrated in schematically in Figure 8. These schematics were constructed from a representative histological image at each age.

3. Discussion

In this study we present evidence that lamellar cross bridges in the annulus fibrosus form as a consequence of vascular regression. This finding contradicts the assumptions of earlier studies that cross bridges form as an adaptive response to changes in the mechanical microenvironment. Our results confirm previous findings that the number of AF lamellae is established early in fetal development and henceforth remains constant (Dahia et al., 2009). Although a small but significant decrease in the number of lamellae was found at 7 months and adult compared to earlier ages (Figure 6), this is likely due to a loss of distinction between the inner AF and NP which commonly occurs with age (Thompson et al., 1990), making lamellae in this region difficult to discriminate. Similarly, the apparent decrease cross bridge penetration depth in the adult posterior AF (Figure 7A) may be due to increased microstructural derangement of the inner lamellae at this age, making cross bridges in this region more difficult to identify.

3.1 Cross Bridges are Present in the Healthy and Degenerate Human AF

While presumed to exist, no previous evidence of cross bridges in the human AF was found in the literature. While it is reasonable to suggest that in the adult sheep the continued presence of cross bridges following vascular regression is attributable to the limited

remodeling capacity of the AF, it cannot be assumed that cross bridges would be present in an adult human disc for the same reason. In this study we have confirmed that cross bridges are indeed present in the human AF, both at skeletal maturity, and in a 53 year old disc exhibiting moderate signs of degeneration. At both ages, cross bridge morphology closely resembled that of those in the adult sheep disc. These findings both highlight the clinical importance of understanding cross bridge function, and confirm the validity of the ovine model for their ongoing study.

3.2 Cross Bridges Form as a Consequence of Vascular Regression

With advancing age there was a clear transition from cross bridges that were associated with blood vessels, to those that were not. Our results clearly demonstrate that there is no increase in the number or penetration depth of cross bridges subsequent to vascular regression. The pattern of vascular regression/cross bridge formation followed a very similar course in the anterior and the posterior AF. The greater density (per lamellae) of blood vessels/cross bridges in the anterior (Figure 6D) perhaps reflects the greater disc height in this region. The increase in the number of cross bridges between fetal day 100 and the neonate that occurs in the anterior (Figures 6B-D) but not in the posterior suggests that the rate of vascularization is different between the two regions, and may also reflect the fact that the peak vascularization (per lamellae) is greater in the anterior region and may take longer to reach.

The avascularity of the adult intervertebral disc is considered to contribute to its limited ability to heal reparatively and complete inability to heal regeneratively. Although the exact reasons for postnatal vascular regression in the AF are not known, mechanical loading may be a contributing factor. From a purely physical perspective, it is possible that the circulatory pressure required to keep blood vessels open simply cannot overcome the large physiological stresses exerted on them by the surrounding extracellular matrix. Indeed, our results clearly show that blood vessel regression commences soon after birth, coincident with the onset of physiological stresses and suggesting a link between the two. One argument against this, however, is the fact that blood vessels are present in the human AF until the third decade of life (Rudert and Tillmann, 1993), indicating the vascular regression is a slowly progressing phenomenon – a finding that is also supported by the results of this study. There is evidence that mechanical factors can mediate vascular biology, by modulating production of anti-angiogenic factors. Tendon fibroblasts cultured under intermittent hydrostatic load secrete increased amounts of endostatin, an anti-angiogenic factor that inhibits endothelial cell migration (Pufe et al., 2003). An alternative, or complementary explanation of vascular regression may be the decrease both in the number of resident cells and, perhaps, decreased metabolism of those that remain, due to reduced matrix synthesis requirements at musculoskeletal maturity. At 3 months, the number of cross bridges staining positive for PECAM-1 was significantly lower in the anterior AF than in the posterior, in contrast to other ages where there was no significant difference between the two regions. This may indicate a disparity in the rate of vascular regression between the anterior and posterior, perhaps attributable to differences in nutritional requirements or the local micromechanical environments.

3.3 Possible Functions of Cross Bridges

While the microarchitecture of the cross bridge network in the ovine disc has been described in detail (Schollum et al., 2008, 2009), very little is known about its effects on mechanical function. Observations from micromechanical studies suggest that cross bridges have at least some tensile strength: under radial strains, cross bridges appear to maintain physical connections between lamellae in the presence of separation at lamellar interfaces (Pezowicz et al., 2006). How these local properties might influence the biomechanical function of AF as a whole is unclear. Additionally, it is unclear whether any such influence would be

relevant at the magnitude of radial strains that occur within the disc structure under physiological loading (Costi et al., 2007; O'Connell et al., 2011; Tzantrizos et al., 2005).

A major characteristic of the degenerating disc is AF delamination (separation of adjacent lamellae). Delaminations may on occasion progress to more severe circumferentially aligned tears (Vernon-Roberts, 1988), which have been shown to affect the mechanics of the intervertebral joint in torsion and bending (Fazzalari et al., 2001; Thompson et al., 2000). Delaminations are evident in the AF from as early as 20 years of age, with their prevalence and severity displaying a positive correlation with age (Videman and Nurminen, 2004). These delaminations and tears might form as a consequence of the increased and non-uniform compressive forces experienced by the AF as the hydrostatic pressure provided by the NP decreases (Adams et al., 1996), or due to fatigue induced microstructural failure at the lamellar interfaces (Iatridis et al., 2005). Enhanced radial integrity resulting from the presence of an interconnected cross bridge network might inhibit the formation of these tears, or, following tear formation, continue to provide some lamellar interconnectivity. Evidence presented here indicates that cross-bridge formation is not of an adaptive mechanobiological nature. Any beneficial mechanical contribution by cross bridges would therefore appear to be coincidental. An alternative hypothesis is that cross bridges act as stress raisers within the lamellar structure, and contribute to microscale failure and tear formation. The AF has a functionally graded composition and architecture designed to evenly distribute and transfer stresses under multiple loading configurations. Cross bridges represent interruptions in this otherwise highly organized structure, and are therefore likely to be associated with localized regions of increased stress.

3.4 Future Work

Ongoing studies should investigate the contribution of cross bridges to AF mechanical function, with a specific focus on describing potential associations with pathological tear formation (either positive or negative). Studies should also investigate potential cellular and biomechanical mechanisms driving postnatal vascular regression.

4 Experimental Procedures

4.1 Sample Preparation

Cadaveric human lumbar spines from two donors aged 25 and 53 years were acquired from National Disease Research Interchange (Philadelphia, USA). T12-L1 vertebra-disc-vertebra spine segments were isolated using a reciprocating saw, and posterior bone elements removed. Ovine lumbar spines were obtained from multiple sources (see acknowledgments) with Institutional Animal Care and Use Committee approval. Donor animals spanned six ages (each $n = 4$): fetal day (F)75, F100, neonate (full term is approximately 150 days gestation), 3 months, 7 months and skeletally mature adult (3.5-5 years). L1-2 spine segments were isolated, and posterior bone elements were removed using miniature bone shears (pre-natal segments) or a reciprocating saw (postnatal segments). Human and ovine spine segments were then fixed in buffered 10% formalin, then decalcified in formic acid/ethylenediaminetetraacetic acid (Formical-2000; Decal Chemical Corporation, Tallman, NY). Fixation time ranged from one to seven days, and decalcification time ranged from two to 14 days, depending on the sample age and size. Complete decalcification was confirmed radiographically. Fetal samples were processed into paraffin en-bloc. For human and postnatal ovine samples, a mid-sagittal slab was obtained using a straight-edged trimming knife. Human samples were graded macroscopically for degenerative condition (Thompson et al., 1990). Mid-sagittal slabs were then processed into paraffin. Histological sections 8 μm thick were cut from the mid-sagittal plane of each sample.

4.2 Immunohistochemistry

Sections (one randomly selected from each sample) were deparaffinized, rehydrated and washed in tris-buffered saline (TBS). Human sections were imaged unstained. For sheep sections, antigen retrieval was performed with proteinase K (Dako; Glostrup, Denmark) for 4 mins at room temperature. Endogenous peroxidase activity was blocked with hydrogen peroxide for 15 mins, and non-specific protein binding was blocked both with 10% normal goat serum containing 2.5% bovine serum albumin and 0.2% Nonidet P40 (BDH Chemicals Ltd; Poole, UK) for 30 mins, and a serum-free blocking agent (Ultra V Block; Thermo Fisher Scientific, Waltham, USA) for 5 mins. Sections were then incubated with an antibody against ovine platelet endothelial cell adhesion molecule (PECAM-1) (Abbiotec; San Diego, USA) at a dilution of 1:800, for 90 minutes at room temperature. PECAM-1 (or CD31) is an endothelial cell marker used to identify blood vessels (Ali et al., 2008). Bound antibody was detected using the UltraVision ONE Detection System (Thermo Fisher Scientific, Waltham, USA), with diaminobenzidine as the chromogen. Between incubation steps sections were washed 4 times with TBS containing 0.2% TWEEN 20 (Sigma Aldrich; St Louis, USA). Finally, sections were cover-slipped with an aqueous mounting medium (Dako; Glostrup, Denmark). Sections of sheep carotid artery were used as positive controls, and normal rabbit IgG antibody (R & D Systems, Inc; Minneapolis, USA) in place of the primary antibody was used to generate negative controls.

4.3 Imaging

Sections were imaged using differential interference contrast (DIC) microscopy (Eclipse 90i; Nikon Corporation, Tokyo, Japan) at the following objective magnifications: 40× for F75 and F100, 20× for neonates, and 10× for 3 months through adult. The microscope was equipped with a motorized stage enabling automated capture and composition of the entire anterior or posterior AF. Fetal samples were also imaged under cross-polarized light, as pilot studies indicated that the AF lamellar structure was not easily discernable under DIC imaging at these ages.

4.4 Quantification

Each composite AF image (anterior and posterior) was analyzed to determine the following six parameters:

1. The total number of distinct lamellae.
2. The total number of lamellar cross bridges. A cross bridge was defined as any radially oriented interruption in a lamella occupying at least 50 percent of its thickness. Where a cross bridge appeared to traverse multiple lamellae consecutively, each of those lamellae was counted as a single cross bridge (e.g. a cross bridge traversing 3 lamellae consecutively is counted as 3 cross bridges).
3. The number of cross bridges per lamellae (total cross bridges divided by total lamellae).
4. The total number of lamellar cross bridges with any part staining positive for PECAM-1.
5. The maximum depth of penetration inward from the AF periphery of cross bridges (the deepest lamellae with a cross bridge divided by the total number of lamellae).
6. The maximum depth of penetration inward from the AF periphery of cross bridges staining positive for PECAM-1.

To qualitatively illustrate the age-related changes in distribution of cross bridges that stained positively and negatively for PECAM-1, a map of the anterior AF was created for a

representative sample at each age. This was done by tracing the outline of the AF using imaging software (Photoshop CS4, Adobe Systems; San Jose, USA) and the positions of all cross bridges were then marked using either a black dot (PECAM-1 positive) or a white dot (PECAM-1 negative).

4.5 Statistical Analysis

One way analyses of variance (ANOVAs) followed by post-hoc Tukey's tests were used to determine differences in each measured parameter between ages. Paired Student's t tests were used to examine differences in measured parameters between the anterior and posterior AF. Significance was defined as $p < 0.05$.

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Figure 1.

Cross bridges (arrows) in the human AF. A. Anterior and B. posterior AF of a human 25 year old T12-L1 disc, Thompson grade 2 (bars = 200µm). C. Anterior and D. posterior AF of a 53 year old T12-L1 disc, Thompson grade 3 (bars = 100µm). DIC imaging; unstained, rehydrated sections. In all images, the disc periphery is to the left and the nucleus pulposus is to the right (both outside the field of view).

Figure 2. Ovine AF at F100. A. Lamellar structure in the anterior AF (polarized light, bar = 200 μ m;). B. High magnification view showing PECAM-1 positive cross bridges in the anterior AF (DIC; bar = 20 μ m). C. Lamellar structure in the posterior AF (polarized light; bar = 200 μ m). D. High magnification view showing PECAM-1 positive cross bridges in the posterior AF (DIC; bar = 20 μ m). Anti-PECAM-1 stain. P = disc periphery; N = nucleus pulposus; arrows = cross bridges.

Figure 3.

Neonate ovine AF. A. Low magnification view of the anterior AF. B. Higher magnification view showing single and C. multi-lamellar cross bridges (arrows) staining positive for PECAM-1. D. Low magnification view of the posterior AF. E. Higher magnification view showing a PECAM-1 positive cross bridge (arrow) with open lumen. F. Example (arrow) of a blood vessel within and running parallel to a lamellar interface. DIC imaging; anti-PECAM-1 stain. For panels A and D, bar = 100 μ m; for panels B, C, E and F, bar = 20 μ m.

Figure 4.

Three month ovine AF. A. A multi-lamellar cross bridge extends to a depth of approximately 15 lamellae inwards from the disc periphery (black arrows = PECAM-1 positive cross bridges; white arrows = PECAM-1 negative cross bridges). B. Over the first 9 lamellae, the cross bridge is associated with a blood vessel, as evidenced by positive PECAM-1 staining and open lumen (*). C. Over the subsequent 6 lamellae, PECAM-1 staining and open lumen are absent; however the cross bridge remains, indicating the former path of the blood vessel. DIC imaging; anti PECAM-1 stain; all bars = 100µm.

Figure 5. Seven month and adult ovine AF. A. Cross bridges (arrows) in the anterior and B. posterior AF at 7 months. C. Cross bridges (arrows) in the anterior and B. posterior AF in adult samples. DIC imaging; anti PECAM-1 stain; all bars = 100 μ m.

Figure 6.

A. Age-associated changes in the total number of distinct lamellae in the anterior and posterior AF; * indicates significant difference with respect to fetal day 100 and 3 months; + indicates significant difference with respect to fetal day 100; # indicates significant difference between anterior and posterior. B. Age-associated changes in the total number of cross bridges in the anterior AF. C. Age-associated changes in the total number of cross bridges in the posterior AF. D. Comparison of the total number of cross bridges in the anterior and posterior AF, normalized by the total number of distinct lamellae. E. Age-associated changes in the percentage of PECAM-1 positive cross bridges in the anterior and posterior AF. For panels B through E, 'f', 'n', '3', '7' and 'a' indicate significant difference with respect to fetal day 100, newborn, 3 month, 7 month and adult samples respectively; # indicates significant difference between anterior and posterior at that age.

Figure 7.

A. Age-associated changes in the penetration depth of cross bridges (total) in the anterior and posterior AF. B. Age-associated changes in the penetration depth of PECAM-1 positive cross bridges in the anterior and posterior AF. 'f', 'n', '3', '7' and 'a' indicate significant difference with respect to fetal day 100, newborn, 3 month, 7 month and adult samples respectively; # indicates significant difference between anterior and posterior at that age.

Figure 8.

Schematic representation of age-associated changes in cross bridge distribution in constructed from representative anterior sheep AF samples (AF= dark gray shaded). Vascular regression is represented by the transition from PECAM-1 positive (black dots) to PECAM-1 negative (white dots). 'P' indicates location of anterior disc periphery.