Large Cohort Investigation of Nerve and Blood Vessel Ingrowth into Human Intervertebral Discs

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Introduction: It is speculated that the ingrowth of nerves and blood vessels into the usually aneural and avascular intervertebral disc (IVD) is involved in the production of chronic low back pain. Previous studies have identified the growth of nerves alongside blood vessels within the inner annulus fibrosus (AF) and in some cases within the nucleus pulposus (NP) of degenerate IVD’s. Freemont et al., (1997) found that in-growing nerve fibres were GAP43 positive indicating that they were actively growing, as well as producing nociceptive neurotransmitters such as Substance P and Calcitonin Gene Related Peptide [1]. These findings led to the hypothesis that nerves within the degenerate disc may play a role in the transmission of pain. Following on from this, recent studies within this area have identified increases at both gene and protein level of factors known to promote neoinnervation and angiogenesis such as NGF, BDNF and VEGF [2,3]. This study aimed to further explore the presence and localisation of nerve and blood vessels in a substantially larger cohort of patient samples than previously investigated. In order to address this controversial topic, two nerve markers which have been previously used within IVD studies were used to confirm the presence of nervous tissue within IVD’s.

Methods:
Human IVD tissue was obtained from surgery or post mortem examination with informed consent of the patients or relatives. Ethical approval was obtained from Sheffield Research Ethics Committee (09/H1308/70). IVD tissue was obtained from 100 patients undergoing microdiscectomy for nerve root compression, cauda equina syndrome or sciatica and 28 post-mortem samples from 26 individuals. Tissue consisting of AF and NP was fixed in 10% neutral buffered formalin, and processed to paraffin wax. Following embedding, 4µm tissue sections were taken for haematoxylin and eosin (H&E) staining, stained sections were evaluated independently by two researchers to determine the extent of degenerative tissue changes based on previously published
criteria [4]. Immunohistochemistry was performed on serial 10µm tissue sections to identify the presence of nerve markers; Neurofilament 200 (NF200) and Protein Gene Product 9.5 (PGP9.5), and the blood vessel marker CD31, thus allowing the investigation of co-localisation of nerves and blood vessels within the IVD tissues. The presence of nerves within IVD tissue was observed and verified by two researchers (ALAB and CLLM).

**Results:**
Immunohistochemistry results demonstrated positive staining for NF200 in 63% of human IVD tissues investigated thus far. Immunopositivity for NF200 was identified within OAF, IAF and within the NP of IVDs from varying grades of degeneration (Figure 1A/B). In many cases, immunopositive nervous tissue followed the tracks of the IVD lamella, yet in some cases, nerves were seen to protrude across the lamellar ridges or extracellular matrix within the NP towards NP cells, thus suggesting that NP cells are producing nerve attracting factors. Nerves which were identified deep within the NP of PM samples, were always within close proximity to fissures, similarly nerves within herniated tissue, were also close to fissures (Figure 1B) or in areas where decreased matrix was seen. The morphology of the nerves varied on the orientation of the nerves in the section, for example in Figure 1A, nerve fibres and processes are visible; whereas in Figure 1D a cross section of tissue reveals a bundle of nerves stained with PGP9.5. Immunopositivity for PGP9.5 varied throughout the sample cohort, a finding of particular interest was the immunopositivity of NP cells themselves in a large group of herniated tissue. In some cases, nerves were found next to blood vessels within the IAF, and also with infiltrating cells in herniated samples which are thought to be of an immune origin. Blood vessels were identified on serial H&E stained sections and further confirmed by immunohistochemistry detecting CD31 (Figure 1E). Further analysis will investigate the localisation of blood vessels to nerves and the frequency of expression within samples.

**Discussion:**
The findings in this study so far showed that nerves and blood vessels are present within not only the outer areas of the AF, but also deep within the NP tissue of herniated and PM IVDs. Nervous tissue was seen mainly close to fissures, suggesting that nerves are able to in grow into the inner regions of the IVD where the matrix composition is compromised, thus agreeing with previous studies by Stefanakis et al., (2011) [5]. Although the majority of nerves were seen to follow the tracks of the tissue, some nerves were seen to cross the lamellar ridges and protrude towards degenerate NP cells. This suggests that those NP cells are expressing factors which may attract growing nerves such as NGF and BDNF, which have already been identified within the NP of both non-degenerate and degenerate NP cells at both gene and protein level [2, 3]. PGP9.5 was the neuronal marker of
choice in many previous studies investigating nerve in growth into the intervertebral disc [1, 5 - 7]. Interestingly, this study identified PGP9.5 expression within many native NP cells within herniated tissues, suggesting these cells could be derived from a neural lineage (Figure 1F). In addition to the NP cells being positive for PGP9.5, what appears to be positive neural tissue was also observed within varying degrees of degenerate IVD tissue (Figure 1C) which agrees with previous studies performed [1, 7]. Interestingly, PGP9.5 staining of human NP cells was not evident within any of the post mortem samples investigated in this study, suggesting that the changes which occur during degeneration may cause NP cells to re-activate factors which may have been switched off after development and maturation of the IVD or that alternative cell types migrate into the IVD during degeneration. Further studies will be performed to confirm that the nerves identified within these tissues are sensory nerve fibres by investigating Substance P and TrkA which we have previously shown to be significantly up regulated by cytokines within the degenerate disc.

**Significance:**
This study addresses the controversial topic of nerve and blood vessel ingrowth into the IVD, in a large number of patient samples. Ongoing studies show that nerves are present within the IAF and NP of degenerate IVDs from patients experiencing pain. These nerves are predominantly found close to fissures which may provide a route into the inner areas of the IVD where the fibrous matrix is disrupted. Unlike other studies, our findings only show nerve association with blood vessels on few occasions. Previous studies have shown human NP cells are able to increase their production of nociceptive factors such as Substance P, which could cause sensitisation of nerves ultimately resulting in discogenic pain.
Figure 1: Immunohistochemistry of nerve and blood vessel markers in human IVD tissue.