The Role of Class 3 Semaphorins in Innervation and Angiogenesis within the Degenerate Human Intervertebral Disc.

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Introduction:
A newly emerging area in regards to innervation and angiogenesis into usually aneural and avascular tissues such as the intervertebral disc (IVD) surrounds the role of class 3 Semaphorins (Semas). Semaphorins are a large family of axonal guidance molecules, known for their ability to cause cytoskeletal changes to nerves and blood vessels to prevent inappropriate entry into usually aneural or avascular tissues. Class 3 Semaphorins signal their response through two prominent Semaphorin receptors: the Neuropilins (NRP) and the Plexins [1, 2]. Sema3A has previously been identified within human IVD samples by immunohistochemistry (IHC) [3], where it was identified at the periphery of the OAF within non-degenerate discs, whilst degenerate samples, showed decreased immunopositivity in the OAF but increased in the NP [3], suggesting a protective role against nerve and blood vessel ingrowth within non-degenerate OAF. This study aimed to firstly, investigate whether class 3 Semaphorins are expressed by native IVD cells at mRNA and protein level. Secondly, to investigate Semaphorin regulation by cytokines which are known to be up-regulated during IVD degeneration, and finally determine whether human NP cells can respond to semaphorins.

Methods:
Human IVD tissue was obtained from patients undergoing microdiscectomy for nerve root compression, cauda equine 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 staining, stained sections were evaluated independently by two researchers to determine the extent of histological degeneration [4].

Real-Time PCR (qRT-PCR) was performed to investigate gene expression of class 3 semaphorins; Sema3A-3F, their receptors Plexin A1-A4 and Neuropilin-1 (NRP-1) and NRP-2 on directly extracted RNA from human NP cells from 52 patients and NP cells from 5 patients cultured in alginate for 2 weeks, prior to treatment for 48hours with IL-1β, IL-6 or TNFα at 0-100ng/mL.
Immunohistochemistry was performed on serial 4 µm tissue sections to identify the presence of Sema3C and its receptors NRP1, NRP2 and PlexinA1. Additionally, the localisation of nerves and blood vessels was investigated in conjunction with Semaphorin positivity in IVD tissues.

Recombinant Sema3A and Sema3C treatments.

Human NP cells were seeded onto an 8 well chamber slide and treated with 0-500ng/mL of recombinant sema3A or Sema3C for 0-24hours, after which cells were fixed in 4% w/v paraformaldehyde/PBS prior to staining with Alexa Fluor® 488 Phalloidin and Hoechst to visualise actin cytoskeletal alterations.

**Results:**

All class 3 Semaphorins and their receptors, except for Sema3B were expressed at mRNA level within human NP cells irrespective of degeneration grade. Sema3A was constitutively expressed in 100% of IVD samples, whilst Sema3C and NRP1 expression increased to 100% in severely degenerate samples as opposed to histologically non-degenerate samples where 75% expressed Sema3C. NRP2 was expressed by all IVD samples regardless of degeneration grade, Plexin A1 expression was present in 95% of severely degenerate samples. Following cytokine treatments only IL-1β was seen to regulate Semaphorin expression: Sema3C was significantly upregulated in response to ≥1pg/mL, whilst ≥100pg/mL Sema3D was also induced.

For the first time this study demonstrated expression of Sema3C (Fig1A), NRP1 (Fig1B), NRP2 (Fig1C) and PlexinA1 (Fig1D) using immunohistochemistry in human IVD tissue. In vitro stimulation of human NP cells by 10ng/ml Sema3A demonstrate a retraction of the cytoskeleton and a loss of F-actin following 5 minutes (Fig2). The percentage of human NP cells demonstrating altered cytoskeleton increased from 0% after 30 second incubation, to 21% after 5 minutes in the presence of Sema3A; indicating further that human NP cells express active receptors for Semaphorins.

**Discussion:**

The role of class 3 Semaphorins within the IVD is a newly emerging area with only one previous study investigating Sema3A in the IVD [3]. Traditionally, Semaphorins are known for their repulsive effects on nerves and blood vessels preventing inappropriate entry into aneural and avascular tissues. Having said this, Sema3C has been identified for its attractive effects on mouse glomerular endothelial cells over a collagen type I gel [5]. This is the first study to identify the presence of all class 3 Semaphorins and their receptors within a large cohort of patients, and report their regulation by cytokines. Semaphorin 3C expression was significantly up regulated approximately 80 fold in response to IL-1β treatment on human NP cells. This suggests that during degeneration, Sema3C expression by
NP cells could be an attractive factor involved in the stimulation of nerve and blood vessel ingrowth into the degenerate disc. Previous studies concentrated on Sema3A which is the most commonly studied molecule from the family, and found that expression of Sema3A was present in non-degenerate discs in the OAF; yet in degenerate discs immunopositivity decreased in the OAF and increased in the NP [3]. The data presented here demonstrates constitutive expression of Sema3A at mRNA level in NP cells regardless of degeneration grade; suggesting that during degeneration Sema3A may become activated in an attempt to inhibit ingrowing nerves. Cytoskeletal studies thus far using Sema3A demonstrate the ability of human NP cells to respond by actively altering their actin cytoskeleton, this could be a potential mechanism by which NP cells are able to detach from their extracellular matrix and respond to chemokines and cytokines within the degenerate environment. Ongoing studies hope to elucidate the nature of Sema3A and Sema3C on endothelial and nerve cells over a matrigel, as well as their localisation to nerves and blood vessels within human IVD tissue.

**Significance:**

Class 3 Semaphorins are of particular interest in the IVD due to their roles in nerve and blood vessel guidance. We have shown human NP cells express a variety of class 3 Semaphorins and their receptors at both gene and protein level. Their regulation by interleukin 1 suggests that cytokines may be involved in the modulation of nerve and blood vessel ingrowth into the degenerate IVD via Semaphorin regulation.
Figure 1: Immunopositivity for Sema3C and its receptors within human IVD tissue.

Figure 2: Visualisation of the actin cytoskeleton of human NP cells, before (A) and after (B) treatment with Sema3A.