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Review

Inflammatory mediators and signalling pathways controlling intervertebral disc degeneration

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Summary. Intervertebral disc (IVD) degeneration (IDD) is one of the major causes of back pain, a condition that represents a serious socio-economic burden. Deeper knowledge of the complex and fine relationship between IVD degeneration, tissue inflammation and pain, appears to be critical to improve the current therapies, which have so far proven themselves ineffective. Upon degeneration, IVD tissues become inflamed, and this inflammatory microenvironment is associated with a cascade of degenerative events that may eventually cause discogenic pain. In particular, several studies have highlighted the major role of a number of pro-inflammatory mediators not only in the onset of the inflammatory condition, but also in the development of IDD in general. In this review, we will present the main pathological events that occur during disc degeneration, focusing on the relationship between the abnormal inflammatory milieu of the degenerating IVD, IDD and the generation of pain. Finally, we will present the current therapies for the treatment of IDD and low back pain, and the perspectives of future, more effective therapies.

Key words: Intervertebral disc degeneration, Inflammatory mediators, Low back pain, Neuro-inflammation

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Introduction

Back pain is a serious public health problem and it has been identified as the single most common cause of disability worldwide by the 2013 Global Burden of Disease Study (Collaborators of Global Burden of Disease Study, 2015). In particular, low back pain (LBP) has been reported as the biggest contributor to the global Years Lived with Disability (Collaborators of Global Burden of Disease Study, 2015). LBP presents with a lifetime prevalence of over 80% in North America (Freburger et al., 2009) and it is estimated that in the United States LBP costs about 100 billion dollars per year for health care services and paid time off from work, resulting in a tremendous socio-economic burden (Katz, 2006). Although LBP etiology is still not completely understood and different anatomical structures can contribute to the onset of pain, it is typically associated with intervertebral disc (IVD) degeneration (Baliga et al., 2015). IVD degeneration (IDD) is considered as an aberrant, pathological, cell-mediated response that leads to progressive structural failure and, often, pain (Adams and Roughley, 2006). Nowadays, the therapeutic strategies to treat IVD degeneration and assuage IDD-related pain include conservative treatments, such as anti-inflammatory drugs. When these treatments prove themselves inefficient, radical surgical interventions (e.g. disc removal and spinal fusion) are considered, even though their aim is not to re-establish the physiological structure and biomechanical properties of the disc but just to remove the source of pain. Recently, researchers have been proposing various alternative biological treatments

capable of preventing further degeneration of the disc and stimulating its regeneration, including gene therapy (Woods et al., 2011; Zhang et al., 2011) and stem cell-based tissue engineering (Werner et al., 2014; O'Connell et al., 2015; Vadalà et al., 2016). Although in the future these novel treatments could be an alternative to actual therapies, clinical management of disc pathologies still remains limited since the biological processes controlling disc development, homeostasis, function and, hence, degeneration are still not completely understood.

The aim of this review is to present the pathological changes that occur in the IVD during its degeneration, highlighting the impact of inflammation in IDD. We will then discuss the major factors involved in the cascade of degradative events, with a particular attention to a number of proinflammatory cytokines that are associated to each step of this process. Finally, we will report the current therapeutic strategies for IDD and LBP, and we will introduce the perspectives of novel biological treatments.

Structure and biology of healthy IVD

The IVD is a complex and heterogeneous, structure made of specialized fibrocartilaginous connective tissue positioned between two adjacent vertebrae. IVD has the function to confer limited flexibility to the body trunk, assure mechanical stability during axial compression and motion, and protect both the spinal cord and spinal nerves. Healthy IVD consists of three structurally distinct, interdependent components: a gelatinous core named nucleus polposus (NP), a lamellar outer ring of fibrous tissue called annulus fibrosus (AF) that surrounds the NP, and two cartilaginous end plates (CEPs) that serves as an interface between the disc and the vertebrae by cranially and caudally covering both the NP and the AF (Raj, 2008).

The NP consists of a highly hydrated proteoglycan-containing gel that incorporates an irregular network of type II collagen and elastin fibers (Adams and Roughley, 2006). Aggrecan is the major proteoglycan in the NP and has a fundamental role in maintaining disc height and turgor against compressive loads by attracting water molecules inside the NP extracellular matrix (ECM) and generating a high swelling pressure (Watanabe et al., 1998). NP fibers are mainly composed by type II collagen but other collagen types are present in smaller amounts and have important functions in maintaining the characteristics of the hydrogel matrix, e.g. type XI collagen is important in the assembly of type II collagen fibers and type IX collagen permits the crosslinking of adjacent collagen fibrils (Kepler et al., 2013a,b). Early in life, the human NP undergoes a substantial change in its cellular composition. Cells in the NP at birth are predominatly of notochordal (NC) origin but they become almost undetectable in the first decade of life. At this point the NP region becomes gradually populated, especially in region close to the CEPs, by a significant majority of chondrocyte-like rounded cells (Risbud et al., 2010). The origin of these chondrocyte-like cells in the mature NP still remains uncertain in humans but the most corroborated hypothesis is that they derive from the embryonic notochord (McCann et al., 2012; Purmessur et al., 2012). In addition, a subpopulation of progenitor cells expressing multipotent cell markers have been identified in both animal healthy IVDs and human degenerated IVDs, even though their origin and functions have not still been determined (Sakai et al., 2012; Marfia et al., 2014).

NP is enclosed by the AF, which prevents the extrusion of NP when excessive loads are applied on the IVD. AF ECM composition varies as a function of intradiscal region. The outer region of AF is composed of a series of concentric lamellae, each formed by parallel bundle of type I collagen fibers. These type I collagen fibers have an oblique orientation of approximately 30 degrees to the longitudinal axis of the spine, alternating their direction between lamellae to form an angle-ply structure that provides tensile strength to the disc. Moving from superficial layers inwards, the AF layers become less well-organized and the inner part of AF appears as a transition tissue between NP and outer AF since it shows a higher content of type II collagen, proteoglycans and water compared to outer AF (Kerr et al., 2016). Cells in the outer part of the AF are fibroblast-like with elongated nuclei that are aligned parallel to the collagen fibers, whereas, moving towards the NP, cells gradually assume a rounded chondrocyte-like phenotype (Adams and Roughley, 2006). Both NP and AF are covered cranially and caudally by CEPs, which consist of a layer of articular cartilage and anchor the IVD to the cortical bone of the vertebral body (bony end plate). The structure formed by the cartilaginous end plate and the bony end plate is known as end plate (EP), and with its resilience properties prevents the load transmitted through the IVD from facturing the bone of the vertebral body (Freemont, 2009). The combination of two adjacent vertebrae, the EPs and the IVD between them, is called spinal motion segment (SMS) and is the functional unit of the spinal column. Cells within the CEPs are similar to articular chondrocytes and secrete type II collagen and a proteoglycan-rich ECM (Kerr et al., 2016). CEPs are also the primary source of nutrition of the IVD.

The healthy, adult IVD is almost entirely avascular and only specialized capillary, beds between the bony end plate and the CEP, provide the limited supply of nutrients and oxygen that arrives inside the IVD via passive diffusion through the CEPs (Urban et al., 2004). Moreover, healthy IVD is typically considered as a poorly innervated organ. Innervation, normally but not exclusively accompanied by vascularization, is restricted to the outer layers of the AF and consist of sensory and sympathetic perivascular nerve fibers. In particular, it has been demonstrated that the sensory fibers that innervate the IVD are small sensory nerves (both peptidergic and non-peptidergic nociceptors), as well as larger fibers forming proprioceptors. These nerves

originate from dorsal root ganglion (DRG) neurons, which contain different types of sensory neurons that project either to the IVD or to the dorsal horn of the spinal cord (Edgar, 2007).

Furthermore, this unique physiological structure without significant innervation and vascularization, as well as the fact that there have been no studies reporting the presence of a resident immune cell population in the healthy disc, have led to the consideration that IVD is an immune-privileged organ (Murai et al., 2010).

IVD degeneration (IDD)

IDD has been reported to begin early in life, often by the second decade (Buckwalter, 1995). Although the precise etiology of IDD is still unclear, degeneration occurs as a natural event of disc aging and an increasing number of studies have shown that this process can be potentially accelerated or exacerbated by the synergistic contribution of environmental and genetic factors (Virtanen et al., 2007; Wang et al., 2016a,b). As for the environmental risk factors, an unhealthy lifestyle (e.g. lack of exercise (Elfering et al., 2002), smoking (Cong et al., 2010; Wang et al., 2012)), mechanical influences and occupational exposures (e.g. heavy lifting (DePalma et al., 2012), vibration (Virtanen et al., 2007), trauma (Heyn et al., 2014; Schroeder et al., 2014)) and infective microorganism infiltration after previous minor trauma (Stirling et al., 2001), have been suggested as contributing causes of IDD. On the other hand, the importance of genetic factors in the development of IVD degeneration has become evident in recent years due to studies that have identified a correlation between polymorphisms of many key genes (e.g. collagen I gene COL1A1 (Tilkeridis et al., 2005; Videman et al., 2009), vitamin-D receptor gene (Videman et al., 1998; Kawaguchi et al., 2002), diverse protease genes linked to matrix degradation (Zhang et al., 2013a, 2015; Zawilla et al., 2014; Liu et al., 2015) and different degrees of IDD (Kepler et al., 2013a,b), as well as have also revealed the existence of familial predisposition to IDD (Kalichman and Hunter, 2008; Battiè et al., 2009). The relative contribution of each of these causes is still currently unknown, but they are all considered as risk factors that may trigger cascades of disrupting events in the IVD microenvironment. During early IDD, the main features that can be observed in a degenerating IVD are the onset of a significant inflammatory condition in the disc tissues, the gradual degradation of the NP and AF matrix, and the reduced viability of resident cells. These pathological modifications may subsequently lead to the aberrant innervation and vascularization of the IVD, and the collapse of the SMS through either the prolapse/herniation of the NP outside a fissured AF or the complete degradation of the NP inside an intact AF (i.e. the so-called black disc). All these events may eventually impair the entire functional properties of the spinal column and consequently cause pain (Smith et al., 2011; Kepler et al., 2013a,b).

Below we will briefly discuss the main degenerative events that occur during IDD. Furthermore, in the following section, we will present the main degenerative factors involved and IDD markers, with a particular attention to the role of the inflammatory micro-environment and the associated proinflammatory cytokines.

Inflammation

IDD is characterized by the early onset of a severe inflammatory milieu both inside the degenerating IVD and in the peridiscal space. A relevant number of studies have been conducted on disc samples with different degrees of degeneration in order to analyze either the expression in the tissue or the secretion upon placement of tissue/cells into culture medium of pro-inflammatory factors during IDD (Takahashi et al., 1996; Shamji et al., 2010). All these gene and protein expression analyses in healthy and degenerated tissues have revealed increased levels of several inflammatory cytokines and chemokines. These inflammatory mediators are produced by resident IVD cells, as well as by circulating immune cells that infiltrate inside the IVD due to the favorable conditions generated during IDD. In particular, in degenerating IVD the infiltration of activated immunocytes, including macrophages, T- and B-cells, and natural killer (NK) cells (Geiss et al., 2007; Kokubo et al., 2008; Murai et al., 2010; Shamji et al., 2010), occurs in response to the expression of a number of chemokines by IVD cells and it is permitted by the loss of structural integrity of the disc ECM (in particular of the outer AF). The presence of inflammatory mediator-producing immune cells within the IVD, which does not contain a resident immune cell population in physiological conditions, enhance inflammation inside the disc tissues. The onset of this severe inflammatory environment inside the degenerating IVD has been demonstrated to trigger a range of pathogenic responses, such as matrix degradation, cell senescence and apoptosis, nerve and vascular ingrowth, which eventually lead to a massive degeneration and may cause pain (Takahashi et al., 1996; Burke et al., 2002; Huang et al., 2008; Shamji et al., 2010). The proinflammatory factors characterizing IDD and their detrimental influence in the cascade of degenerative events characterizing IDD will be further discussed in the next section.

Altered synthesis and degradation of extracellular matrix

The unique structural hallmark of IVD is responsible for its ability in load-bearing and the correct mechanical stress transmission to the adjacent vertebrae. Important changes in the normal homeostasis of the disc ECM cause the loss of its physiological well-defined structure and lead to the functional impairment of IVD. The major processes involved in ECM degeneration are the dysregulation in the synthesis of fundamental matrix

components by IVD cells, as well as the increased production of degradative enzymes.

In the very early stages of degeneration an increasing production of collagen (especially type II) is observable, probably as an attempt to repair the tissue damage. However, during advanced stages of degeneration, the production of type II collagen markedly decreases, while type I collagen synthesis undergoes a significant increase (LeMaitre et al., 2007a,b). Furthermore, an alteration in the distribution of the collagen types within the disc occurs, leading to the loss of distinct structures between the NP and the outer AF: the presence of type II collagen slightly increases in the outer AF, and simultaneously type I collagen begins to form strong collagen fibers also in the NP and inner AF (especially impairing the viscoelastic properties of the NP) (Yao et al., 2013). The situation is further aggravated by the progressive loss, within the NP, of proteoglycans, especially aggrecan, which normally provide the maintenance of disc hydration and turgor, assuring the ability to support axial loadings (Freemont, 2009). This altered condition of disc ECM causes loss of water content and fibrosis of the NP, and an overall disorganization of the functional structure of IVD.

The shift in the balance between anabolic and catabolic factors in the ECM of degenerating IVD involves also the increased expression of degradative enzymes, such as matrix proteases, which further decrease the content of collagens and proteoglycans (Antoniou et al., 1996; Roberts et al., 2000). Indeed, several of these matrix proteases become overexpressed in degenerated disc (Wang et al., 2015a,b).

This cascade of degenerative events is also responsible for cracks and fissures in the AF, loss of disc height, and osteophyte formation on the EPs. Advanced stages of disc degeneration may eventually lead to macroscopic effects including disc herniation and formation of the so-called black disc (Roberts et al., 2006a,b).

Senescence and apoptosis of intervertebral disc cells

The physiological activity of native cells is a fundamental aspect for the maintenance of tissue homeostasis, as well as for the development of correct tissue repair processes after damaging stimuli. This disc cell activity can be regulated by mechanical loading, the onset of an inflammatory microenvironment within the tissue, as well as changes in nutriment and oxygen supply, depending on the cell type (Kohyma et al., 2000; Ha et al., 2006; Walter et al., 2011). When these stimuli persist or occur in an excessive manner, cells may undergo either senescence or apoptosis. In this regard, it has been observed a significant number of both senescent and apoptotic cells in degenerating IVD (Zhao et al., 2006). On one hand, an increased cell senescence inside the IVD tissue contributes to the development of IDD through two mechanisms: directly reducing the number of functional and mitotic cells, as well as enhancing the deleterious microenvironment (Feng et al., 2016). Indeed, senescent cells remain metabolically active and able to interact with neighboring cells, but undergo changes in their phenotype and begin to aberrantly secrete proinflammatory mediators, including cytokines and chemokines, matrix degradative proteases, and specific growth factors, which enhance the inflammatory and degenerative milieu in the degenerating IVD (Berlemann et al., 1998; Acosta et al., 2008, 2013; Munoz-Espin and Serrano, 2014).

On the other hand, apoptosis is the process of programmed cell death that is involved in different physiopathological conditions, from the cell deletion typically occurring during organogenesis, the homeostatic control of cell proliferation and differentiation in adult tissues, as well as the pathogenesis of various diseases (Zhao et al., 2006). It has been observed that during IDD, there is a significant increase in the overall level of cell apoptosis within IVD tissues (Wang et al., 2011). In this context, cell apoptosis simultaneously leads to both the decrease in cell density (Gruber et al., 2000) and the enhancement of the detrimental inflammatory microenvironment (Park et al., 2001). In particular, the debris originating from dead cells, which cannot be rapidly cleared away since the IVD is almost avascular and does not contain native immunocompetent cells, trigger a reaction that, in turn, aggravates the inflammatory condition of the degenerating disc (Hashimoto et al., 1998). Furthermore, it has been suggested that cell death could be associated to cell autophagy, a mechanism through which cells can degrade non-functional proteins and organelles in the intracellular milieu (Klionsky, 2004; Kiriyama and Nochi, 2015). Although it has a physiologically cytoprotective role, dysregulation of autophagy has been associated to several degenerative diseases, including neurodegeneration and osteoarthritis, and other studies will be important to further elucidate its role in IDD (Kiriyama and Nochi, 2015).

Nerve and vascular ingrowth

Healthy IVD is almost aneural and avascular, with only few sensory nerves and blood microvessels projecting into the outer AF, and its structural integrity prevents further innervation and vascularization (Raj, 2008).

Physiologically, proteoglycans of ECM, especially aggrecan, provide an interstitial hydrostatic pressure, which counteract nerve and vessel ingrowth inside the IVD (Wilke et al., 1999; Johnson et al., 2006). Moreover, aggrecan chondroitin sulfate components in the healthy IVD are known to inhibit nerve formation (Johnson et al., 2002). During IDD, loss of structural integrity, due to proteolytic cleavage of aggrecan and the formation of cracks and fissures in the AF, as well as biological and chemical modifications of aggrecan, create a favorable condition for innervation and vascularization (Johnson et al., 2002). The nerve endings

ingrowth, occurring in both inner AF and NP, involves mainly nociceptive and only few proprioceptive fibers, and is supported by simultaneous infiltration of new blood vessels, which provide nutritional factors to the nerves (Freemont et al., 1997, 2002). Indeed, the depletion of proteoglycan from the inner surface of AF fissures decreases the ability of AF to inhibit angiogenesis (Derby et al., 1999; Melrose et al., 2002).

It is currently accepted that the process of innervation and vascularization in IVD degeneration is also related to the action of nerve and vascular growth factors. In particular, during IDD it has been observed an overexpression of neurotrophic and neurotropic factors belonging to the family of neurotrophins (NTs), such as nerve growth factor (NGF) and brain-derived growth factor (BDGF) (Navone et al., 2012). In addition, the process of vascularization, which typically accompanies nerve ingrowth, is also promoted by an increased level of vascular endothelial growth factor (VEGF) during IDD (Lee et al., 2011).

As most of the nerve fibers innervating painful degenerating IVDs show the same histologic profile observable within the outer AF of healthy disc, such as immunopositivity for the substance P (SP), it has been suggested that differences between the two conditions are strictly related to the number, density and spatial organization and not to the type of nerve fibers within the disc (Freemont et al., 1997).

Pain

The structural and functional alterations that occur during IDD may eventually cause pain. However, the association between disc degeneration and clinical symptom profile remains unclear and identification of pain etiology is often complex (Boden et al., 1990; Jensen et al., 1994).

Structural impairments like cracks and fissures in AF may allow the prolapse of NP material and disc herniation, which can lead to a mechanical compression of root ganglion nerves and, hence, cause pain (Kirkaldy-Willis and Farfan, 1982; Boden et al., 1990; Berlemann et al., 1998).

Nevertheless, back pain can also occur during disc degeneration in the absence of herniation and consequent nerve compression. This particular condition is known as discogenic pain and is strictly related to the establishment of an inflammatory microenvironment within the disc and in the peridiscal space. Indeed, several studies have shown that both IVD cells and infiltrated immune cells can secrete proinflammatory cytokines that may irritate the nerve fibers innervating the IVD, as well as their roots (Kokubo et al., 2008; Shamji et al., 2010). Moreover, the evidence that in the degenerated IVD there is an overexpression of the substance P (SP), which is typically involved in the modulation of nociception, suggests a direct involvement of this neuropeptide in discogenic pain (Brown et al., 1997).

Tissue and cellular markers of intervertebral disc degeneration

Expression of inflammatory markers in degenerated IVD

Inflammation is typically considered as a response of the organism to harmful stimuli, such as either infections or tissue injury, but has also a physiological role in maintaining tissue homeostasis. The inflammatory response generally consists of an initial increased production and secretion of proinflammatory mediators that lead to further activation of specific tissue-resident cells and the recruitment of circulating cells (especially immune cells), in order to either resolve the pathological situation or accomplish the homeostatic tissue turnover. Subsequently, when the inflammatory stimuli have been eliminated, the production of anti-inflammatory cytokines by diverse types of cells tends to restore a healthy situation in the tissue (Medzhitov, 2008). If either the damaging stimuli persist or there is a dysregulation in the mechanism of homeostatic inflammation, the inflammatory environment may cause serious damage to the tissues and the whole organism.

Regardless of the cause triggering IDD, in the degenerating IVD and surrounding tissues there is always the early onset of a detrimental inflammatory microenvironment (Molinos et al., 2015), which has a significant role in the cascade of events characterizing every stage of the degeneration. Indeed, a range of inflammatory mediators, such as cytokines and chemokines, have been detected in human IVDs depending on their physiopathological condition. During IDD, several of these factors appear to be overexpressed, characterizing the inflammatory milieu that exacerbates disc degeneration. Several studies have been conducted in order to evaluate the characteristics and the detrimental effects of the inflammatory microenvironment developed during IDD, by identifying the major proinflammatory factors involved and the signaling pathways through which their action is exerted.

In this context, gene and protein expression analysis in both healthy/control and degenerated/pathological tissues have been performed in *in vitro* and *in vivo* models of different species. First of all, the ability of native IVD cells to secrete proinflammatory mediators has been demonstrated. For example, *in vitro* cell stimulation with lipopolysaccharide (LPS), a large molecule of the outer membrane of Gram-negative bacteria that is able to elicit strong immune response in animals, has shown the ability of both control and degenerated disc-derived cells to secrete pro-inflammatory agents, such as interleukin-(IL-)1β, IL-6, IL-8, IL-10, Prostaglandin E2 (PGE2) and granulocyte-macrophage colony-stimulating factor (GM-CSF), in animal (Rand et al., 1997) and human IVD (Burke et al., 2003). Moreover, the regulation of these inflammatory cytokines by disc cells has been found to be responsive to several other environmental factors such as abnormal mechanical loading, injury and smoking (Oda et al.,

2004; Ulrich et al., 2007; Walter et al., 2011). The expression of inflammatory factors can also be regulated in an autocrine and paracrine manner by the presence of the same or other inflammatory mediators, and, interestingly, this regulation has been reported to change depending on the physiopathological condition of the cells. Indeed, Le Maitre and colleagues, showed that cells derived from degenerated IVDs respond to IL-1 exposure with a further increase in IL-1 gene expression, whereas cells from non-degenerated discs respond with a decreased expression of IL-1. This result suggests that an healthy homeostatic response to IL-1 is replaced by a positive feedback loop in the degenerating IVD (LeMaitre et al., 2005).

The main proinflammatory mediators that have been correlated with IDD include TNF-α, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, IL-20, IFN-γ, PGE2, as well as a number of different chemokines, and their over-expression in the degenerating IVD has been demonstrated to trigger a range of pathogenic responses, such as matrix degradation, cell senescence and apoptosis, aberrant disc innervation and vascularization, which eventually lead to a massive degeneration and may cause pain (Takahashi et al., 1996; Shamji et al., 2010; Burke et al., 2002; Huang et al., 2008). The inflammatory cytokines that have been studied the most are certaintly IL-1 (in two different isoforms, IL-1α and IL-1β) and TNF-α, along with their associated enzymes and receptors. Some evidence has proved significant increased levels in gene and protein expression of IL-1 and TNF-α in degenerated and herniated intervertebral discs (LeMaitre et al., 2005, 2007a-d), as well as in the epidural space of herniated disc (typically through the analysis of epidural lavages) (Cuéllar et al., 2013). Moreover, mRNA and protein levels of IL-1β and TNF-α have been demonstrated to be upregulated with increasing IVD age/degeneration (Bachmeier et al., 2007; Park et al., 2011). In particular, this over-expression is higher in the NP compared to the AF, and in symptomatic (i.e. painful) discs compared to asymptomatic ones (Weiler et al., 2005; Park et al., 2011). Both IL-1β and TNF-α can be produced by resident IVD cells, as well as infiltrating immunocytes, such as monocytes/macrophages, dendritic cells, B lymphocytes and NK cells (Séguin et al., 2005; Le Maitre et al., 2005, 2007a-d). These and other evidence have highlighted the involvement of IL-1 and TNF-α in disc degeneration, herniation and even discogenic pain. Although IL-1 and TNF-α are, by far, the most studied cytokines, several other pro-inflammatory mediators have been demonstrated to be overexpressed during IVD degeneration (Akyol et al., 2010). A major role in IDD has been assigned to IL-6, a cytokine that acts mainly through binding to its receptors and can be produced both by IVD cells and by infiltrating immune cells, such as macrophages and T-cells (Rand et al., 1997). This proinflammatory cytokine is overexpressed in degenerated and even more in herniated IVDs, as demonstrated by discographic lavage of herniated discs or enzyme-linked immunosorbent assay analysis of cerebrospinal fluid (Andrade et al., 2013). IL-17 is another proinflammatory cytokine that has been found to be overexpressed in degenerated and to a greater extent in herniated IVDs (Shamji et al., 2010). Upon stimulation with IL-23, IL-17 can be produced by Th17 cells and other lymphocytes, as well as by neutrophils, mast cells and even disc cells, as recently demonstrated (Gruber et al., 2013). Another inflammatory mediator associated with IDD is interferongamma (IFN-γ), a type II interferon. IFN-γ can be produced and secreted by TH1, TC and NK cells, as well as macrophages, myeloid cells and dendritic cells. Although the detrimental effects of an increased production of IFN-γ are still not completely understood, its overexpression in degenerated IVDs, and especially in painful degenerated IVDs, may be associated with the generation of pain (Shamji et al., 2010).

These proinflammatory cytokines are either directly or indirectly associated to the upregulation of other inflammatory cytokines (Gruber et al., 2013), the chemoattraction and activation of immune cells into the IVD tissue (Kepler et al., 2013a,b), the downregulation of specific disc cell genes (LeMaitre et al., 2005), the upregulation of proteases that are capable of degerading the disc matrix (Wang et al., 2011a,b), the induction of disc cell senescence and apoptosis, the production of neurogenic and angiogenic growth factors (Abe et al., 2007), and even the generation of discogenic pain (Murata et al., 2006). The role of these proinflammatory cytokines in the cascade of degradative events during IDD will be discussed more in detail below.

The consideration that some of the inflammatory cytokines overexpressed during IDD, e.g. IL-4, IL-17, IFN-γ, were classically associated to a number of immune cells led to the hypothesis that native IVD cells were not the sole contributors to the deleterious inflammatory microenvironment found in degenerating IVDs. In support of this theory, several studies demonstrated the presence of a number of immune cell types, including monocyte-derived macrophages, natural killer (NK) cells, activated T-cells and activated B cells, in degenerated and herniated IVDs compared to non-degenerated controls (Geiss et al., 2007; Kokubo et al., 2008; Shamji et al., 2010; Murai et al., 2012). The limited infiltration of immune cells inside the IVD, especially in the AF, may have a beneficial effect in some cases. When micro-lesions, caused by hyper-physiological loading or low nutriment supply, occur in the IVD, a subpopulation of immune cells could be necessary in the early production of proinflammatory cytokines and in the elimination of cell debris, as well as the subsequent secretion of anti-inflammatory cytokines in order to restore the healthy disc structure (Sun et al., 2013). In addition, the accumulation of immune cells in the peridiscal zone can also have a beneficial influence in the absorption of the herniated NP, since their presence can contribute to the enhanced production of matrix metalloproteinases, which play an essential role

in spontaneous regression of disc materials (Doita et al., 2001). However, the massive infiltration of inflammatory cells might be detrimental.

The pathological structural changes of a degenerated disc, from matrix degradation and AF fissures to disc herniation, permit the infiltration into IVD tissues of immune cells. Moreover, this immune cell infiltration is guided by a number of chemokines that are produced in the inflammatory milieu of the degenerating IVD.

In this context, the analysis of degenerated and prolapsed disc tissue specimens and cultured cells have demonstrated elevated levels of many different chemokines, including monocyte chemotactic protein (MCP)-1/CCL2, macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, regulated and normal T cell expressed and secreted (RANTES)/CCL5, MCP-3/CCL7, MCP-4/CCL13, MIP-3α/CCL20, neutrophil-activating protein (NAP)-3/CXCL1, IL-8/CXCL8, monokine induced by gamma interferon (MIG)/CXCL9 and interferon gamma-induced protein (IP)-10/CXCL10. All these overexpressed chemokines can act as chemoattractants to a range of circulating and peridiscal immune cells (Ahn et al., 2002; Kawaguchi et al., 2002; Lee et al., 2011; Phillips et al., 2013, 2015).

Furthermore, several studies have demonstrated a tight connection between the increased production of these chemokines by native IVD cells and the overexpression of proinflammatory cytokines.

For example, it has been reported that isolated NP cells treated with either IL-1β or TNF-α increase CCL3 expression, and the regulation of CCL3 expression by these two cytokines appears to act through MAPK, NF-kB/p65 and C/EBPβ signalling pathways. This overexpression of CCL3, which has been positively correlated with the grade of degeneration and is higher in herniated tissue compared to degenerated but contained IVDs, can, in turn, promote CCR1-dependent macrophage migration inside the disc, further enhancing the degenerative inflammatory microenvironment (Wang et al., 2013a,b). Similarly, the expression of both RANTES, which has an important role in recruiting leukocytes, and IL-1β appears to be significantly higher in painful IVDs compared to degenerated painless and non-degenerated discs, and RANTES levels positively correlate with IL-1β (Pattappa et al., 2014). Interestingly, disc cells cultured in alginate beads upregulate the expression of RANTES upon treatment with both IL-1β and TNF-α (Kepler et al., 2013a,b). Moreover, treatment of NP cells with TNF-α and IL-17, alone or in combination, seems to enhance CCL20 secretion in a dose-dependent manner (Zhang et al., 2013a,b). The CCL20 receptor CCR6 is specifically expressed on the surface of Th17 cells, and is associated with Th17 infiltration (Pène et al., 2008). Since the infiltration of Th17 cells in degenerative IVD has been speculated due to the presence of IL-17 in herniated discs (Shamji et al., 2010), the CCL20-CCR6 system could be involved in the trafficking of IL-17-producing cells. The tight relation between proinflammatory cytokines and chemokines has been also demonstrated by Phillips and colleagues, who reported that stimulation of cultured NP cells from both degenerated, prolapsed and non-degenerated (post-mortem) IVDs with IL-1β induces a dose-dependent increased release of several chemokines, such as MCP-1, MIP-1α, MIP-1β, RANTES, MCP-3, CXCL1, IL-8, CXCL9 and CXCL10 (Phillips et al., 2015).

In particular, the IL-1β-induced overexpression of IL-8, a pro-angiogenic chemokine that has been reported to be increased in degenerated IVDs (with particularly higher levels in painful IDD than in herniation) (Philipps et al., 2013; Zhang et al., 2016), may be involved in the observed vascularization of degenerated IVDs, which is associated with discogenic pain. Indeed, higher levels of IL-8, as well as TNF-α, can be found in painful degenerated IVDs compared to disc herniation (Lee et al., 2009a,b), and co-neutralization of IL-8 and TNF-α significantly improved symptoms of mechanical hyperalgesia in both a disc autograft and a spinal nerve ligation model (Takada et al., 2012).

This evidence supports the idea that the proinflammatory cytokines, which are overexpressed in degenerating discs, stimulate the increased production of chemokines by IVD native cells. This proinflammatory microenvironment, in association with other favourable conditions including degraded AF matrix and aggrecan decreased expression in the NP, leads to immunocyte infiltration, as well as innervation and vascularization of IVD tissues. This, in turn, enhances the inflammatory milieu within the tissues, aggravating the cascade of degenerative events, and may eventually cause discogenic pain.

Influence of proinflammatory mediators on IVD matrix degradation

Structural changes of the IVD matrix and its overall degradation are the primary effects of IDD. These are mainly caused by a dysregulated production of fundamental ECM components by native IVD cells, the massive production of several matrix proteases by disc cells and infiltrating immune cells, as well as a general increase of the disc cells catabolism. In particular, the primary proteases implicated in IDD belong to the families of the matrix metalloproteinases (including MMP1, -2, -3, -7, -9, -13), aggrecanases/ADAMTS (including ADAMTS4, -5), and, with a lower impact, cathepsins (including cathepsins K, D, L) and serine protease High Temperature Requirement Factor A1 (HTRA1). The increased expression of these proteases has been observed in degenerated and herniated IVDs (Roberts et al., 2000; Ariga et al., 2001; Tiaden et al., 2012). In particular, MMPs are extracellular calcium-dependent, zinc-containing endopeptidase, which regulate the turnover and the composition of ECM's proteins. MMPs are synthetized in a latent form and need to be activated by other enzymes. Therefore, MMPs act directly in matrix degradation by proteolytic

cutting of some fundamental components, and indirectly by activation of other MMPs, creating a detrimental microenvironment in ECM (Matsui et al., 1998; Crean et al., 1997). Moreover, among ADAMTS, ADAMTS-4 and ADAMTS-5 are also known as aggrecanase-1 and aggrecanase-2, respectively, because they can specifically cleave proteoglycans, with a particular specificity for aggrecan (Troeberg and Nagase, 2012). It appears clear that these matrix proteases may play a major role in IVD ECM degradation.

Interestingly, it has been reported that inflammatory mediators, such as IL-1, IL-6, TNF-α, induce the overexpression of several of these matrix proteases, as well as regulating a number of genes associated to ECM component production by IVD cells. Cultured IVD cells exposed to either IL-1α or IL-1β have shown an increased expression of MMP1, MMP2, MMP3, MMP9, MMP13, ADAMTS4, ADAMTS5, syndecan 4, and iNOS, and concomitant decreased levels of the main ECM components (aggrecan, collagen type I and collagen type II), with more pronounced effects in NP cells than AF cells (Shen et al., 2003; LeMaitre et al., 2005; Wang et al., 2011a,b; Zhao et al., 2011; Buser et al., 2014). Moreover, it has been demonstrated that both IL-1α and IL-1β decrease the expression of the SOX-6 gene in chondrocyte-like cells of non-degenerated IVDs, thus dysregulating their chondrogenic phenotype and inhibiting the expression of genes for collagen I, collagen II and aggrecan (LeMaitre et al., 2005). Similar studies conducted on either IVD tissue or isolated disc cells have demonstrated that somministration of TNF-α leads to a decreased expression and synthesis of collagen type II and aggrecan and an increased expression of several proteases, including MMP1, MMP3, MMP9, MMP13, ADAMTS4, and ADAMTS5 (Séguin et al., 2005, 2006). Furthermore, high levels of both osteopontin (OPN), a protein normally involved in both cell attachment and calcification of mineralized tissue, and CD44, one of the receptors through which OPN exerts its action, were measured in human mesenchymal stem cells (MSCs) isolated from degenerated intervertebral discs. These high levels directly correlated with the Thompson grade of the samples and this evidence suggests that overexpression of OPN and CD44 might exacerbate ECM degeneration and/or mineralization (Marfia et al., 2015). Researchers have reported a number of different mechanisms through which proinflammatory cytokines induce the up-regulation of matrix proteases. For example, IL-1β and TNF-α act on NP cells through the modulation of syndecan-4 (SDC4) via the NF-kB pathway to upregulate the expression of ADAMTS-5 (LeMaitre et al., 2005). Another mechanism through which TNF-α, and probably IL-1β, induce the overexpression of catabolic molecules, such as ADAMTS-5, MMP13, and cyclooxygenase-2 (COX-2), is the expression of prolyl hydroxylases-3 (PHD-3) that, in turn, promotes NF-kB-mediated disc cell catabolism (Fujita et al., 2012). Moreover, it has been shown that TNF-α post-translationally induces increased MMP-2 activity by controlling MMP-14 expression via the extracellular signal-regulated kinases (ERK) pathway (Séguin et al., 2008). Interestingly, the effect of TNF-α on the overexpression of some metalloproteinases, in particular MMP3 and MMP9, appears to be less pronounced compared to the effect of IL-1β (Millward-Sadler et al., 2009). This seems to be confirmed by the fact that treatments against IL-1b were shown to inhibit matrix degradation while TNF-α blockers had no significant effects (Hoyland et al., 2008). In another study, human NP cells exposed to IL-6 revealed an impaired expression of collagen II and aggrecan, as well as an increased production of MMP3, and these changes were exacerbated by the additional exposure to either IL-1β or TNF-α (Studer et al., 2011). Furthermore, it has been reported that IL-17 upregulates the expression of ADAMTS-7 by NP cells. It has been hypothesized that IL-17 induces the production of TNF-α, which, alone or in combination with IL-17, stimulates the production of ADAMTS-7 via activation of the NF-kB pathway (Wang et al., 2015a,b; Lai et al., 2014).

This evidence highlights that the proinflammatory mediators overexpressed during IDD have a major role in disc ECM degradation, through a direct regulatory action on the expression of both genes associated to fundamental matrix components and degradative matrix proteases.

Influence of proinflammatory mediators on intervertebral disc cell senescence and death

Inflammatory mediators have also been shown to have a role in other important aspects observable during disc degeneration, which are the dysregulation of genes related to physiological cell proliferation (Wang et al., 2013a,b), as well as disc cells senescence (Purmessur et al., 2013) and apoptosis (Yang et al., 2015).

A study conducted by Wang and colleagues shows that NP cells treated with IL-1β and TNF-α exhibit an increased activity and a general dysregulation of specific components of the Notch signalling pathway, which has been associated to disc cell proliferation and differentiation (Mead and Yutzey, 2009; Hiyama et al., 2011). The action of IL-1β and TNF-α appears to be exerted through the MAPK and NF-kB pathways, since cell treatment with NF-κB and MAPK inhibitors abolished the inductive effect of these cytokines. This observation well correlates with the evidence that the expression levels of both these two cytokines and some of the Notch signalling pathway genes and receptors significantly increase in degenerated discs compared to non-degenerate discs (Wang et al., 2013a,b).

Proinflammatory cytokines are related not only to potentially detrimental dysregulation in the expression of genes and molecules related to cell proliferation, but also to the increased senescence of disc cells. In patients with IDD, senescence-associated β-galactosidase (SA-b-Gal) positive cells can be found in both NP and AF, and they

have a preference to aggregate in clusters (Roberts et al., 2006a,b). Moreover, it has been seen that the number of SA-b-Gal positive IVD cells and the decrease of cell replicative potential (measured by the number of Ki67-positive cells) positively correlates to the grade of disc degeneration (Gruber et al., 2007, 2009; LeMaitre et al., 2007a-d). Compared to healthy disc cells, senescent cells of degenerating discs alter their pattern of secretion and irremediably change the IVD microenvironment. Indeed they secrete several proinflammatory factors, such as TNF-α, IL-1β, IL-6, and chemokines and matrix proteases, which, in turn, promote the senescence of neighbouring cells and the infiltration of immune cells into the IVD, reinforcing the inflammatory microenvironment in a positive feedback loop (Munoz-Espin and Serrano, 2014; Roberts et al., 2006a,b). The induction of stress-induced premature senescence (SIPS) by pro-inflmmatory mediators has been demonstrated by a number of studies. The exposure to TNF-α of bovine discs in a whole-organ culture model leads to an increased number of SA-b-Gal positive cells (Purmessur et al., 2013), and similar results have been obtained upon the treatment of cultured rat NP cells with IL-1β and TNF-α (Markova et al., 2013). Even though the exploration of the signalling network underlying cell senescence is still in its first stages, the literature suggests possible signalling pathways. For example, the exposure of cultured rabbit NP cells to TNF-α leads to apoptosis by activating the Wnt/β-catenin signaling pathway (Ye et al., 2011), a pathway known to be related to cell senescence and matrix metalloproteinase expression in IVD (Hiyama et al., 2010).

Another evidence of the dysregulation of normal cell metabolism occurring during disc degeneration is the significant death of IVD cells. The decreased number of viable cells is evidently a major factor in the inability of restoring a physiological condition in a degenerating disc. The involvement of proinflammatory cytokines in the increased level of apoptotic disc cells has been recently assessed by different research groups. Zhang and collegues demonstrated through Giemsa staining, TdT-mediated dUTP-biotin nick end-labeling (TUNEL) and Annexin V/PI double-staining flow cytometry that the *in vitro* treatment of rabbit NP cells with IL-1β induces cell apoptosis. Moreover, they demonstrated that the antagonist proliferative action of insulin-like growth factor (IGF)-1 is able to significantly reduce the IL-1β-induced cell apoptosis (Zhang et al., 2013a,b).

This evidence supports the idea that growth factors such as IGF-1 and transforming growth factor (TGF)-β1 strongly stimulate the proliferation of NP cells in humans (Zhang et al., 2006; Pratsinis et al., 2012). IL-1β-induced apoptosis was also confirmed in both rat AF (Wang et al., 2014) and NP cells (Yang et al., 2015). It has also been demonstrated that TNF-α induces apoptosis on human NP cells. Moreover, the transfection of NP cells with an inhibitor of miR-138-5p, a microRNA (miRNA) that is upregulated in degenerative NP tissues and is hence supposed to be involved in IDD, decreased the number of TNF-α-induced apoptotic cells. This suggests an interconnection between the apoptotic effect of TNF-α and this miRNA (Wang et al., 2016a,b).

Furthermore, proinflammatory cytokines have a role in the induction of increased cell autophagy, which has been supposed to be related to a certain grade of apoptosis in degenerating discs (Shen et al., 2011). A study conducted by Gruber and collegues demonstrated that the exposure of human AF 3D-cultured cells to IL-1β and TNF-α resulted in the increased expression of autophagy-related genes (Gruber et al., 2015).

It is clear that further studies on the signalling pathways through which the proinflammatory mediators overexpressed in degenerative IVD exert their effects on disc cells will be necessary in order to evaluate effective therapies to inhibit the deleterious effects of the inflammatory microenvironment and restore the physiological activity of disc cells.

Influence of proinflammatory mediators on IVD innervation and vascularisation

It has been proposed that an important cause of the nerve ingrowth into painful degenerated disc is the release of "neurogenic" factors belonging to the family of neurotrophins (NTs), such as NGF, BDNF and neurotropin-3 (NT3). For example, it has been demonstrated that NGF and BDNF directly influence the development, differentiation, and chemotaxis of neuronal cells, as well as fibroblast activation (Bonini et al., 2003; Garcia-Cosamalon et al., 2010), and both exert their functions as a result of the binding with high-affinity receptors (Purmessur et al., 2008). Neurotrophins are involved in this process also indirectly, supporting degradation of ECM. Indeed, it has been demonstrated that the upregulation of NGF in IVD may lead to an increase of MMP expression, which is a key step in the development of damage in AF and EPs that allows innervation (Kao et al., 2014, 2015).

Although the constitutive expression of NGF and BDNF in healthy IVD is controversial, with studies showing either a moderate concentration (Binch et al., 2014), low concentration (Abe et al., 2007) or an undetectable presence (Freemont et al., 2002) of these two neurotrophins, it has been demonstrated that significantly higher levels can be found in painful degenerated IVDs. In particular, NGF and two of its receptors, tropomyosin receptor kinase A (TrkA) and p75 neurotrophin receptor (p75NTR), are expressed at increased levels in painful IVDs in parallel with the hyperinnervation (Freemont et al., 2002; Purmessur et al., 2008; Navone et al., 2012). In addition, the increase of BDNF and one of its receptors, tropomyosin receptor kinase B (TrkB), directly correlates with the degree of degeneration (Purmessur et al., 2008).

NGF not only stimulates innervation but also acts as a chemotactic factor for endothelial cells and, hence, can be considered a major agent in both nerve and vascular growth (Nico et al., 2008). Moreover, endothelial cells

have been shown to produce a number of MMPs in capillary sprouts during inflammation, wound healing and tumour growth (Handsley and Edwards, 2005; Van Hinsbergh et al., 2006). Thus, it was hypothesized that endothelial cells promote innervation not only producing neurogenic factors, such as NGF, but also inducing the pathological degradation of the IVD matrix and the formation of AF fissures (Freemont et al., 2002; Johnson et al., 2002).

Initially, higher levels of NGF in degenerated, innervated IVDs were supposed to be due to its production by blood microvessels that often accompany nerve fibers. Indeed, these blood vessels express NGF and, in turn, nerves express TrkA, which is associated with pathways promoting neurite growth and survival (Freemont et al., 2002). Currently, it is known that even disc cells, in particular during degenerative conditions, have the ability to produce nerve-promoting factors that can induce nerve ingrowth inside the disc (Johnson et al., 2006). Indeed, both NGF and BDNF, along with their receptors TrkA and TrkB, are highly expressed by IVD cells at every stage of degeneration (Navone et al., 2012), but only BDNF level seems to have a positive correlation with the degree of degeneration (Gruber et al., 2008; Purmessur et al., 2008). Moreover, an additional source of neurotrophic factors for nerve and vascular ingrowth in the degenerative disc, are the immune cells. In particular, NGF is produced and secreted by macrophages, lymphocytes, eosinophils and mast cells, which have been found in degenerated and, particularly, in herniated IVD (Bonini et al., 2003; Kokubo et al., 2008). It has also been reported that NGF, overexpressed in degenerating IVD, may act directly on macrophages, eosinophils, B- and T-cells, and mast cells to induce their proliferation, activation and release of several proinflammatory cytokines (Thacker et al., 2007). This indicates a tight functional inter-relationship between neurotrophins and proinflammatory cytokines.

In parallel to the overproduction of neurogenic factors by several types of cells during disc degeneration, it has been demonstrated an increased expression of angiogenic factors in innervated, painful IVDs. These factors, which induce the proliferation of blood vessels that comigrate with the nerve fibers inside the degenerating IVD, include pleiotrophin and VEGF, which are directly produced by disc cells (Johnson et al., 2007; Lee et al., 2011). For example, it has been seen that VEGF acts in concert with NGF, sharing the Ras/ERK and P13K/Akt intracellular signalling pathways, and these two growth factors affect the survival and proliferation of nerve and endothelial cells (Nico et al., 2008).

Regarding the implication of inflammatory mediators, several studies reported the influence of different cytokines on the expression of neurotrophic and angiogenic factors by IVD and peridiscal cells. For example, Abe and collegues demonstrated that IL-1β and TNF-α upregulate NGF mRNA expression and secretion in cadaveric human AF and NP cells, cultured either in monolayer or in alginated beads (Abe et al., 2007), while another study showed that IL-1β exposure of IVD cells in three-dimensional culture significantly increased the expression of neurotrophins including BDNF and neurotrophin 3 (Gruber et al., 2012). Moreover, the treatment of native NP cells with IL-1β induced a significant overexpression of NGF, VEGF and SP, whereas the treatment with IL-6 induced an increase only in VEGF. In the same study, human dermal microvascular endothelial cells, used as a model for the endothelial cells of the infiltrating blood vessels, showed a significant upregulation of NT3 upon exposure to TNF-α (Binch et al., 2014). Interestingly, it was demonstrated that stimulation of cultured human NP cells with IL-1β leads to significant increases in NGF and BDNF gene expression, whereas treatment with TNFα was associated with an upregulation of substance P expression only (Purmessur et al., 2008). Since the neuropeptide SP also induces the expression of inflammatory mediators including IL-1β, TNF-α, IL-6 and IL-8 in both NP and AF cells, through a process started by the phosphorylation of p38-MAPK and ERK1/2 (Kepler et al., 2015), and enhances the RANTES-induced chemotaxis and activation of monocytes/macrophages (Chernova et al., 2009), it is clear that elevated levels of SP function in an autocrine and paracrine manner to further enhance the inflammatory reaction in the IVD, acting as a link between inflammation, disc degeneration and pain (Kepler et al., 2015a,b; Koerner et al., 2016).

Influence of proinflammatory mediators on IDD-related back pain

Substance P, as well as neurotrophins, has been related not only to nerve and vascular ingrowth inside the degenerating IVD but also to the onset of discogenic pain.

On one hand, the substance P has a major role in the development of discogenic pain by acting directly on the nerve fibers innervating the degenerating IVD and the nerve roots from the DRG (Koerner et al., 2016). On the other hand, neurotrophins such as NGF and BDNF may be responsible for the generation of pain in symptomatic IVDs by both inducing the synthesis of nociceptive neuropeptides and directly acting on the nerve fibers. It has been demonstrated that nerves exposed to NGF increase the production of nociceptive neuropeptides, such as SP and calcitonin gene-related peptide (CGRP) (Malcangio et al., 1997; Pezet et al., 2011). Moreover, NGF also promotes the expression, by neuronal cells, of a number of receptors and membrane channels that are associated with ischemic and inflammatory pain, including a type of pH sensitive Na^+ channel, named acid-sensing ion channel 3 (ASIC3) (Priestley et al., 2002; Mamet et al., 2003; Navone et al., 2012). In neuronal cells of both DRG and the innervating fibers inside the IVD, fast excitatory signals, as well as slow peptidergic neurotransmission, can be modulated by the

action of BDNF (Merighi et al., 2008), suggesting the role of this neutotrophin in nociception associated to degenerated IVD.

Furthermore, discogenic pain has been associated to the action of several proinflammatory cytonkines overexpressed during IDD. This is not only due to the cytokine-induced overproduction of NGF and BDNF, but also to other direct mechanisms that are still not well defined. These proinflammatory cytonkines, including IL-1, IL-6, and TNF-α, seem to directly induce apoptosis of neurons, as well as upregulate the expression of transduction molecules involved in hyperalgesia (Ebbinghaus et al., 2012).

For example, TNF-α and IL-6 seem to exert an apoptotic action on DRG cells (Murata et al., 2011), whereas it was demonstrated that the exposure of DRG cells to TNF-α induces allodynia and hyperalgesia responses (Murata et al., 2006). In cultured DRG neurons, treatments with IL-1β upregulate the expression of transient receptor potential cation channel subfamily V member 1 (TRPV-1), a transduction molecule involved in thermal hyperalgesia (Ebbinghaus et al., 2012).

Possible therapeutical/prognostic approach to counteract IVD neuroinflammation

Current therapies

Current therapies for IVD degeneration are symptomatic treatments mainly aimed to reduce back pain, which normally are a combination of one or more approaches including both physical therapy and pharmacotherapy. In particular, the latter consists of both the administration of nonsteroidal anti-inflammatory drugs and epidural steroid injections, such as the injection of corticosteroids, which tend to reduce the inflammatory processes by inhibiting the prostaglandin metabolism. Unfortunately, there are several drawbacks to these approaches. First of all, some contraindications for the drugs administered during these conservative treatments exist, as well as the possibility of therapeutic failure or a relapsed pain. For example, steroid drugs have no tissue specificity, affect not only inflammation but may affect the physiological homeostasis and metabolism of the entire organism, and their sensitivity varies among individuals (Ramamoorthy and Cidlowski, 2013). Furthermore, these symptomatic treatments are only conservatives. Usually, these conservative therapies should be administered during the first weeks from the onset of symptoms. Increasing sciatic pain, restriction of movements and muscle weakness, or opioid-resistant pain constitute absolute surgical indications (Legrand et al., 2007). In particular, surgical intervention is an option when non-operative medical management fails to adequately relieve individual-specific, intolerable pain during the activities of daily living. However, surgical procedures including discectomy and spinal fusion neither re-establish nor preserve the function of the IVD, their aim is simply to eradicate the source of pain (Di Martino et al., 2005). These considerations lead to the obvious need for new treatments, through which it would be possible to retard IVD degeneration and even regenerate the decayed tissue structures. Indeed, several research groups are working on different biological approaches that belong to the administration of agents that directly target specific inflammatory mediators and reduce the detrimental microenvironment within the degenerating IVD, the modulation of the expression of different genes through gene therapy, and the stimulation of IVD regeneration through either cell-based therapy or tissue engineering.

Novel anti-inflammatory drugs

As for the clinical use of nonsteroidal anti-inflammatory drugs and epidural steroid injections, the attempt to control inflammation through the use of regulating agents, which can be administered either orally or locally close to IVD tissues, has been long considered one of the most direct approaches to treat IDD. In particular, as the knowledge about the role of inflammatory mediators in IDD is increasing, a number of studies reported the achievement of pain relief on both animal and human models through the treatment with specific proinflammatory cytokine inhibitors and blockers. For example, Ohtori and collegues reported that the epidural application to the spinal nerves of either a TNF-α inhibitor or an anti-IL-6R monoclonal antibody resulted in pain relief in patients with sciatica (Ohtori et al., 2012a,b). Similar results were reported by Genevay and collegues that showed a significant improvement in leg pain after subcutaneous injections of a human antibody against TNF-α (Genevay et al., 2012). Another example of molecule aimed at modulating a specific target, which is considered to have a major role in inflammation during IDD, is Resolvin D1 (RvD1), a potent lipid mediator with anti-inflammatory properties. Intrathecal injection of RvD1 showed a potent analgesic effect, both inhibiting the up-regulation of TNF-α and IL-1β, and increasing IL-10 and TGF-β1 release, and attenuated the expression of NF-ϰB/p65 and p-ERK in a dose-dependent manner (Liu et al., 2016). Other research groups focused on the administration of Fullerol, a polyhydroxylated derivative of fullerene, which possesses good biocompatibility and excellent efficiency in eliminating reactive free radicals (Injac et al., 2013). In this context, Fullerol demonstrated anti-inflammatory properties in TNF-α-treated mouse DRG tissue and neurons, by reducing cellular apoptosis, suppressing reactive oxygen species (ROS) activity, and inhibiting the expression of IL-1β, IL-6, COX-2, and PGE2 in a dose-dependent manner. Fullerol-treated cells also exhibited the upregulation of anti-oxidative enzyme genes superoxide dismutase 2 (SOD2) and catalase (Liu et al., 2013). Moreover, Sato and colleagues evaluated the effect of anti-receptor activator of NF-kB ligand (RANKL) antibodies on sensory nerves innervating

injured IVD. It has been reported that RANKL expression is elevated in animal models of pain or intervertebral disc herniation, and in this paper the authors showed that anti-RANKL antibody both suppressed CGRP expression in DRG neurons and decreased TNF-a and IL-6 levels in injured IVD (Sato et al., 2015). Furthermore, the study of drugs that could inhibit the voltage-dependent sodium channel has been conducted, as these molecules could play a critical neuroprotective role in degenerative diseases. For example, Lidocaine decreased both ASIC3 expression in DRG neurons and pain associated with IDD in a rat lumbar disc herniation model (Ohtori et al., 2006). Despite the promising results obtained by these studies, the treatment of IDD through injected molecules still has some drawbacks. First of all, the route of administration is critical. On one hand, a systematic delivery could not be optimal since these drugs are often non-tissue specific and the IVD have a low grade of vascularization, even when the degeneration begins. On the other hand, a local administration through puncturing could itself cause IDD, although recent studies report alternative routes for drug delivery within the IVD (Vadalà et al., 2013a,b). Moreover, the method of administration of these pharmacological agents appears problematic since IDD is a chronic condition and these injected molecules normally have short biological half-lives, hence needing repeated administration for an effective treatment.

Gene therapy

In this context, gene therapy could be a promising solution to achieve a more prolonged therapeutic effect. Gene therapy gives the possibility to locally modulate the expression of a specific gene and, hence, the production of its related protein (Vadalà et al., 2007). One of the first studies to propose gene therapy applied to IDD was the work by Wehling and collegues, which suggested the transduction of bovine chondrocytic end plate cells with the gene responsible for the production of the IL-1 receptor antagonist (IL-1Ra) through a retrovirus vector (Wehling et al., 1997). This cell transfection and the consecutive injection of transfected cells in degenerated NP explants aimed to reduce the IL-1-mediated matrix degradation, as suggested by the positive results that showed a decreased expression of a number of proteases, such as MMP3, for two weeks after the treatment (Le Maitre et al., 2007a-d). Another approach was the *in vivo* TGF-β1 transfection of human IVD cells, which enhanced proteoglycan synthesis and collagen production (Tan et al., 2003; Lee et al., 2008). Moreover, Liu and collegues examined the effects of TGF-β3, connective tissue growth factor (CTGF) and tissue inhibitor of metalloproteinase 1 (TIMP1) gene transduction using a single lentiviral vector on a *in vivo* rabbit IDD model. They reported that this transduction promoted synthesis of aggrecan and type II collagen in degenerative IVDs (Liu et al., 2015a,b). Several studies have been conducted in order to assess the effectiveness of gene therapy for IDD treatments and the efficacy and safety of viral and non-viral vectors are rapidly increasing, even though the clinical setting of gene therapy is still limited by safety concerns of the gene transfer vectors, such as misplaced injections and possibility of oncogenesis (Feng et al., 2015; Liu and Wang, 2015). Furthermore, inflammatory mediators form a complex regulatory network and it is not easy to discriminate the action of a single cytokine. For this reason, the use of one or few inhibitors against specific proinflammatory cytokines may not be the most effective solution (Goupille et al., 2007).

Cell-based therapy

Recently, attention has been focused on cell-based therapy, through which it would be possible to stimulate the regeneration of intervertebral disc. In the last few years, among the numerous cell types proposed for this purpose, mesenchymal stem cells (MSCs) have proved the best candidate, also in relation to the possibility of autologous transplantation, due to their ability to differentiate in several cell types and to interfere with the inflammatory microenvironment, as cytokines-releasing factor (Prockop and Oh, 2012).

Furthermore, MSCs are able to migrate to injured tissues and take part in the regeneration process by means of the secretion of growth factors, cytokines and chemokines (Meirelles et al., 2009).

Normally, autologous transplantation of cells in degenerated discs is performed by injecting MSCs in the NP, but potentially transplanted cells may migrate to other non-specific sites (Orozco et al., 2011). Indeed, Vadalà and colleagues observed the osteophytes formation caused by the MSCs migration, in rabbit models. (Vadalà et al., 2012). In order to overcome this problem, cells are primarily grown and embedded in collagen scaffolds and subsequently implanted in the disc (Yoshikawa et al., 2010). A number of studies have demonstrated that the MSCs transplantation could benefit the IDD, both stimulating disc regeneration and exerting analgesic effects in treated patients. An evidence of this action has been proved by Sobajima et al. in rabbit models, in which the MSCs transplantation promoted an upregulation of ECM synthesis (Sobajima et al., 2008). Furthermore, in canine models it has been observed that mesenchymal stem cells are able to increase the synthesis of type II collagen and decrease the apoptosis rate of disc cells, improving disc degenerating condition (Serigano et al., 2010). In a number of recent clinical studies, patients treated with transplantation of MSCs from autologous bone marrow have shown a rapid and marked pain reduction. This was also confirmed by a 1 year follow-up, in which disc rehydration in several patients was observed (Pettine et al., 2014; Orozco et al., 2011). However, these studies did not prove an anatomical improvement in every degenerated disc, whose structural composition, such as the IVD height, was not recovered. Despite all these

promising data, the mechanisms of action underlying the interaction between MSCs and the degenerated disc cells are still not clear. Some authors describe several models to explain the contribution of MSCs in disc regeneration. Hiyama and collegues have observed that MSCs transplanted in discal regions in canine models could restore FasL expression, both differentiating in FasL-expressing cells and modulating FasL expression in NP cells (Hiyama et al., 2008). In murine models of injured lungs, it was observed that MSCs are able to secrete chemical modulators such as IL-1Ra and to increase the expression of anti-inflammatory proteins, such as TSG-6 (Ortiz et al., 2007; Lee et al., 2009a,b). Overall, these data suggest a potential immune-modulatory effect of MSCs, which may be exploited in novel cell-based therapies for IDD management (Tremolada et al., 2014).

Conclusions

Nowadays, IDD-associated back pain is a serious socio-economic burden and current therapies are not effective in the disease management. In the last decade, several studies have highlighted the major role of inflammation in the development of IDD and its associated symptoms. In particular, the overexpression of specific proinflammatory mediators and the onset of an inflammatory milieu are responsible for the cascade of degenerative events that may eventually cause pain. Although much progress has been made in understanding the processes involved in IDD and the pivotal role of inflammation further studies will be necessary to better comprehend the pathways through which proinflammatory mediators contribute to IVD degeneration and BP, in order to identify novel targets for treating symptomatic disc diseases and design effective therapies capable of inducing a beneficial tissue regeneration.

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