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TNF α -stimulated gene product (TSG-6) and its binding protein, I α I, in the human intervertebral disc: new molecules for the disc

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Abstract Inflammation and irritation of the nerve roots has been indicated as an important factor in the pain associated with symptomatic disc herniations. Tumour necrosis factor α (TNF α) is now believed to be involved in this pathway. TNF α causes connective tissue cells in culture to synthesise a glycoprotein, TNF α -stimulated gene-6 (TSG-6). TSG-6 is found in inflammatory diseases of related connective tissues, such as articular cartilage in rheumatoid arthritis, but is not present in unaffected individuals. In order to determine if TSG-6 occurred in intervertebral disc (and cartilage endplate), we have investigated the presence of TSG-6 and its binding protein, inter- α -inhibitor (I α I), in 58 herniated and 15 non-herniated discs. Immunostaining for the cytokines, IL-1 α , IL-1 β and TNF α , has also been carried out. We have demonstrated that both TSG-6

and I α I occur commonly in human intervertebral disc matrix with at least some TSG-6 in 98% of discs studied and I α I in all of them. Staining for TSG-6 was greatest in herniated discs, particularly close to blood vessels. I α I immunostaining was frequently widespread throughout the disc but there was little in the cartilage endplate. It has been proposed that these molecules have widespread effects, including extracellular matrix stabilisation, down-regulation of the protease network and reduction of inflammation. Hence, the occurrence of TSG-6 and I α I in disc tissue could have implications in the aetiopathogenesis and future therapeutics of intervertebral disc disease.

Keywords TSG-6 · I α I · Carrier proteins · Tumour necrosis factor · Intervertebral disc displacement · Inflammation mediators

Introduction

There is currently much attention focussed on the inflammatory potential of intervertebral disc tissue [1, 24, 28]. It is suggested that the cytokine known as tumour necrosis factor α (TNF α) may be a significant player in this pathway [10]. Indeed, antibodies to TNF α are being used in clinical trials with encouraging results [11]. TNF α -stimulated gene-6 (TSG-6) is a 35kD glycoprotein that is considered to be a marker of inflammation [20,

33]. It is normally absent or produced at very low levels by cells of mesenchymal origin, but they can be induced to secrete TSG-6 when stimulated by TNF α or other pro-inflammatory mediators such as prostaglandin E₂ and IL-1, both in vitro and in vivo [5, 16, 20]. Furthermore, TSG-6 is found in synovial fluid and joint tissues of patients with rheumatoid arthritis (RA) or osteoarthritis (OA) but not in unaffected individuals [2, 30].

TSG-6's role in the inflammatory cascade is unknown, but several different functions for this

molecule have been proposed. Extracellular matrix remodelling is a key feature of the known sites of TSG-6 expression, indicating that it might participate in this process [20]. Indeed, TSG-6 has been shown to associate with many matrix components. For instance, it interacts with the glycosaminoglycans, hyaluronan (HA) [3, 13, 14] and chondroitin-4-sulphate [25], as well as the hyaluronan-binding region of aggrecan [26], the most common proteoglycan found in disc. In addition, TSG-6 is up-regulated in the matrix surrounding osteoarthritis-like lesions in STR/ort mice, with decreased levels of aggrecan being seen in areas strongly expressing this protein [23]. It is detectable in the chondrocyte pericellular matrix of these mice, prior to development of osteoarthritic lesions, and thus might be an early marker of disease. It also stimulates the proliferation of vascular smooth muscle cells [34], and TSG-6 has been detected in the inflamed blood vessel walls of RA and OA synovium [2]. In addition, TSG-6 has anti-inflammatory effects *in vivo*, being a potent inhibitor of neutrophil migration [7, 32], and it has been found to be chondro-protective in mice models of arthritis [8, 21]. Since angiogenesis and inflammation are both believed to be significant in disc herniation patients with sciatica [22], we hypothesised that TSG-6 could be important in the aetiopathogenesis of this disorder.

TSG-6 is known to form a stable, ~120 kDa, complex with inter- α -inhibitor (I α I), a 180 kDa serine protease inhibitor found in serum [23, 31]. I α I-TSG-6 complexes of this size have been detected in the synovial fluids of arthritis patients [30]. Recent work has indicated that I α I-TSG-6 complexes act as intermediates in the covalent attachment of the heavy chains (HC) of I α I to hyaluronan [6] (Rugg and Day, unpublished data), possibly stabilising hyaluronan in the matrix. Complexes of HC-HA have been detected in synovial fluids from arthritic patients and may correlate with disease severity [12]. I α I has anti-plasmin activity which is greatly enhanced when bound to TSG-6 (via a non-covalent interaction [7]), and this is likely to down-regulate matrix metalloproteinase (MMP) activation (which is known to be higher in degenerate and herniated discs than in normals [4]). Indeed, it has been suggested recently that TSG-6 can have a protective role against matrix degradation, at least in cartilage, and that transgenic mice that constitutively express TSG-6 in cartilage are more resistant to developing arthritis than control animals [8]. In addition, there is evidence that TSG-6 may form part of a negative feedback loop capable of down-regulating the inflammatory response [7, 20, 32, 33]. In this study we investigated, therefore, whether TSG-6 or I α I was present in normal and diseased intervertebral discs.

Materials and methods

Samples were obtained of 58 discs from 54 patients (aged 16–76 years, mean 41.2 years $\pm$ 12 years) who underwent surgery for removal of herniated intervertebral discs, as permitted by the local research ethics committee. For comparison, discs were also obtained from ten patients with other disc pathologies: discogenic back pain ($n=9$, aged 27–50 years, mean 39.9 years $\pm$ 9.2 years) and one with a fractured vertebra (21 years), and five discs were obtained within 24–36 h postmortem (aged 21–73 years, mean 51.2 years $\pm$ 19 years). These samples were graded macroscopically for their degree of degeneration, using a modification of the Thompson scheme [29]. One disc was grade I; five were grade II; three were grade III; two were grade IV; one was grade V; and three were un-graded. All samples were dissected, some being snap-frozen for immunohistochemistry and some paraffin-embedded, for standard histology with haematoxylin and eosin staining. Articular cartilage was obtained from ankle joints of 10-year-olds and 14-year olds and from osteoarthritic knee joints (from individuals aged 64 years, 72 years, 73 years and 77 years) for comparison.

Immunohistochemistry was performed for TSG-6, I α I and TNF α , IL-1 α and IL-1 β . Monoclonal antibodies were used against TNF α (clone 68B6A3, Biosource), IL-1 β (clone 6E10, Biogenesis) and TSG-6 (Q75 [15]) at dilutions of 40 μ g/ml, 6 μ g/ml and 5.3 μ g/ml, respectively. Anti-human polyclonal antibodies raised in rabbits were also used against TSG-6 [2], I α I (Dako) and IL-1 α (Biogenesis) at dilutions of 1/750, 1/25 and 1/200, respectively. Seven-micron-thick frozen sections were fixed in 10% (volume of solute per volume of solvent, v/v) formalin in phosphate-buffered saline (PBS), except for sections to be stained with the polyclonal TSG-6 antibody, which were fixed in cold acetone [2] and for I α I, which were fixed in freshly prepared 4% paraformaldehyde. Sections were incubated with the primary antibodies diluted in PBS. The endogenous peroxidase activity was blocked with 1% (v/v) hydrogen peroxide in methanol, before labelling with a biotinylated secondary antibody and then peroxidase-labelled biotin-streptavidin complex (Vectastain Elite, Vector Laboratories, Peterborough, UK). Both the secondary antibody and the complex contained 10% (v/v) normal human serum. Diaminobenzidine was used as the colourimetric stain, and sometimes Mayer's haematoxylin was used as a counterstain. For controls, adjacent sections were incubated with PBS or an anti-mouse immunoglobulin G (IgG) for monoclonal antibodies, and normal rabbit serum for polyclonal antibodies, in place of the primary antibodies. None of these demonstrated any staining. All histology was assessed by an observer blinded to the clinical status of the sample.

Results

There was immunostaining for both TSG-6 and I α I in 98% and 100% of surgical disc samples, respectively, but the extent of staining was very variable. Both TSG-6 antibodies demonstrated similar staining. There was definite matrix staining, but in limited locations in some specimens and in many samples a few cells were positively stained (Fig. 1). The most staining for TSG-6 was seen in herniated discs. Where blood vessels were present they were frequently immunopositive, and disc cells close to the blood vessels were often more immunopositive than those further from them (Fig. 1b). In the few samples that contained cartilage endplate, TSG-6 immunoreactivity was markedly less than for the disc. Occasional cells were stained, but little matrix staining was observed (Fig. 2). The boundary region between the disc and endplate, however, was strongly stained.

Staining for I α I was more widespread than for TSG-6, often occurring throughout large regions or the entire matrix in a homogenous pattern (Figs. 2 and 3), particularly towards the nucleus in intact samples or in the case of herniated samples, where the morphology resembled that of the nucleus pulposus. There were also areas of disc matrix, however, which were totally negative, most commonly in the outer annulus. Anterior

longitudinal ligament, where present, was positively stained. The staining of cells was also variable, ranging from being negative, to the same intensity as the surrounding matrix, to more intense (Fig. 2). The pattern of matrix staining seen in disc for I α I was very different from that seen in hyaline cartilage, where it was predominantly negative, whether in the cartilage endplate or in articular cartilage (Fig. 3). In articular cartilage, staining was limited to a thin, intensely stained band at the surface in healthy, intact cartilage (Fig. 3c) but was more widespread in degenerated tissue from osteoarthritic patients (Fig. 3d).

Autopsy samples also demonstrated immunostaining for both TSG-6 and I α I but to a lesser extent than the surgical samples. However, the pattern was similar, with more staining in the nucleus than the annulus for I α I, but none in the cartilage endplate.

Positive immunostaining for the pro-inflammatory cytokines, TNF α and IL-1, was also found in surgical and autopsy samples. This was both within cells of blood vessels (where present) and in the intervertebral disc cells, and frequently also within the perivascular and pericellular matrix. Staining co-localised to some extent with that for TSG-6, particularly in the case of TNF α . IL-1 β was present in all the herniated and control, non-herniated discs, but to varying degrees.

Fig. 1 Sections of discs immunostained for TSG-6 (using Q75 antibody) demonstrated cell-associated staining (*arrows*) **a, b** particularly in the vicinity of blood vessels (*BV, arrow-head*). The matrix staining for I α I was extensive and homogenous in many samples **c**. **d** Normal serum control with no staining of the matrix or cells (*arrows*). **a, c, d** 32-year-old male with disc herniation **b** 65-year-old male with disc herniation. Magnification $\times 200$

Fig. 2 Immunoreactivity for I α I was often homogenous throughout the disc matrix **a–c** with the cells (*arrows*) sometimes being positively and sometimes negatively stained. **d** Normal serum control. **a, d** 50-year-old female with discogenic back pain; **b** 32-year-old male with disc herniation. **c** 65-year-old male with disc herniation. Magnification $\times 200$

IL-1 α was found in fewer samples, i.e., only 80% of herniated disc samples and 70% of controls (non-herniated surgical discs and autopsy samples) examined. Immunostaining for TNF α was present in 88% of disc herniation samples and 70% of controls. There was one sample that had no staining for TSG-6, and this also had no staining for TNF α , but it did for I α I, IL-1 α and IL-1 β .

Discussion

In this study we report for the first time the immunolocalisation of TSG-6 and I α I in human intervertebral disc. As has been described previously, TSG-6 is not normally present in healthy adult tissues elsewhere in the body but is produced in response to inflammatory mediators [20]. There is a clear association between TSG-6 expression and inflammation, with TSG-6 being found in the serum, synovial fluid, cartilage and synovium of patients with various forms of arthritis [2, 30]. The presence of TSG-6 in so many disc samples, therefore, suggests that inflammatory mediators have been present at some stage, albeit transiently. Indeed immunopositivity for TNF α was present in the majority of the herniated samples. It is to be expected that TNF α would not remain in an active form in the tissue for long, whereas TSG-6 might remain bound within disc extracellular matrix for a longer period (and I α I even longer

still, once covalently attached to HA). Hence, the immunostaining of TSG-6 (or I α I) could represent more of an historical perspective of the presence of inflammatory mediators than TNF α would.

Widespread immunopositivity for I α I was observed, staining large areas of matrix, particularly in the nucleus (Fig. 2). Serine proteinase inhibitors have been reported previously in intervertebral disc. However, they were mostly of much lower molecular weights (for example 12–14 kDa or 54 kDa [18]) than the I α I found in the present study, although I α I with a molecular weight of 120–250 kDa has been demonstrated by Western blotting previously in canine discs [19]. The source of the I α I in disc was not investigated in this study: some cell-associated staining was seen, suggesting that the disc cells have the ability to synthesise I α I. However, the possibility remains that it could also diffuse in from the vasculature in adjacent tissues. The distribution of I α I found in the disc differed considerably from that observed in articular cartilage, in which it is immunolocalised to a very discrete but heavily stained band at the surface of the cartilage (Fig. 3c). This distribution suggests that it diffuses into the cartilage, since a molecule of the size of I α I (approximately 180 kDa) could only penetrate the surface layer of healthy cartilage [17]. The disc, in contrast, may be more permeable to large molecules, since serum proteins of similar molecular weights have been reported to occur throughout the human intervertebral disc matrix previously [9]. Further

Fig. 3 Hyaline cartilage, whether cartilage endplate (CEP) **a, b** or articular cartilage **c–e** showed less immunoreactivity for both TSG-6 (Q75) **a** and IxI **b–d** than did the intervertebral disc. Note the thicker band of surface staining for IxI in the cartilage **d** in a 73-year-old with osteoarthritis than in young, healthy cartilage (**c** 14-year-old). **a** Disc 32-year-old male with herniation. **b** Disc 65-year-old male with herniation **e** Normal serum control for IxI (14-year-old). Magnification **a** $\times 125$, **b** $\times 200$, **c–e** $\times 30$

study of young vs old, healthy vs degenerated, and vascularised vs non-vascularised discs is required to provide more information on this.

Whilst TSG-6 expression is associated with inflammation and is induced by pro-inflammatory cytokines such as TNF α , IL-1 and IL-6 [20], it seems that its function may be protective. TSG-6 could initiate a negative-feedback mechanism to “switch off” or interrupt the inflammatory cascade, by inhibiting destructive enzymes by binding to IxI [7, 33]. Although IxI is a relatively weak serine protease inhibitor in its own right and its inhibition of plasmin, at least, is significantly increased by binding to TSG-6 [7, 32]. Plasmin has an important role in the activation of matrix metalloproteinases (MMPs) [27], and these are known to have a significant presence in intervertebral disc, particularly in herniation and degeneration [4]. Modulation of their activation is, therefore, likely to be very important.

There is great complexity in the control of matrix turnover and remodelling in disc. Data from this study indicate a further layer of complexity. Matrix degradation may result from failure in any one of many pathways

involving pro-inflammatory cytokines, inflammatory mediators or proteinases (whether serine/metalloproteinases or other enzyme families), all of which interlink via several loops and feedbacks. In addition to TSG-6 and IxI’s possible influence on protease activity, they may also be important in the regulation of matrix remodelling and stability via their interactions with other matrix components [2, 20]. For example, covalent attachment of the heavy chains of IxI to hyaluronan may provide additional cross-linking of disc matrix.

This study should be considered a starting point for further investigation. It has demonstrated the presence of two molecules previously unlocalised in intervertebral disc, each with great potential to influence matrix turnover and inflammation. Thus, both TSG-6 and IxI provide further targets for pharmacological influence. For example, the high levels of TSG-6 expressed in transgenic mice appeared as effective as anti-TNF α treatment in a model of collagen-induced arthritis [21]. Hence, TSG-6 could be used as a therapy in its own right. What is needed now is to study these molecules, their synthesis and the control of their activity, in well-defined model systems to determine their exact function and how they can be modified.

Conclusions

TSG-6 and its binding protein, IxI, are present in human intervertebral discs, particularly from patients with disc

prolapses. TSG-6 is induced by pro-inflammatory cytokines such as TNF α and IL-1. Whilst its function is not clear, particularly in the disc, it is associated elsewhere in the body with extracellular remodelling, diminishing matrix breakdown and acting as a negative feedback inhibiting the inflammatory cascade. Hence the presence of TSG-6 and I α I in disc could have implications for disc

turnover in health and disease and also in the aetio-pathogenesis and treatments of disc disorders such as disc herniation.

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