TSG-6: a multifunctional protein associated with inflammation

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Summary
TSG-6 expression is upregulated in many cell types in response to a variety of proinflammatory mediators and growth factors. This protein is detected in several inflammatory disease states (e.g. rheumatoid arthritis) and in the context of inflammation-like processes, such as ovulation, and is often associated with extracellular matrix remodelling. TSG-6 has anti-inflammatory and chondroprotective effects in various models of inflammation and arthritis, which suggest that it is a component of a negative feedback loop capable of downregulating the inflammatory response. Growing evidence also indicates that TSG-6 acts as a crucial factor in ovulation by influencing the expansion of the hyaluronan-rich cumulus extracellular matrix in the pre-ovulatory follicle. TSG-6 is a member of the Link module superfamily and binds to hyaluronan (a vital component of extracellular matrix), as well as other glycosaminoglycans, via its Link module. In addition, TSG-6 forms both covalent and non-covalent complexes with inter-α-inhibitor (a serine protease inhibitor present at high levels in serum) and potentiates its anti-plasmin activity.

Key words: TSG-6, Hyaluronan, Inter-α-inhibitor, Ovulation, Inflammation, Arthritis

Introduction
Tumor necrosis factor (TNF)-stimulated gene 6 (TSG-6), which maps to human chromosome 2q23.3 (Lee et al., 1993a; Nentwich et al., 2002), was originally identified as a cDNA derived from TNF-treated human fibroblasts (Lee et al., 1990; Lee et al., 1992). TSG-6 genes from other mammalian species, including mouse (Fülöp et al., 1997) and rabbit (Feng and Liau, 1993), have also been sequenced, and the encoded proteins show a high degree of sequence conservation (e.g. human and murine TSG-6 are >94% identical). Recently, a gene with similarity to TSG-6 was identified in lymphocyte-like cells from a lamprey (Mayer et al., 2002). The synthesis of TSG-6 mRNA and protein is tightly regulated in a wide variety of cell types (see below). The protein [also known as TNF-induced protein 6 or tnfip6 (Glant et al., 2002)] is not constitutively present in healthy adult tissues but is produced in response to inflammatory mediators and, not surprisingly, is detected in the context of many inflammatory diseases (see Fig. 1) (reviewed in Wisniewski and Vilcek, 1997). It is also becoming clear that TSG-6 is produced in inflammation-like processes, such as ovulation (Fülöp et al., 1997) and cervical ripening (Fujimoto et al., 2002), where its expression is probably induced by prostaglandin E2 (PGE2) (Fujimoto et al., 2002; Ochner et al., 2003). Growth factors [e.g. transforming growth factor β (TGFβ), epidermal growth factor (EGF) and fibroblast growth factor (FGF)] upregulate TSG-6 synthesis in some cell types (Feng and Liau, 1993; Maier et al., 1996; Ye et al., 1997), and it appears in vascular smooth muscle cells (vSMCs) following injury and mechanical strain (Ye et al., 1997; Lee et al., 2001).

Extracellular matrix (ECM) remodelling is a key feature of most, if not all, the known sites of TSG-6 expression, indicating that it might participate in this process. Indeed, TSG-6 interacts with the ECM glycosaminolycan (GAG) hyaluronan (HA) (Kohda et al., 1996), as well as two other ECM components, chondroitin-4-sulphate (C4S) and aggrecan (Parkar and Day, 1997; Parkar et al., 1998) and the serine protease inhibitor inter-α-inhibitor (IαI) (see Fig. 2) (Wisniewski et al., 1994; Wisniewski et al., 1996; Mukhopadhyay et al., 2001; Getting et al., 2002). Here we discuss these interactions and how they contribute to the multifunctional nature of this interesting protein.

Properties and functions of TSG-6
TSG-6 structure and ligand-binding properties
TSG-6 is an ~35 kDa secreted protein composed mainly of contiguous Link and CUB modules (see Fig. 3); on the basis of our structural and modelling studies, these can be defined as residues 37-128 and 129-250 in the preprotein, respectively (Kohda et al., 1996; Nentwich et al., 2002). Link modules (Day and Prestwich, 2002) are found in many proteins that interact with the ubiquitous GAG HA, which is a vital constituent of ECM (Tammi et al., 2002). NMR spectroscopy has revealed that the Link module of TSG-6 comprises two triple-stranded antiparallel β-sheets and two α-helices arranged around a large, well defined hydrophobic core (see Fig. 3) (Kohda et al., 1996) (C. D. Blundell, I. D. Campbell and A.J.D., unpublished). Studies using a recombinant TSG-6 Link module (termed Link_TSG6) (Day et al., 1996) have revealed that this domain binds to HA, C4S (Parkar and Day, 1997) and the G1-domain of the proteoglycan aggrecan (Parkar et al., 1998). Our original data, which demonstrated that HA binding to Link_TSG6 could not be competed by heparin, led us to assume that TSG-6 did not bind this GAG (Parkar and Day,
reported that procollagen C-proteinase enhancer promotes tolloid proteinase activity and thereby controls collagen assembly through its two CUB modules (Hulmes et al., 1997).

The structure of the TSG-6 CUB module (see Fig. 3) has been modelled (Nentwich et al., 2002) on the basis of the coordinates from three spermadhesins, each comprising a single CUB module that has a structure related to a jellyroll fold (Romão et al., 1997; Varela et al., 1997). Two allelic variants of TSG-6 exist owing to a G/A dimorphism at nucleotide 431, which results in an Arg/Gln alteration in the CUB module (residue 144 of the preprotein) (Nentwich et al., 2002). The newly described Gln144 allele is the most common in Caucasians (>75% are A431 homozygotes), but as yet no functional differences have been identified between the two TSG-6 variants when they are expressed in Drosophila cells (Nentwich et al., 2002; Getting et al., 2002).

**Regulation of TSG-6 expression**

Although there is little or no constitutive expression of TSG-6 in unstimulated cells or tissues, it is produced in response to a wide range of factors (see Table 1). The rapid upregulation of TSG-6 in the presence of the pro-inflammatory cytokines TNF and IL-1 is consistent with its involvement in inflammatory processes. Indeed, high levels of TSG-6 protein have been detected in the sera from patients who have bacterial sepsis and systemic lupus erythematosus (Lee et al., 1993b; Wisniewski and Vilcek, 1997), in mucosal smooth muscle cells (mSMCs) from individuals who have inflammatory bowel disease (C. A. de la Motte, V. C. Hascall, A.J.D. and S. A. Strong, unpublished) and in the joint tissues and synovial fluids (and to a lesser extent the sera) of patients who have various forms of arthritis (Wisniewski et al., 1993; Lee et al., 1993b; Bayliss et al., 2001) (see Fig. 1). TSG-6 mRNA and protein are also produced in cumulus oocyte complexes (COCs) following the induction of ovulation (Fülöp et al., 1997; Yoshioka et al., 2000; Carrette et al., 2001; Mukhopadhyay et al., 2001) and by cervical SMCs (cSMCs) treated with PGE2, which promotes cervical ripening (Fujimoto et al., 2002): both of these

**Fig. 1.** TSG-6 expression. TSG-6 is expressed in the context of inflammatory diseases and inflammation-like processes and has also been detected during development (see references in text and Table 1).

**Fig. 2.** TSG-6 properties and functions. TSG-6 binds specifically to GAGs, including HA, C4S and heparin and to the proteoglycan aggregan. It also interacts, both covalently and non-covalently, with Itd: covalent complexes may be involved in crosslinking HA chains, whereas non-covalent interactions potentiate the anti-plasmin activity of Itd. TSG-6 has been implicated in cell proliferation, the inhibition of neutrophil migration and the regulation of CD44. The mechanisms of these activities remain to be elucidated.
TSG-6: a multifunctional protein

response to vascular injury (Ye et al., 1997). A recent study, using microarray technology, identified TSG-6 as one of a very small number of genes upregulated (~four-fold) in human arterial SMCs in response to mechanical strain (Feng et al., 1999; Lee et al., 2001). These conditions also differentially modulated the synthesis of vascular proteoglycans (resulting in elevated levels of versican, biglycan and perlecan and reduced levels of decorin) and caused increased aggregation of versican with HA (Lee et al., 2001). Following a biomechanical stimulus, the arterial ECM thus appears to undergo a highly coordinated reorganisation in which TSG-6 might participate.

TSG-6 expression is differentially regulated depending on the cell type (Table 1). For example, although IL-1 and TNF are strong inducers of TSG-6 expression in many cell types, IL-1 has no such effect on peripheral blood monocytes (PBMCs) and vSMCs are unresponsive to TNF. Furthermore, among the cell types and stimuli tested, there is considerable variation in the kinetics of TSG-6 production and its sensitivity to protein synthesis inhibitors. This suggests that several distinct pathways regulate TSG-6 expression. For example, cycloheximide does not inhibit TSG-6 transcription in response to TNF, IL-1 or PGE2 (Feng and Liau, 1993; Lee et al., 1993a; Fujimoto et al., 2002) but abrogates growth-factor-induced expression of TSG-6 (Feng and Liau, 1993; Ye et al., 1997). Furthermore, although the upregulation of TSG-6 in response to most stimuli is rapid and relatively short lived, some factors [e.g. TGFβ (Feng and Liau, 1993; Maier et al., 1996) and PGE2 (Fujimoto et al., 2002)] invoke a delayed and more prolonged response. Studies of the TSG-6 promoter have identified NF-IL6 and AP-1 sites as being amongst the elements involved in the regulation of both TNF- and IL-1-induced TSG-6 expression, although these cytokines are not thought to act through identical pathways (Lee et al., 1993a; Klampfer et al., 1994; Wisniewski and Vilecek, 1997). PGE2 probably targets different regulatory elements (Fujimoto et al., 2002).

Recent studies employing DNA microarray technology and/or proteomics have revealed novel sites of TSG-6 expression (see Table 1). For example, constitutive expression of TSG-6 mRNA is 5.3-fold higher in gingival fibroblasts compared with periodontal ligament fibroblasts (Han and Amar, 2002). This could be significant, since gingival and periodontal ligament fibroblasts display distinct activities during the maintenance of tissue integrity and in inflammatory disease, which may result from the differential expression of specific genes. Microarray analysis has also identified a significant number of distinct genes as being induced or repressed in neutrophils after a 4 hour incubation with LPS: TSG-6 mRNA was increased up to 7.1-fold in this context (Fessler et al., 2002; Malcolm et al., 2003). Similarly, TSG-6 is one of 28 mRNAs upregulated during TNF-driven maturation of monocyte-derived dendritic cells (Le Naour et al., 2001). Mikita et al. used microarray technology to identify 127 genes upregulated following LPS treatment of human THP-1-derived macrophages (Mikita et al., 2001). In this case TSG-6 mRNA was upregulated 5.9-fold 1 hour after LPS treatment and 8.9-fold after 6 hours. However, this effect was delayed in lipid-loaded macrophages, which exhibit a phenotype similar to that of the foam cell macrophages believed to play a major role in the pathology of atherosclerosis. Infection with the intracellular pathogen *Chlamydia pneumoniae* causes acute respiratory illnesses, such

![Fig. 3. Structure of human TSG-6. A representation of the modular structure of the mature TSG-6 protein is shown with amino acid positions indicated on the basis of the preprotein sequence (Lee et al., 1992): contiguous Link and CUB (Complement subcomponents C1r/C1s, Uggf, BMP-1) modules are flanked by a 19 amino-acid N-terminal sequence and a 27 amino-acid C-terminal sequence. The refined solution structure of the Link module (C. D. Blundell, I. D. Campbell and A.J.D., unpublished; PDB accession code 1o7b) and a refined solution structure of the CUB domain (Nentwich et al., 2002) are depicted below. The five amino acids identified as being involved in HA binding (Mahoney et al., 2001) are highlighted in purple on the Link module and the polymorphic residue (position 144) (Nentwich et al., 2002) is marked on the CUB module. The point of connection between the Link and CUB modules is indicated by a dashed line (their relative orientations remain to be determined) and the locations of the N- and C-terminal sequences, which are of unknown structure, are indicated.](image-url)
## Table 1. Expression patterns of TSG-6

| Cell/tissue type                  | Species       | Stimulus                       | mRNA* | Protein* | Maximal mRNA expression | Onset of mRNA expression | Reference                |
|----------------------------------|---------------|--------------------------------|-------|----------|-------------------------|--------------------------|--------------------------|
| Foreskin fibroblast cell line    | Human         | No treatment                   | 0     | 0        | +++                     | 4 hours                  | 1.5 hours                | Lee et al., 1990; Lee et al., 1992; Lee et al., 1993a; Wisniewski et al., 1993 |
|                                  |               | TNF                            | +++   | +++      |                         |                          |                          |
|                                  |               | IL-1β                          | +/−   | ++/−     | +++                     | 4 hours                  |                          |
|                                  |               | TGFβ                           | +     | +/−      | +++                     | 4 hours                  |                          |
|                                  |               | PMA                            |       |          | +++                    |                          |                          |
|                                  |               | A23187                         |       |          | +                      |                          |                          |
|                                  |               | Poly(I).poly(C)                |       |          | +                      |                          |                          |
|                                  |               | IL-6                           | 0     |          | +                      |                          |                          |
|                                  |               | EGF                            | 0     |          | +                      |                          |                          |
|                                  |               | PDGF                           | 0     |          | +                      |                          |                          |
| Cultured gingival fibroblasts    | Human         | No treatment                   | ++    |          | +                       |                          | Han and Amar, 2002       |
| Skin fibroblast cell line        | Human         | No treatment                   | 0     |          | +++                    |                          | Seidita et al., 2000    |
|                                  |               | γ-irradiation                  |       |          | +                      |                          |                          |
|                                  |               | PALA                           |       |          | +++                    |                          |                          |
| PBMCs                            | Human         | No treatment                   | 0     |          | +                      |                          | Lee et al., 1992; Lee et al., 1993a; Lee et al., 1993b; Wisniewski et al., 1993 |
|                                  |               | TNF                            | ++    |          | +++                    | 4 hours                  |                          |
|                                  |               | LPS                            | ++++  | +++      | +++                    | 48 hours                 |                          |
|                                  |               | IL-1                           | 0     | 0        | +++                    | 3 hours                  |                          |
|                                  |               | ConA                           | +/−   | +/−      | +++                    | 48 hours                 |                          |
|                                  |               | PHA                            |       |          | +++                    |                          |                          |
| THP-1-derived macrophages        | Human         | No treatment                   | 0     |          | +++                    | 1 hour                   | Mikita et al., 2001     |
|                                  |               | LPS                            | ++++  |          |                         |                          |                          |
| Neutrophils                      | Human         | No treatment                   | 0     |          | +++                    |                          | Fessler et al., 2002; Malcolm et al., 2003 |
| Cultured immature myeloid         | Human         | No treatment                   | 0     |          | +++                    |                          | Le Naour et al., 2001   |
| dendritic cells                  |               | TNF                            | ++++  |          |                         |                          |                          |
| Renal proximal tubular epithelial | Human         | No treatment                   | 0     | 0        | +++                    | 3 hours                  | Janssen et al., 2001    |
| cell line                        |               | IL-1                           | ++++  | +++      | +++                    | 48 hours                 |                          |
|                                  |               | Glucose                       | ++++  | +++      | +++                    | 48 hours                 |                          |
| Microvascular endothelial cell   | Human         | No treatment                   | 0     |          | +++                    |                          | Coombes and Mahony, 2001 |
| line                             |               | Chlamydia pneumoniae           | +++   |          |                         |                          |                          |
| Cultured articular chondrocytes   | Human         | No treatment                   | +/-   | 0        | +++                    | >32 hours                | 2 hours; Margerie et al., 1997 |
|                                  |               | TNF                            | +++   | ++       | +++                    | 16 hours                 | 4 hours                 |
|                                  |               | IL-1                           | ++++  | +++      | +++                    | 16 hours                 | 4 hours                 |
|                                  |               | TGFβ                           | +++   | +++      | +                      |                          |                          |
|                                  |               | PDGF-AA                        | +++   | +++      | +                      |                          |                          |
|                                  |               | IL-6                           | ++    |          | +                      |                          |                          |
|                                  |               | FGF                            | +     |          | +                      |                          |                          |
|                                  |               | IL-1+TGFβ                      | ++++  | +++      | +++                    | 4 hours                  |                          |
|                                  |               | PMA                            | ++++  |          | +++                    |                          |                          |
|                                  |               | A23187                         | ++++  |          | +++                    |                          |                          |
| Cultured cartilage explants      | Human         | TNF                            | +++   |          | +++                    |                          | Maier et al., 1996      |
|                                  |               | IL-1                           | +++   |          | +++                    |                          |                          |
| Cultured articular synoviocytes   | Human (RA)    | No treatment                   | ++    |          | +++                    |                          | Wisniewski et al., 1993 |
|                                  |               | IL-1                           | ++++  | +++      | +++                    |                          |                          |
|                                  |               | TNF                            | ++++  | +++      | +++                    |                          |                          |
| Cultured arterial smooth muscle   | Human         | No treatment                   | 0     |          | +++                    |                          | Lee et al., 2001         |
| cells                            |               | Mechanical strain              | ++++  |          | +++                    |                          |                          |
| Cultured cervical smooth muscle   | Human         | No treatment                   | 0     | 0        | +++                    | 6 hours                  | Fujimoto et al., 2002   |
| cells                            |               | TNF                            | ++++  | +++      | 6 hours                | 0.5 hours                |                          |
|                                  |               | PGE2                           | +     |          | +                      | 24 hours                 | 6 hours                 |
| Vascular smooth muscle cells     | Rabbit        | Serum                          | ++++  |          | +++                    | 4 hours                  | 2 hours                 | Feng and Liao, 1993 |
|                                  |               | FGF-1                          | ++++  |          | +++                    | 2-4 hours                | 2 hours                 | Ye et al., 1997 |
|                                  |               | EGF                            | ++++  |          | +++                    | 4 hours                  | 2 hours                 | Ye et al., 1997 |
|                                  |               | TGFβ                           | ++++  |          | +++                    | 24 hours                 | 2 hours                 | Ye et al., 1997 |
|                                  |               | PDGF-BB                        | ++++  |          | +++                    | 4 hours                  | 2 hours                 |
|                                  |               | IL-1                           | +/−   |          | +++                    |                          |                          |
|                                  |               | TNF                            | 0     |          | +++                    |                          |                          |
| Vascular smooth muscle cells     | Rat           | Uninjured                      | 0     |          | +++                    | 2 weeks                  | Ye et al., 1997         |
|                                  |               | Injured                        | ++++  |          |                         |                          |                          |
as pneumonia and bronchitis, and has been implicated in chronic conditions including atherosclerosis and coronary heart disease in humans. TSG-6 is one of ~20 genes that are significantly upregulated (~two-fold induction) in C. pneumoniae-infected human microvascular endothelial cells (Coombes and Mahony, 2001), which suggests that it might be involved in the pathology of vascular and respiratory diseases.

Reversible and irreversible p53-mediated G1 cell cycle arrest can be induced in human fibroblasts by the antimetabolite N-phosphoacetyl-L-aspartate (PALA) and γ-irradiation, respectively. cDNA representational difference analysis has revealed that TSG-6 mRNA is substantially upregulated in embryonic skin fibroblasts by PALA, but not γ-irradiation, indicating that TSG-6 might be a novel component of the reversible arrest pathway (Seidita et al., 2000). Furthermore, the absence of TSG-6 expression in p53-defective cells suggests that TSG-6 is directly controlled by p53. p21WAF1/CIP1 is a downstream effector in p53-mediated G1 arrest, but can also be upregulated and drive apoptosis in a p53-independent manner. Wu et al. have used adenovirus-vector-mediated transduction of p53 (rAd-p53) or p21WAF1/CIP1 (rAd-p21) to mimic p53-dependent and independent upregulation, respectively, of p21WAF1/CIP1 in human ovarian cancer cell lines (Wu et al., 2002). TSG-6 is not induced by rAd-p53 but is significantly upregulated within 4-8 hours of rAd-p21 infection. p53-independent apoptosis might thus be another process in which TSG-6 functions.

TSG-6 and inter-α-inhibitor

The first detailed study of TSG-6 expression (Lee et al., 1992) detected a ~120 kDa TSG-6-immunoreactive species in the culture supernatants of TNF-stimulated fibroblasts in addition to the expected ~35 kDa protein. Analysis of recombinant human (rh)TSG-6 expressed using baculovirus revealed that this 120 kDa species is a stable, probably covalent, complex comprising TSG-6 and a serum protein (Wisniewski et al., 1992). The latter was identified as IαI, one of a family of closely related serine protease inhibitors (Wisniewski et al., 1994). IαI consists of three polypeptides [heavy chain 1 (HC1), heavy chain 2 (HC2) and bikunin] linked by a chondroitin sulphate moiety that originates from a glycosidic linkage to Ser-10 of bikunin (Enghild et al., 1999); it is the bikunin component that is responsible for the protease inhibitory effects of IαI. Co-incubation of IαI purified from human serum with rhTSG-6 gave rise to a 120 kDa complex, and microsequencing of this chondroitinase ABC-sensitive species revealed the presence of TSG-6 together with bikunin and HC2 (Wisniewski et al., 1994). Although they noted that the total mass of these components is greater than 120 kDa, Wisniewski et al. hypothesised that TSG-6 replaces HC1 of IαI in a trans-esterification reaction (Wisniewski et al., 1994).

TSG-6–IαI complexes have been seen in physiological samples, indicating that they do occur in vivo. Wisniewski et al. detected a ~120 kDa TSG-6–IαI complex in the synovial fluids of arthritic patients (Wisniewski et al., 1993; Wisniewski et al., 1994), which was assumed to have the composition detailed above (i.e. TSG-6, HC2 and bikunin linked by chondroitin sulphate), although this was not shown experimentally. In contrast, Mukhopadhyay et al. have identified a ~125 kDa TSG-6–IαI complex (Mukhopadhyay et al., 2001), which was insensitive to chondroitinase ABC, in the ECM of ovulated COCs from murine fallopian tubes. Mass spectrometry of peptides derived from this species indicated that it contains TSG-6, HC1 and HC2 (but no bikunin) and probably represents two complexes, HC1-TSG-6 and HC2-TSG-6, because TSG-6 has a molecular weight of ~35 kDa and each of the heavy chains is ~83 kDa. These complexes are sensitive to mild NaOH treatment and, thus, might contain ester linkages. Nentwich et al. showed that rhTSG-6 expressed by Drosophila cells and IαI purified from human serum form an ~120 kDa complex after just 30 seconds at 37°C (Nentwich et al., 2002). Furthermore, this complex has the same composition as that detected in murine COCs (M. S. Rugg, A.

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**Table 1. Continued**

| Cell/tissue type | Species          | Stimulus       | mRNA* | Protein* | Maximal mRNA expression | Onset of mRNA expression | Reference  |
|------------------|------------------|----------------|-------|----------|-------------------------|-------------------------|------------|
| Ovarian cancer cell lines | Human | No treatment | p53 transduction | p21WAF1/CIP1 transduction | 0 | 0 | ++ | Wu et al., 2002 |
| Cumulus oocyte complex | Mouse | hCG-induced ovulation | ++++ | ++++ | 3 hours | 1 hour | Fulop et al., 1997 |
| Ovary (cumulus mass and granulosa cells) | Rat | hCG-induced ovulation | ++++ | ++++ | 4 hours | 2 hours | Yoshioka et al., 2000 |
| Skeletal muscle | Rabbit foetus | | ++++ | ++++ | 6 hours | 3 hours | Feng and Liau, 1993 |
| Oesophagus | (25 day gestation) | | ++++ | ++++ | 6 hours | 3 hours | |
| Heart | | | +++ | +++ | 6 hours | 3 hours | |
| Lung | | | 0 | 0 | 6 hours | 3 hours | |
| Liver | | | 0 | 0 | 6 hours | 3 hours | |
| Kidney | | | 0 | 0 | 6 hours | 3 hours | |
| Aorta | | | 0 | 0 | 6 hours | 3 hours | |
| Brain | | | 0 | 0 | 6 hours | 3 hours | |
| Placenta | | | 0 | 0 | 6 hours | 3 hours | |

*Values are consistent within each respective study.
C. Willis, E. Fries and A.J.D., unpublished data). Colocalisation of TSG-6 and Itzl in vivo is, therefore, likely to give rise to rapid complex formation. However, it seems possible that several covalent TSG-6·Itzl complexes, which have different compositions and structures, might exist.

Itzl, like TSG-6, is a HA-binding protein: this was first reported by Sandson et al., who found that HA recovered from pathological synovial fluids is tightly bound to Itzl (Sandson et al., 1965). More recent studies have shown that the heavy chains of Itzl (HC1 and HC2) and heavy chain 3 (HC3) from the related protein pre-α-inhibitor (PαI) (which are collectively termed serum-derived HA-associated proteins or SHAPs) can form covalent complexes with HA, probably owing to substitution of chondroitin sulphate by HA in trans-esterification reactions (Yoneda et al., 1990; Huang et al., 1993; Zhao et al., 1995); isolated HA and Itzl do not interact covalently, indicating that a serum factor catalyses this process (Haung et al., 1993). HA acts as a vital structural component of connective tissues as well as contributing to processes such as immune cell trafficking and intracellular signalling (Tammi et al., 2002). Its synthesis is upregulated by TNF and IL-1 and its levels are elevated in the joints of patients with rheumatoid arthritis (RA) (Hamerman and Wood, 1984; Butler et al., 1988). Itzl is barely detectable in normal synovial fluids but occurs at elevated levels in disease [probably originating from serum (Becker and Sandson, 1971)], and high levels of SHAPs have been detected in osteoarthritis (OA) and RA synovial fluids (Kida et al., 1999). The ability of TSG-6 to interact with both HA and Itzl, and its upregulation in inflammatory situations, suggests that it might somehow influence the formation of HA·Itzl complexes and thus be important for regulating ECM remodelling and/or assembly.

As mentioned above, Itzl is a protease inhibitor, but although it is present in human serum at ~0.45 mg/ml and has been reported to inactivate a broad range of serine proteases (including trypsin, neutrophil elastase and plasmin), its activity is relatively low and its physiological relevance in this regard is not clear (Potempa et al., 1989; Salier et al., 1996). Wisniewski et al. used an in vitro plasmin assay to show that the very modest anti-plasmin activity of Itzl is potentiated by TSG-6 (which alone has virtually no effect on plasmin) (Wisniewski et al., 1996), although TSG-6 does not modulate the inhibition of other proteases (trypsin, neutrophil elastase and urokinase) by Itzl. Janssen et al. have found that stimulation of TSG-6 expression in renal epithelial cells (which constitutively produce the components of PαI, i.e. bikunin and HC3) reduces plasmin activity in culture supernatants, an effect that is abolished by TSG-6 immunoneutralisation (Janssen et al., 2001). Recently, we have shown that recombinant Link/TSG6 alone can potentiate the anti-plasmin activity of Itzl, although it cannot form a covalent complex with Itzl (Getting et al., 2002). Therefore, covalent complex formation is not necessary for TSG-6 to modulate Itzl activity. Furthermore, the TSG-6·Itzl complexes that we have characterised (Mukhopadhyay et al., 2001) (M. S. Rugg, A. C. Willis, E. Fries and A.J.D., unpublished data) do not contain the bikunin chain and, therefore, would not be expected to exhibit any serine protease inhibitory activity.

TSG-6 thus appears to influence Itzl at two different levels (see Fig. 2). Firstly, through the formation of one or more type of covalent complex with Itzl, TSG-6 might somehow contribute to the formation of Itzl·HA complexes in the ECM. Secondly, as a result of non-covalent interactions, TSG-6 enhances the anti-plasmin activity of Itzl and thus might modulate the protease network. This could have significant physiological relevance during inflammatory processes, in which ECM remodelling and the regulation of protease activity are key features.

TSG-6 in arthritis

Given that TSG-6 levels are elevated in the synovial fluids, and to a lesser extent the sera, of patients who have various forms of arthritis (Wisniewski et al., 1993), how might it influence these conditions? TSG-6 expression can be induced in cultured articular synoviocytes by IL-1 and TNF (Wisniewski et al., 1993) and in articular chondrocytes by IL-1, TNF, PDGF and TGFβ (Maier et al., 1996; Margerie et al., 1997). There is also good evidence that TSG-6 is produced locally in the synovium and cartilage of OA and RA joints (Bayliss et al., 2001). For example, TSG-6 has been detected in the blood vessel walls of inflamed synovium and within the pannus region of patients with arthritis. Its presence in these locations is consistent with the involvement of TSG-6 in cell proliferation (Ye et al., 1997) and/or ECM remodelling. Indeed, TSG-6 expression is upregulated in the ECM surrounding OA-like lesions in STR/orf mice (which develop a natural form of OA), decreased levels of aggregcan being detected in areas strongly expressing TSG-6 (Flannely et al., 2001). TSG-6 might, therefore, compete with aggregcan for binding to HA in vivo as it does in vitro (Parkar et al., 1998). Furthermore TSG-6 is detectable in the chondrocyte pericellular matrix of young STR/orf mice, prior to the development of OA lesions and, thus, may be an early marker for disease.

Mapping to human chromosome 2q23.3, TSG-6 lies within the 2q12-q35 region identified as harbouring an OA susceptibility locus (Nentwich et al. (Nentwich et al., 2002) and references therein). Nentwich et al. have, therefore, typed panels of 400 OA patients and 400 controls for the TSG-6 G431A single nucleotide polymorphism described above (Nentwich et al., 2002). Although this dimorphism did not prove to be a marker for OA in the population studied, this does not rule out the possibility that other, as-yet-unidentified, variants of TSG-6 contribute to OA susceptibility.

Several mouse models have been used to investigate the role(s) of TSG-6 in arthritis. Collagen-induced arthritis (CIA) is an autoimmune polyarthritis inducible in susceptible mouse strains by immunisation with type II collagen and has a histopathology similar to that of human RA, including synovitis, followed by cartilage destruction and bone erosion with eventual loss of joint function. Mindrescu et al. have used this model to test the effects of systemic recombinant TSG-6 and of TSG-6 produced locally by T cells in the arthritic joints of transgenic mice (Mindrescu et al., 2000; Mindrescu et al., 2002). Both approaches reduced disease incidence and caused potent inhibition of inflammation and joint destruction. The transgenic mice showed the greater amelioration (as well as delayed onset) of symptoms, and the improvement was comparable to that seen with anti-TNF antibody treatment. Local expression of TSG-6 in arthritic joints could, therefore, limit inflammation and thereby protect cartilage and bone. Note that, although treatment of CIA-affected mice with
rhTSG-6 caused significant reduction in the levels of antibodies against type II collagen, no such effect was seen in the TSG-6 transgenic mice, which suggests that TSG-6 does not affect the immune response to type II collagen. This idea is supported by the fact that there is no evidence for altered cytokine production or inhibition of T cell activity in response to type II collagen in TSG-6 transgenic mice with CIA (Mindrescu et al., 2002).

Bárdos et al. have used mice with proteoglycan-induced arthritis (PGIA) as a model for human RA to determine the effects of systemic TSG-6 administration (Bárdos et al., 2001). Intravenous injection of severely arthritic mice with recombinant murine TSG-6 (rmTSG-6) caused a dramatic reduction in joint edema and, in long-term treatment, inhibited cartilage degradation and bone erosion. However, it did not delay the onset or reduce the incidence of PGIA nor did it alter the production of various pro- and anti-inflammatory cytokines, the levels of antibodies to PGs or PG-specific T cell responses.

CD44 is the major cell surface receptor for HA (Tammi et al., 2002), and CD44-HA interactions contribute to leukocyte rolling during inflammation (Mohamadzadeh et al., 1998; Puré and Cuff, 2001). Pro-inflammatory cytokines upregulate HA expression on the vascular endothelium and induce the HA-binding capacity of CD44+ leukocytes, thereby promoting the contribution of HA-CD44 interactions to leukocyte migration. Since TSG-6 also binds HA, TSG-6 might mediate its anti-inflammatory effects by blocking this interaction. However, Mindrescu et al. saw no correlation between the extent of T lymphocyte infiltration into the joints of TSG-6 transgenic mice with CIA and the severity of disease (Mindrescu et al., 2002), and Bárdos et al. observed that although TSG-6 can compete with CD44 for binding to HA in vitro (albeit at very high TSG-6 concentrations), this does not appear to be the case in vivo (Bárdos et al., 2001). These data suggest that the anti-inflammatory effect of TSG-6 in arthritis is unlikely to be due entirely to inhibition of T lymphocyte influx, although this could be a contributory mechanism. Moreover, recent work has shown that pre-incubation of HA with TSG-6 or Link_TSG6 enhances and/or induces the binding of CD44+ cells to HA, both under static and flow conditions, which suggests that TSG-6 promotes lymphocyte adhesion/migration (J. Lesley, K. Mikecz and A.J.D., unpublished).

Bárdos et al. also observed that a single intra-articular injection of rmTSG-6 into the acutely inflamed joints of mice with antigen-induced arthritis (AIA; a model for monoarticular arthritis) has a strong chondroprotective effect, which lasts for 5 to 7 days (Bárdos et al., 2001). The absence of aggregcan fragments from treated joints indicated that the matrix metalloproteinases (MMPs) that degrade cartilage proteoglycans under inflammatory conditions were inhibited. To investigate the chondroprotective effect, Glant et al. generated transgenic mice that express murine TSG-6 specifically in cartilage (Glant et al., 2002). The induction of AIA in these mice results in severe joint inflammation, but their cartilage remains intact for at least 1 week (control mice suffered major damage from day 5) and both loss of aggregcan and accumulation of MMP-generated fragments are reduced. Furthermore, after 4-5 weeks, TSG-6 transgenic mice are free of local inflammation and their cartilage is almost fully repaired (which is not the case in controls). Glant et al. hypothesise that potentiation of the anti-plasmin effect of IαI by TSG-6 is responsible (Glant et al., 2002), as plasmin is involved in the activation of cartilage MMPs, which have matrix-degrading activity (MMPs 1, 2, 3, 9 and 14) (reviewed in Murphy et al., 1999), and can also participate in the activation of aggrecanases [Glant et al. (Glant et al., 2002) and references therein]. Bárdos et al. observed that TSG-6 injected into mice accumulates at sites of inflammation (probably by binding HA) (Bárdos et al., 2001), which would allow it to entrap IαI from the serum. In this regard, both TSG-6–IαI and HA-IαI complexes have been detected in the synovial fluids of patients who have arthritis (Becker and Sandson, 1971; Winsiewski et al., 1993), although the roles of these species are not clear.

**TSG-6 as a regulator of inflammation**

The importance of the plasmin/plasminogen activator system in regulating the protease network associated with inflammation led Winsiewski et al. to investigate the effect of TSG-6 on a mouse air pouch model of acute inflammation (Winsiewski et al., 1996). The cells lining an air pouch resemble those in the synovial lining of a joint, and the introduction of a proinflammatory stimulus (e.g. carrageenan or IL-1) produces local effects similar to synovitis. Co-injection of rhTSG-6 significantly reduces neutrophil infiltration. Winsiewski et al. detected a 120 kDa, TSG-6-immunoreactive species (Winsiewski et al., 1996), assumed to be the TSG-6–IαI complex described previously (Winsiewski et al., 1994), in air pouch exudates and found that two single-site mutants of rhTSG-6 that exhibited little or no potentiation of the anti-plasmin effect of IαI (although they formed stable TSG-6–IαI complexes) showed reduced or no anti-inflammatory activity. This led them to hypothesise that TSG-6 inhibits neutrophil migration by modulating the protease network in conjunction with IαI.

Recent work has revealed that the anti-inflammatory effect of TSG-6 is mediated by its Link module (Getting et al., 2002). In mouse air pouch models of IL-1- or zymosan-induced acute inflammation, equivalent doses of rhTSG-6 and Link_TSG6 inhibit neutrophil influx to similar extents. Link_TSG6 also significantly reduces levels of the inflammatory mediators KC, TNF and PGE2 in air pouch exudates. Analysis of Link_TSG6 mutants revealed that the anti-inflammatory effects of TSG-6 in vivo are likely to be independent of its ability to bind HA or potentiate IαI action (Getting et al., 2002). This study, therefore, casts doubt on the hypothesis that the anti-inflammatory effect of TSG-6 is mediated through downregulation of the protease network. We also observed that Link_TSG6 exerts similar neutrophil-inhibitory effects in different models of inflammation and regardless of its route of administration (i.e. into the inflammatory site or intravenously). It thus seems likely that TSG-6 acts via the circulation to influence a fundamental process of neutrophil extravasation. Preliminary evidence suggests that TSG-6 modulates the adhesion of neutrophils to the endothelium (Cao et al., 2002). The timely resolution of leukocyte extravasation is essential to prevent damage to healthy tissue (e.g. by the toxic enzymes and free radicals released by neutrophils), and this depends on the activation of localised anti-inflammatory systems (Perretti, 1997).
Whatever its precise mechanism of action, it seems likely that TSG-6 is an endogenous inhibitor of inflammation that forms part of a negative feedback loop.

**TSG-6 in ovulation**

Mammalian ovulation is a highly regulated, inflammation-like process promoted by the midcycle luteinising hormone (LH) surge and dependent on the temporal and spatial expression of specific genes (reviewed in Richards et al., 2002). Ovulation is initiated in a responsive preovulatory follicle, which comprises several layers of granulosa cells lining a central cavity that contains the COC (an oocyte surrounded by closely adherent cumulus cells) and follicular fluid. Upon ovulatory stimulation the compact COC expands, and this is accompanied by permeabilisation of the blood/follicle barrier, allowing ingress of large serum proteins. Ultimately, the expanded COC is released through the ruptured follicle wall and enters the oviduct. The ovulatory process has been studied in detail in mice, where it can be initiated, for example, by administration of follicle-stimulating hormone (FSH) or human chorionic gonadotropin (hCG; which is functionally analogous to LH).

In mice, ovulation occurs ~14 hours after a LH surge, and during this time the COC undergoes a 20- to 40-fold increase in volume (Chen et al., 1990) owing to the formation of an extensive mucoclastic matrix between the cumulus cells of the COC; this is necessary for successful ovulation and fertilisation (Chen et al., 1993). The major component of the cumulus ECM (cECM) is HA, which is produced by the cumulus and granulosa cells (Eppig, 1979; Chen et al., 1990; Salustri et al., 1992) (reviewed by Salustri and Fülöp, 1998; Richards et al., 2002). However, additional molecules are required for effective incorporation of newly synthesised HA into the cECM. In particular, serum is essential for this process because it contains IαI, which diffuses into the follicular fluid during ovulation and is critical for organising and stabilising the expanding matrix (Chen et al., 1992; Camaioni et al., 1993). The heavy chains of IαI are infertile and exhibit impaired ovulation with impaired HA synthesis (Sato et al., 1999). More recently, the bikunin protease activity in this context.

Note that, in addition to matrix expansion, ovulation involves regulation of matrix degradation, which results in rupture of the COC and led Ocshner et al. to suggest that TSG-6 might play an essential role in the formation or function of the cECM (Ocshner et al., 2003). Varani et al. have shown that growth differentiation factor 9 (GDF-9), a member of the TGFβ superfamily secreted by oocytes, upregulates the expression of COX-2, HAS-2, pentraxin 3 (Ptx3) and TSG-6 (Varani et al., 2002). GDF-9-knockout mice are infertile, whereas mice null for Ptx3 exhibit female subfertility (Elvin et al., 1999; Varani et al., 2002).

TSG-6 protein is present in the cECM, where it colocalises with HA and IαI; TSG-6 occurs both as a free protein and in complexes with IαI (Carrette et al., 2001; Mukhopadhyay et al., 2001). The HC1-TSG-6 and HC2-TSG-6 complexes identified in the COC (see above) form interactions with HA that are resistant to detergent, heat and reducing agents but are disrupted by treatment with NaOH (Mukhopadhyay et al., 2001). Given their likely covalent association with HA, these complexes might be involved in crosslinking of HA chains, through HA binding to the TSG-6 Link module. They could thus participate in matrix assembly within the COC. The free TSG-6 detected in the cECM could associate non-covalently with HA and/or intact or bikunin-containing fragments of IαI. Note that, in addition to matrix expansion, ovulation involves regulated matrix degradation, which results in rupture of the follicle at the surface of the ovary to allow release of the COC into the oviduct. Various metalloproteinases and cathepsins have been implicated in this process (reviewed in Richards et al., 2002), and TSG-6 in association with IαI might regulate protease activity in this context.

**Conclusion/perspectives**

We conclude that TSG-6 is a multi-functional molecule with a complex and tightly regulated expression profile. Because TSG-6 was identified and shown to be expressed in response to TNF and IL-1, its involvement in inflammatory processes has been of major interest. In particular, the upregulation of TSG-6 at sites of ECM remodelling, for example, in arthritic joints and pre-ovulatory follicles, is consistent with its importance in this aspect of inflammation. Although TSG-6 is produced in many pathological situations, its normal physiological function appears to be in the context of...
ovulation. Recent work in this field has provided novel insights into the complex interplay between TSG-6, IxI and HA and suggests that TSG-6 participates in HA crosslinking and/or catalyses the transfer of IxI heavy chains to HA; the generation of TSG-6-knockout mice should help to clarify these issues. The mechanism(s) by which TSG-6 interacts covalently with IxI and the precise structures of the complexes formed will be the subject of future study.

We now know that the ability of TSG-6 to potentiate the antiplasmin activity of IxI is due to the non-covalent association of these proteins. Again, the nature of the complex formed and the mechanism(s) involved remain to be elucidated, as does its physiological relevance. There is evidence that the chondroprotective effect of TSG-6 in arthritis is due to its inhibition of MMPs and aggrecanases that damage cartilage, although further work is required to confirm whether this is via its effect on plasmin activity. However, downregulation of the protease network does not appear to account for the ability of TSG-6 to act as a potent inhibitor of acute inflammation: this probably occurs via a mechanism that involves neither IxI nor HA. Future research will focus on the influence of TSG-6 on the interactions that contribute to leukocyte migration. Indeed, it has been shown recently that TSG-6 enhances the interaction of HA with CD44 on the surface of lymphocyte cell lines and thus may differentially modulate the adhesion/migration of specific leukocyte subpopulations (J. Lesley, K. Mikecz and A.J.D., unpublished data).

To date, all the properties of TSG-6 that have been studied in detail can be attributed to its Link module, and research into the role of its CUB module is clearly needed. In the future, studies on the structural basis of TSG-6–ligand interactions, coupled with exploration of new in vivo and in vitro models, should further our understanding of this exciting and important protein.

**Note added in proof**

Following the acceptance of this Commentary for publication, further progress has been made towards understanding the role of TSG-6 in ovulation. A recent paper reports that immunoprecipitation of TSG-6 from follicular fluid inhibits the covalent coupling of IxI heavy chains to HA, but that other components of the follicular fluid are also necessary for this transfer reaction (Jessen and Ødum, 2003). More importantly, characterization of female TSG-6-knockout mice has revealed that they do not form the cumulus matrix that is required for successful ovulation/fertilization of oocytes and that they are completely sterile (Fülöp et al., 2003). These defects in TSG-6−/− females result, at least in part, from failure to transfer IxI heavy chains onto HA during COC mucification, thus establishing the absolute requirement of TSG-6 for this transfer process.

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