Review

Inter-α-inhibitor, hyaluronan and inflammation*

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Inter-α-inhibitor is an abundant plasma protein whose physiological function is only now beginning to be revealed. It consists of three polypeptides: two heavy chains and one light chain called bikunin. Bikunin, which has antiproteolytic activity, carries a chondroitin sulphate chain to which the heavy chains are covalently linked. The heavy chains can be transferred from inter-α-inhibitor to hyaluronan molecules and become covalently linked. This reaction seems to be mediated by TSG-6, a protein secreted by various cells upon stimulation by inflammatory cytokines. Inter-α-inhibitor has been shown to be required for the stabilization of the cumulus cell-oocyte complex during the expansion that occurs prior to ovulation. Hyaluronan-linked heavy chains in the extracellular matrix of this cellular complex have recently been shown to be tightly bound to TSG-6. Since TSG-6 binds to hyaluronan, its complex with heavy chains could stabilize the extracellular matrix by cross-linking hyaluronan molecules. Heavy chains linked to hyaluronan molecules have also been found in inflamed tissues. The physiological role of these complexes is not known but there are indications that they might protect hyaluronan against fragmentation by reactive oxygen species. TSG-6 also binds to bikunin thereby enhancing its antiplasmin activity. Taken together, these results suggest that inter-α-inhibitor is an anti-inflammatory agent which is activated by TSG-6.

In the 1960’s a novel trypsin inhibitor was isolated from human plasma and found to occur at a concentration of about 0.5 mg/ml. Upon paper electrophoresis the protein ran in

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the middle of the α-region and was therefore
given the name inter-α-trypsin inhibitor (IαI)
(Heide et al., 1965; Steinbuch & Loeb, 1961).
However, since trypsin does not normally oc-
cur in plasma, the trypsin part of the name
was later dropped. IαI was found to be a poor
inhibitor of other more physiologically rele-
vant proteases such as elastase and kallikrein
(Potempa et al., 1989), and its function re-
mained unknown for many years after its dis-
covery.

**STRUCTURE OF INTER-α-INHIBITOR**

IαI consists of three different polypeptides:
two homologous heavy chains of about 75 kDa
(HC1 and 2) and one light chain of 16 kDa
(bikunin). There is another plasma protein
closely related to IαI, called pre-α-inhibitor,
which consists of one bikunin molecule and
one heavy chain (Fig. 1); this heavy chain —
HC3 — is homologous to those of IαI. Bikunin
bears a chondroitin sulphate chain and the
C-terminal α-carboxyl group of the heavy
chains of both inter- and pre-α-inhibitor is
esterified with an internal, unsulphated
GalNAc residue of this polysaccharide (Fig. 2)
(Morelle et al., 1994). (For a review on IαI see
Salier et al., 1996). Bikunin, which also occurs
in free form in plasma, accounts for the prote-
ase inhibitory activity of IαI (Wachter &
Hochstrasser, 1981). As shown in Fig. 3, the
polypeptide of bikunin contains two protease
domains of the Kunitz type. Each of these has
antiproteolytic activity but with different
specificities. (For a review on bikunin see
Fries & Blom, 2000). The crystal structure of
bikunin has been determined and docking
analysis has indicated that domain I sterically
hinders binding of proteases larger than
trypsin to domain II (Xu et al., 1998). Indeed,
proteolytically released domain II has a sev-
eral-fold higher inhibitory activity against fac-
tor Xa and plasma kallikrein than intact biku-
nin (Morishita et al., 1994).

**BIOSYNTHESIS OF INTER- AND
PRE-α-INHIBITOR**

Inter- and pre-α-inhibitor are synthesized by
hepatocytes and their assembly has been stud-
ied by pulse-chase experiments with isolated cells (Sjöberg & Fries, 1992; Thøgersen & Enghild, 1995). Both the heavy chains and bikunin are synthesized as precursors with C- and N-terminal extensions, respectively (Fig. 4). Upon arrival in the Golgi complex, the chondroitin sulphate chain of the bikunin precursor is elongated and the C-terminal extension of the heavy chains cleaved off. The new C-terminal carboxyl group of the heavy chains then immediately becomes covalently linked to the chondroitin sulphate chain of the bikunin precursor. Coupling can also be achieved with recombinant proteins expressed in COS-cells (Blom et al., 1997). Experiments with this system indicate that the C-terminal extension mediates the coupling reaction (Thuveson & Fries, 1999). The N-terminal extension of the bikunin precursor is released by proteolytic cleavage late in the Golgi complex and/or secretory vesicles (Bratt et al., 1993). This part of the precursor, which gives rise to the plasma protein $\alpha_1$-microglobulin, is not essential for coupling (Kaczmarczyk et al., 2002) and there is no known functional relationship between the two proteins.

**Figure 3. Schematic representation of the structure of bikunin.**

Bikunin consists of a polypeptide of 16 kDa, a chondroitin sulphate chain of 7 kDa and an N-linked oligosaccharide of 2 kDa. The amino-acid residues are depicted as circles in red and sugar residues as open squares; sulphated sugar residues are in gray. The polypeptide contains two proteinase inhibitor domains of the Kunitz type.

**Figure 4. Biosynthesis of pre-$\alpha$-inhibitor.**

Bikunin (in red) is synthesized as a precursor with an N-terminal extension containing the plasma protein $\alpha_1$-microglobulin. In the Golgi complex, the precursor acquires a chondroitin sulphate chain (indicated with CS). The heavy chain (in blue) is also synthesized as a precursor with a C-terminal extension, which is cleaved off in the Golgi complex. Concurrently, the chondroitin sulphate chain of the bikunin precursor is covalently linked to the new C-terminus of the heavy chain yielding the structure shown in Fig. 2. When the complex reaches the secretory vesicles, the bikunin precursor is proteolytically cleaved resulting in the release of the $\alpha_1$-microglobulin moiety and the formation of mature pre-$\alpha$-inhibitor. Inter-$\alpha$-inhibitor is assembled in a similar way.
OVULATION AND INTER-\(\alpha\)-INHIBITOR

In fully grown follicles the oocytes form a complex with a compact layer of surrounding cells called cumulus cells. Upon ovulatory stimulation, the cumulus cells start producing a hyaluronan-rich extracellular matrix, which leads to a many-fold expansion of the volume of the cumulus cell-oocyte complex (COC). This expansion seems to facilitate the detachment of the COC from the follicle wall and its extrusion upon ovulation. Furthermore, the expanded extracellular matrix seems crucial for the capture of the COC by oviductal fimbriae as well as for successful fertilization (Chen et al., 1993). It was found many years ago that in the presence of serum and follicle stimulating hormone, the preovulatory expansion of the COC could occur also in vitro (Chen et al., 1990; Eppig, 1980). In 1992 it was reported that \(\text{I}\alpha\text{I} \) (or pre-\(\alpha\)-inhibitor) is the essential component of the added serum (Chen et al., 1992). It was subsequently shown that the permeability of the capillaries surrounding the follicles increases upon ovulatory stimulation leading to an influx of \(\text{I}\alpha\text{I} \) (Powers et al., 1995). In addition, immunofluorescence microscopy showed the appearance of heavy chains in the COCs indicating a direct structural role for this protein (Chen et al., 1994). These findings were recently corroborated through the study of mice lacking bikunin: female animals were found to be infertile with small, non-expanded COCs, and the defect could be alleviated through injection of \(\text{I}\alpha\text{I} \) (Zhuo et al., 2001).

HYALURONAN AND \(\text{I}\alpha\text{I} \)

Various cells in culture, as for example fibroblasts, have been found to produce a pericellular, gel-like structure, or coat, which can be visualized by the addition of particles, such as erythrocytes, to the medium (Clarris & Fraser, 1968). This coat contains hyaluronan, as shown by the fact that it will disappear upon treatment with hyaluronidase. If the culture medium contains serum, the coat will reform after removal of the enzyme. \(\text{I}\alpha\text{I} \) has been shown to be the component of serum that is essential for coat formation (Blom et al., 1995). When analyzing hyaluronan from fibroblasts in culture, Sugahara and coworkers found covalently linked heavy chains which were derived from the medium (Zhao et al., 1995). Heavy chains covalently linked to hyaluronan have also been detected in the synovial fluid of rheumatoid arthritis patients (Hutadilok et al., 1988). Analysis of this material by mass spectrometry showed that the heavy chains were linked to hyaluronan through the same kind of ester bond as had earlier been shown to exist between chondroitin sulphate and heavy chains in inter-\(\alpha\)-inhibitor (Zhao et al., 1995) and pre-\(\alpha\)-inhibitor (Enghild et al., 1991).

TSG-6 AND \(\text{I}\alpha\text{I} \)

The TSG-6 gene (the tumour necrosis factor-stimulated gene 6) codes for a 35 kDa protein secreted by various cells, such as fibroblasts, upon stimulation by the proinflammatory cytokines tumour necrosis factor or interleukin 1. (For a review see Wisniewski & Vilcek, 1997). The N-terminal half of the TSG-6 protein (the link module) binds hyaluronan, and its amino-acid sequence shows 36–40% identity with members of the hyaladherin family, including the lymphocyte homing/hyaluronan receptor CD44, cartilage link protein, aggrecan, and versican (Lee et al., 1992). High levels of TSG-6 have been found in synovial fluid of patients with rheumatoid arthritis. When analyzing the TSG-6 protein in this material by immunoblotting, Wisniewski et al. (1994) detected not only a 35 kDa band but also one of 120 kDa. Amino acid sequencing indicated that this band contained equimolar amounts of TSG-6, bikunin and one of the \(\text{I}\alpha\text{I} \) heavy chains (HC2). Furthermore, they found that the 120 kDa complex would
form when recombinant TSG-6 and isolated IcI were incubated at 37°C. Based on these observations they suggested that TSG-6 displaces HC1, possibly through a transesterification reaction. They noted, however, that the apparent molecular mass of the complex was not consistent with it containing both bikunin, TSG-6 and one heavy chain. They therefore proposed that it had undergone proteolytic modification. In a later study of the extracellular matrix of COCs, Mukhopadhyay et al. (2001) detected a 125 kDa complex, which, based on mass spectrometry, was found to contain HC1, HC2 and TSG-6, but not bikunin. They concluded that the presence of both heavy chains in the analyzed material probably reflected the occurrence of two different TSG-6-containing complexes: one with HC1 and another with HC2. Since TSG-6 binds hyaluronan, the complex between TSG-6 and heavy chain could act as a cross link between different hyaluronan molecules thereby stabilizing the extracellular matrix of the COCs. Furthermore, some of the hyaluronan molecules could be anchored to the cells by binding to proteins such as CD44 (Fig. 5).

In search for a function of the protease inhibitory activity of IcI, Wisniewski et al. (1996) discovered that TSG-6 potentiates its antiplasmin activity. They also found that injection of TSG-6 suppresses infiltration of neutrophils into inflamed tissue and suggested that the cause of this effect was inhibition of plasmin by bikunin. In a subsequent study by Day and coworkers based on the analysis of recombinant link modules mutated at different sites, it was found, however, that potentiation of antiplasmin activity did not correlate with inhibition of neutrophil migration (Getting et al., 2002).

CONCLUSIONS

The identification of a complex between the heavy chains of IcI and TSG-6 in the hyaluronan-rich extracellular matrix of COCs provides a simple explanation for the role of IcI in the stabilization of the COCs. However, the general function of IcI, whose plasma level is stable and equal in males and females, is still unclear. Ovulation has a number of characteristics typical of an inflammatory reaction (Espey, 1994) and it is therefore conceivable that IcI is in fact an inflammatory agent. Certain observations suggest that its activity is regulated by TSG-6, whose expression is clearly controlled by inflammatory signals. Thus, it was recently reported that in a model system, TSG-6 mediates the transfer of heavy chains from IcI to hyaluronan (Jessen & Ødum, 2003). The physiological significance of this transfer is unclear, but in vitro experiments have shown that the heavy chains protect hyaluronan against cleavage by reactive oxygen species (Hutadilok et al., 1988). Therefore, the role of the heavy chains could be to contain the destructive effect of activated neutrophils. Plasmin plays an important role in inflammation, and the fact that the anti-
plasmin activity of IαI is enhanced by TSG-6 lends further support to the idea that IαI is an antiinflammatory agent.

Analysis of proteins expressed by fibroblasts cultured on plastic has led to the conclusion that these cells are in a state resembling inflammation (Iyer et al., 1999). It is possible that the hyaluronan-containing coat seen on fibroblasts and other cells also exists in inflamed tissue where it could facilitate cell migration (Camenisch et al., 2000). Unlike the hyaluronan of the COCs, that extracted from fibroblasts has not been reported to contain TSG-6. It is therefore not clear how the heavy chains could stabilize the pericellular coat on these cells. Possibly, they might bind to other proteins yet to be discovered. Interestingly, comparative sequence analysis has indicated that the heavy chains contain a von Willebrandt factor A domain, which in other proteins has been shown to bind to collagens (Bork & Rohde, 1991).
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