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SERPINB3, apoptosis and autoimmunity

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ARTICLE INFO

ABSTRACT

SERPINB3 (Squamous Cell Carcinoma Antigen, SCCA1) is a member of the ov-serpins, a serine protease inhibitors family expressed in many cell types including normal epithelium, leukocytes, tumors of epithelial origin and primary liver cancer. Several studies, carried out in vitro and in vivo, have documented an important role of SERPINB3 in the modulation of programmed cell death by different mechanisms, both in inflammatory processes and in cancer. SERPINB3 significantly attenuates apoptosis by contrasting cytochrome c release from the mitochondria and by antichemotactic effect for NK cells. Mechanisms involved in apoptosis induction and regulation play a key role in the balance between cell proliferation and death. Imbalance of this equilibrium may contribute to the development of autoimmune diseases, as defective apoptosis of immune cells leads to deregulated autoreactive cell proliferation. Since defective programmed cell death represents a critical feature of autoimmunity, the involvement of SERPINB3 in this pathological field deserves further studies.

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Contents

1. Introduction

The serpins belong to a family of serine protease inhibitors, consisting in a large group of homologous glycoproteins, described for the first time in 1980 [1]. The members of this family evolved to inhibit protease with common structural features and inhibitory mechanisms [2]. Based on phylogenetic analysis, the serpins can be classified in 16 groups, or "clades". Human serpins are divided into nine clades (A–I) in which clade B serpins, also termed ovoalbumin serpins (ov-serpins), are included [3]. Over 70 serpin structures have been

identified and these data, along with a large amount of biochemical and biophysical information, reveal that inhibitory serpins are "suicide" or "single use" inhibitors that use a unique and extensive conformational change to inhibit proteases [4].

Leucocyte and complement serine proteases play a role as effectors of the immune response by killing or destroying infected or abnormal cells. Members of the serpin superfamily of protease inhibitors regulate such proteases to prevent the premature death of the immune cells and tissue damage. The vertebrate clade B serpins, including SERPINB1, which inhibits neutrophyl elastase and cathepsin G, SERPINB6 responsible for inhibiting cathepsin G and SERPINB9 a regulator of granzyme B, reside in cells of both the innate and the adaptative immune system. [4]. This review will be focused on

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SERPINB3 (Squamous Cell Carcinoma Antigen, SCCA1) and will explore recent findings supporting its role in the modulation of programmed cell death, a critical feature of autoimmunity.

2. Ov-serpin/clade B serpin family

2.1. Ov-serpin molecular characteristics

The ov-serpin family clade was originally proposed on the basis of similarity of amino acid sequence (39–50%), common structural features (lack of the N- and C-terminal extension regions, common to other serpins and existence of serine residue), lack of the signal sequence, found in other serpins and similar gene organization. The human ov-serpins/clade B serpins family consists of 13 members, including chicken ovalbumin, plasminogen actyivator-2 (PAI-2, SERPINB2), Squamous Cell Carcinoma Antigen (SCCA1, SERPINB3 and SCCA2, SERPINB4) and elastase inhibitor (MNEI, SERPINB1) [5].

Serpin molecules are evolutionarily old, being detectable in bacteria and in some viruses. In contrast, the genes of ov-serpins/ clade B family have evolved recently from a common ancestral gene, being not detectable in *Caenorhabditis elegans* or *Drosophila melanogaster*. Among the genes of the 13 members of the clade B family, three localize on chromosome 6p25 (SERPINB1, B6 and B9) and the remaining genes localize on chromosome 18q21.3 (SERPINB2, B3, B4, B5, B7, B8, B10, B11, B12 and B13) [2].

2.2. Structure and mechanisms of inhibition

Serpins act as inhibitors of serine and cysteine proteases, although some of them have evolved into different non-inhibitory functions. Serpin superfamily proteins share a common tertiary structure consisting of nine α-helices (A–I), three β-sheets (A–C) and a reactive-site loop (RSL). The RSL is involved in the initial interaction with the target protease, which recognizes this structure as a substrate and cleaves its sequence between two residues termed P1 (N-terminal) and P1′ (C-terminal). The divergent functions or the specificity of target proteases mainly depend on the variety of RSLs. The structure of the serpin superfamily is metastable, changing from the "stressed" (S) form to the "relaxed" form (R). When the binding of RSL to target proteases and subsequent cleavage occur, the stressed form of the serpin changes to the relaxed form, loosing its inhibitory activity [2,3]. Following RSL cleavage, the serpin-protease interaction may proceed along either of the following two pathways: the "inhibitory pathway", where the cleavage of the RSL leads to a rapid conformational change in the serpin before the deacylation step in the protease's catalytic cycle. In this case the covalent bond between the serpin P1 and the protease's catalytic serine remains intact. In the "substrate pathway", the RSL insertion occurs after protease deacylation and the serpin becomes a true protease substrate; the P1 – P1′ bond is cleaved and the protease is released active, while the serpin is rendered inactive [3]. Non-inhibitory serpins have evolved by ceasing to act as protease inhibitors and by acquiring the ability to interact with other molecules, exerting new functions.

2.3. Clade B serpin and the immune system

In humans, ten out of the 13 genes of clade B are located on chromosome 18 [2]. SERPINB2 (plasminogen activator inhibitor 2, PAI-2) is a serpin found in monocytes, macrophages and in leukocytes in response to inflammatory mediators [6]. SERPINB10 has been found in bone marrow in the developing blood cells of the monocyte lineage and its expression is reduced after mitogen-induced maturation of monocyte cell lines, suggesting an involvement in monocyte development [7].

SERPINB1, SERPINB6 and SERPINB9 are encoded by genes located in a separate cluster on chromosome 6. SERPINB1 is an inhibitor of neutrophil elastase present in cells of the myeloid lineage, particularly granulocytes and macrophages. It participates in phagocytosis by degrading bacterial components and is also released in small amounts into the extracellular space during inflammation, suggesting a role in leukocyte migration and in protecting the cell from proteases released into the cytoplasm during stress or phagocytosis [3]. SERPINB6 is an intracellular serpin expressed primarily in myeloid cells, platelets, endothelial and epithelial cells. It has been proposed that this serpin protects host cells from cathepsin G, which may be released into the cytoplasm when the cell is under stress, or following granule–phagosome fusion during an inflammatory reaction [8]. SERPINB9 is an intracellular inhibitor of the cytotoxic protease granzyme B (GrB) which is expressed primarily in cytotoxic lymphocytes (CL), comprising cytotoxic T lymphocytes and natural killer (NK) cells [9]. SERPINB9 may maintain cytotoxic T lymphocyte (CTL) effector cell lifespan and its overexpression in primary CTL enhances their ability to kill target cells, suggesting that this serpin provides protection from mislocalized GrB released into the cytoplasm during degranulation.

3. SERPINB3 and autoimmunity

3.1. General features

SERPINB3 and SERPINB4 were originally purified from squamous cell carcinoma of the uterine cervix, as the major component of the TA-4 antigen [10]. The two isoforms, sharing similar molecular weight, were initially defined as neutral form (pI>6.2) and acidic form (pI>6.2). Whereas the neutral form was found in the cytoplasm of normal and some malignant squamous cells, the acid form was found predominantly in the cytoplasm of tumor cells. Genomic sequencing revealed that the two isoforms are encode by two separate, but highly homologous (92% at amino acid level), genes. The deduced pI values and molecular weight of the translation products suggested that the neutral form was encoded by the original squamous cell carcinoma antigen (SCCA) gene (SCCA1, SERPINB3) and the acidic form was encoded by a new gene (SCCA2, SERPINB4) [11]. It has been shown that SERPINB3 and SERPINB4 have distinct properties and substrates; SERPINB3 inhibits papain-like cystein proteases such as papain, cathepsin-S, -K and -L, whereas SERPINB4 inhibits cathepsin G, human mast cell chymase and Der p 1 and Der f 1 [12]. Specificities are the result of a difference in their RSL sequence, in which only 7 amino acid residues among 13 (54%) are identical. SERPINB3 and SERPINB4 are co-expressed in normal tissues: epithelium of the tongue, esophagus, tonsil, cervix uterine, Hassal's corpuscles of the thymus and some areas of the skin. SCCA was also detected in saliva, respiratory secretions and amniotic fluid samples from healthy individuals [13]. However, the biological roles of these two isoforms are currently under investigation.

Although it was initially reported that SCCA proteins are cytosolic proteins, passively released from cancer cells [14], additional localization–secretory, cytoplasmic and nuclear–have been subsequently described, expanding the potential range of physiological functions of this molecule [15].

Serum concentration of SCCA has been found elevated in patients with various kinds of squamous cell carcinoma and in patients with psoriasis [16]. Psoriasis is a T-lymphocyte-mediated inflammatory disease of the skin in which IgG and complement deposition in upper epidermidis, as expression of autoantigen–autoantibody interactions, has also been described. The most prominent epidermal protein identified in IgG binding to autoantigens in a recent study has been SCCA, while the other identified autoantigens included arginase 1, enolase 1 and keratin 10 [17].

In patients with systemic scleroderma SCCA, especially the complexed form with circulating IgM, has been found significantly increased in the group of patients with lung fibrosis and similar trend has been observed in patients with diffuse skin involvement [18].

These data indicate that SCCA is up-regulated in scleroderma with fibrotic involvement, suggesting that this protein might be involved in tissue remodeling alterations.

3.2. Involvement in apoptosis

Programmed cell death is conserved throughout evolution as a strategy employed by multicellular organisms to regulate a wide range of physiological processes, including embryo development, tissue remodelling, immune system development and cellular responses to infection, and tumorigenesis. Cell death can be triggered by a variety of stimuli that can ultimately lead to apoptosis, characterized by typical cellular changes, frequently resulting in chromatin fragmentation and condensation, membrane blebbing and collapse of the nucleus [19]. Within the immune system, tumor necrosis factor-α (TNF-α) is an important effector of programmed cell death, playing a major role in immune defence against infections and cancer. The initial events involved in TNF-induced cell death occur through the 55-kDa TNF receptor with subsequent signal transduction events [20]. However, the specific cell death pathway(s) triggered by TNF and their relationships with other effector-induced apoptotic cell death mechanisms are still under investigation.

A number of serpins have demonstrated a protective role against apoptosis. Serine proteinases are indeed implicated in cell death induced by TNF, while their inhibitors protect tumor cells from the cytotoxic effects of TNF. Members of the ov-serpin superfamily, such as PAI-2, commonly act as proteinase inhibitors and sometime interact with multiple proteinase targets [21].

CrmA is a cowpox virus gene product that was originally identified as a serpin that inhibits the interleukin-1β converting enzyme (ICE), an Asp-specific protease, which proteolytically processes pro-interleukin-1β to yield mature, active interleukin-1β. CrmA, as a member of the serpin family of protease inhibitors, inhibits ICE by forming an active site-directed complex. CrmA was shown to block Fas-induced apoptosis, suggesting that it might function as an inhibitor of CTL-mediated killing, since Fas is one of the two effector pathways utilized

in this process. As a consequence, CrmA could block the lethal apoptotic cascade induced by CTLs, allowing virus infected cells to overcome immune surveillance [22].

SERPINB3, typically found over-expressed in cancer cells of epithelial origin [13,23] and of the liver [24], significantly attenuates apoptosis mediated by anti-cancer drugs or TNF-α allowing tumor growth [25]. Inhibition of drug-induced apoptosis was shown to be directly related to the level of expression of SERPINB3 in cancer cells. Transfection of antisense SERPINB3 cDNA into SCC Ag-1 positive tumor cell line cells (SKGIIIa) resulted in the inhibition of the serpin expression and it also significantly increased susceptibility of these cells to drug-induced apoptosis, supporting the role of SERPINB3 in apoptosis resistance [25]. The molecular target location of SERPINB3 in the apoptotic pathway was suggested upstream caspase-3, as proven by the decrease of caspase-3 activity upon apoptosis induction with TNF-α in SCC Ag-1 cDNA transduced PCI-51 cells [26]. TNF-α exposure was also shown to induce up-regulation of the expression of SERPINB3 and this could represent protective mechanisms from TNF-α induced apoptosis [27]. These findings could represent a mechanism by which SERPINB3 contributes to the defence system protecting from apoptotic death mediated by immune killer cells. The inhibitory functions of SERPINB3 were not restricted to a single squamous cell carcinoma cell line (PCI-51), because SERPINB3 cDNA transduction to non-squamous cell line (i.e. K562) gave analogous results [26]. Inhibition of apoptosis in tumor cells by transduction of SERPINB3 cDNA and increased susceptibility to apoptosis by transfection of the antisense cDNA were not impressive, when compared with the change in the protein level of the serpin and this could depend on the possibility that SERPINB3 inhibits only part of the pathways induced by apoptotic signals. Therefore, SERPINB3 may be considered as one of the multiple cellular factors involved in the regulation of apoptosis.

Concerning the mechanism of action of TNF-α in cell death, the combination of TNF-α with its receptor-1 initiates the apoptosis signaling cascade by the activation of caspase 2 and caspase 8 and/or mitogen activated protein kinase signaling. Cathepsin G (serine protease) and cathepsin B (cysteine protease) have been implicated
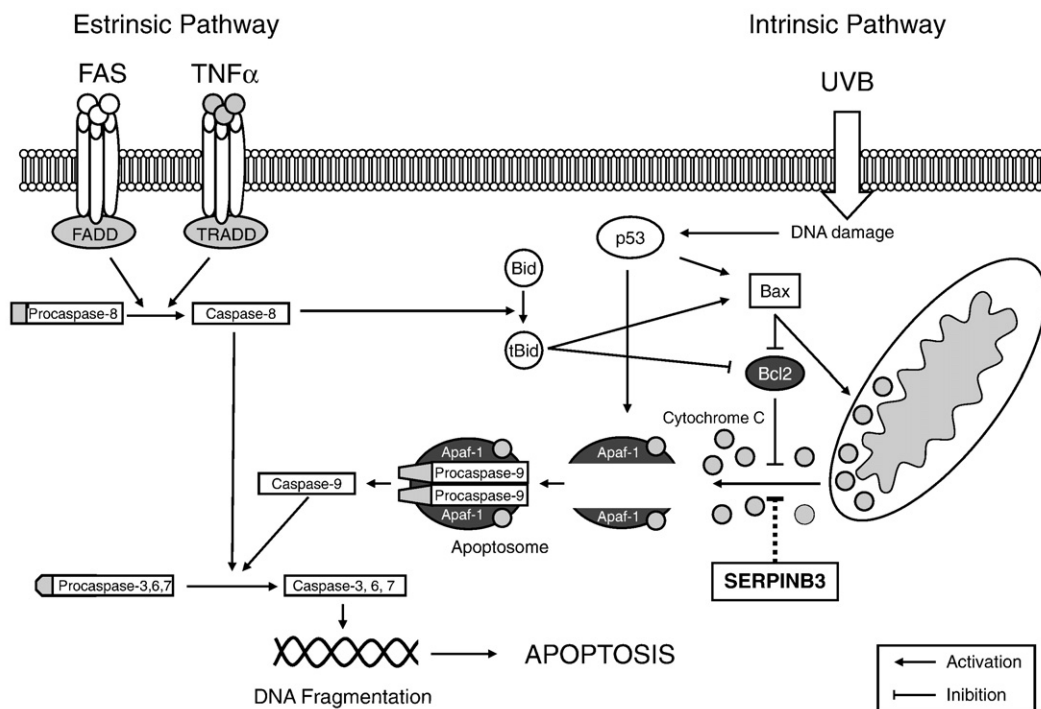
Fig. 1. Hypothetical model of the main pathway involved in apoptosis inhibition by SERPINB3. The specific molecular target of the serpin remains still unknown, however its location has been defined upstream caspase-3, with supporting evidences of cytochrome c release inhibition by mithocondria.

in TNF-α-induced apoptotic signaling pathways [28]. Furthermore, cathepsin D (aspartic protease) and cathepsin B induce cytochrome c release from mitochondria, which is responsible for caspase 9 activation, resulting in the activation of caspase 3. SERPINB3 was shown to prevent TNF-α induced cell death *in vitro* by contrasting cytochrome c release from the mitochondria (Fig. 1) [29].

Apoptosis induced by radiation treatment was also significantly suppressed in 293T cells genetically engineered for SERPINB3, being these cells protected from this apoptotic stimulus. In the 293T cells radiation preferentially triggers the activation of an apoptotic pathway involving caspase 3 and caspase 9 activity and SERPINB3 was involved in targeting this apoptotic cascade. The p38 MAPK molecule, a member of the MAPK family involved in apoptosis when activated in the phosphorylated form, was found less phosphorylated in SERPINB3 transfected cells, not only after radiation treatment but also before treatment. Moreover, phosphorylated MKK3/MKK6, which is the active form of MAPK kinase and works upstream of p38 MAPK, was suppressed after radiation in the SCC Ag cDNA-transfected cells, while in the control cells was increased after radiation. On the basis of these findings, it has been suggested that the proapototic effect of p38 MAPK was reduced by SERPINB3 at the step of phosphorylation of p38 MAPK and/or MKK3/MKK6 [30].

Additional studies indicate that *in vitro* retroviral infection of tumor cells with antisense constructs suppressing expression of SERPINB3 resulted in inhibition of cellular growth and in increase of apoptotic cell death. The number of infiltrating large mononuclear cells was increased by suppression of this serpin and infiltration of immune cells into tumor site was blocked by extracellular SERPINB3. Because chemotaxis of NK cells can be inhibited by SERPINB3 in a dose dependent manner, an inhibitory function against migration of NK cells has been proposed. The mechanism of the antichemotactic effect of secreted SERPINB3 is still unclear. No direct binding of SERPINB3 to NK cells could be detected, however, because antichemotactic activity was lost with mutation of the hinge region (GST-SCC Ag1-A341R) or variable region (GST-SCC Ag1-F352A) of the reactive-site loop, this structure appeared to be important for the antichemotactic effect. Since the RSL is the region where serpins react with target proteases, secreted SERPINB3 may inactivate the protease involved in the migration of NK cells [25].

Recently, inhibition of UV-induced apoptosis by SERPINB3 has been documented. This serpin is expressed in psoriatic epidermis, characterized by abnormal cellular proliferation and differentiation. Its up-regulation was shown to suppress c-Jun NH$_2$-terminal kinase-1 (JNK1) and to block UV-induced apoptosis. The observed anti-apoptotic activity seems to be independent of proteinase-inhibitory activity, because these molecules have specific inhibition profiles for cysteine proteinases. After UV irradiation SERPINB3 was translocated into the nucleus via binding with the active form of the JNK molecule (p-JNK). Translocation was inhibited by a peptide inhibitor of JNKs, TAT-containing JNK-interacting protein-1 (JIP1) peptide, suggesting that SERPINB3 requires direct or indirect association with JNK1 for nuclear translocation after UV irradiation. The results suggested that the kinase activity of JNK1 was specifically regulated by SERPINB3 [31].

Taken together these results, obtained *in vitro* and *in vivo*, document an important role of SERPINB3 in the modulation of the programmed cell death by different mechanisms, both in inflammatory processes and in cancer.

4. Autoimmunity and apoptosis

Apoptosis plays a central role in the immune system, being involved in the maintenance of self-tolerance and in homeostatic control of lymphocyte populations. Cells undergoing apoptotic cell death are morphologically and biochemically distinct from healthy cells and are characterized by nuclear and cytoplasmic shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation,

increased calcium flux and loss of membrane asymmetry. Mechanisms which control apoptosis induction and regulation play a key role in the balance between cell proliferation and death. Imbalance of these mechanisms may contribute to the development of autoimmune diseases, as defective apoptosis of immune cells leads to deregulated autoreactive cell proliferation [32].

Apoptosis is important for the maintenance of both central and peripheral tolerance, typical features of a healthy immune system. Furthermore, apoptosis enables the removal of activated and clonally-expanded lymphocytes generated during the course of an immune response. While some mechanisms regulate the deletion of auto-reactive T and B lymphocytes and the contraction of clonally expanded populations, the underlying molecular events driving the apoptotic pathways are similar. A relevant number of studies have linked the failure to achieve programmed cell death or to clear apoptotic cells to autoimmunity. This deregulation of apoptosis contributes to auto-immune responses by two ways. One is the failure to terminate immune responses and to control autoreactive lymphocytes affecting their switch-off system and lead to broken immunological tolerance with survival of B and T clones involved in autoimmune response. The other is the exposure of self-antigens in an inflammatory extent that can initiate immune responses against them. Although defects in apoptosis propagate autoimmunity and significantly contribute to disease susceptibility, an interruption of multiple immunoregulatory mechanisms is required for full disease penetrance [33].

During apoptosis, autoantigens normally sequestered inside a healthy cell are redistributed to the surface of apoptotic cells. This redistribution can have several consequences. First, autoantigens that are normally sequestered within a cell become concentrated in packages on the surface of apoptotic cells. These packages once released, could prime an immune response to the antigens they contain, if not cleared away properly. Second, the redistribution of autoantigens during apoptosis may create a situation where self-antigens might be presented in a novel context, such as viral antigens complex or epitope modifications. Third, once tolerance has been broken, intracellular autoantigens may become accessible to circulating autoantibodies, where they may opsonize and increase the immunogenicity of apoptotic cells, or cause localized inflammation and tissue damage [34,35].

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease characterized by the production of pathogenic autoantibodies to a spectrum of nuclear antigens. Apoptotic phenomena play a role in the development of SLE and apoptotic cells have been proposed as a possible source of nuclear SLE autoantigen. Recent data documented that TNF blockade induces PBMCs that have adapted to long-term TNF exposure to undergo apoptosis and this may suggest increased antigen presentation under these circumstances, driving autoantibody formation [36]. Defect in the clearance machinery and a subsequent overload of apoptotic cells is a potential mechanism for the break-down of self-tolerance in SLE [37,38]. A common mechanism for autoimmune response is a secondary necrosis of non-ingested apoptotic cells, favoring responses against themselves. The link between SLE and apoptotic cell clearance is emphasized by the fact that immunization with dendritic cells loaded with apoptotic cells can induce autoantibodies, but sustained disease only occurs in susceptible situations [40]. An increased production of apoptotic bodies with a strong reduction of serum DNase activity, which is involved in their clearance, has been detected in SLE patients. At present, a detailed analysis of DNase gene mutations in SLE is lacking and it is unknown whether these mutations could represent a diagnostic marker for SLE development. In summary, SLE is a complex disorder in which defects in apoptosis and impaired clearance are strong contributing factors for susceptibility, onset and severity of the disease.

The role of SERPINB3 in autoimmune disorders has been only partially evaluated. In SLE patients with nephrotic syndrome an increase of the SERPINB3 has been reported in serum, not related with

SLE activity but ascribed to renal failure [39]. Recent data obtained in our laboratories indicate that SERPINB3 was expressed on the surface of B lymphocytes in the majority of normal subjects but in none of SLE patients [40]. Since a direct correlation of surface SERPINB3 with the memory B cell marker CD27 was documented, a possible involvement of SERPINB3 in B cell defects in SLE could be speculated. Additional studies are needed to further explore the role of this serpin in the B cell maturation process and its contribution to the deregulation of B cell reactivity.

5. Concluding remarks

Recent progress in understanding the biological role of human SERPINB3 has been reported. The structure and inhibitory mechanisms of the human ov-serpin/clade B serpin family have been described. Focus has been addressed to general features of SERPINB3, to its inhibitory role in programmed cell death and to its effect on immune system components. The involvement of the SERPINB3 in autoimmunity process deserves further studies.

Take-home messages

- SERPINB3 is a serine protease inhibitor that can affect immune system components
- Apoptosis plays a central role in the immune system, being involved in the maintenance of self-tolerance and in the homeostatic control of lymphocyte populations.
- Deregulation of apoptosis contributes to autoimmune responses
- SERPINB3 is involved in the regulation of apoptosis by different mechanisms

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